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Epidemiology of bovine infectious abortion and perinatal mortality in Flanders

Contribution of *Anaplasma phagocytophilum* and *Chlamydiae*

Hans Van Loo

Dissertation submitted in fulfillment of the requirements
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Promoters:
Prof. dr. B. Pardon
Prof. dr. G. Opsomer
Dr. O. Bogado Pascottini

Department of Internal Medicine, Reproduction and Population Medicine
Faculty of Veterinary Medicine
Ghent University

GHENT UNIVERSITY

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Hans Van Loo (2023)

Department of Internal Medicine, Reproduction and Population Medicine

Faculty of Veterinary Medicine,

Ghent University,

Salisburylaan 133, 9820 Merelbeke, Belgium.

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PROMOTERS

Prof. dr. Bart Pardon

Faculty of Veterinary Medicine, Ghent University, Belgium

Prof. dr. Geert Opsomer

Faculty of Veterinary Medicine, Ghent University, Belgium

Dr. Osvaldo Bogado Pascottini

Faculty of Veterinary Medicine, Ghent University, Belgium

MEMBERS OF THE EXAMINATION COMMITTEE

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Laurent Delooz, DVM

Association Régionale de Santé et d'Identification Animales, Belgium

Dr. Stefaan Ribbens

Dierengezondheidszorg Vlaanderen, Belgium

Prof. Dr. Daisy Vanrompay

Faculty of Bioscience Engineering, Department of Animal Sciences and Aquatic Ecology, Ghent University, Belgium

Teach thy tongue to say 'I do not know', and thou shalt progress
(Mosheh ben Maimon - Maimonides)

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List of Abbreviations

A. fumigatus	*Aspergillus fumigatus*
A. phagocytophilum	*Anaplasma phagocytophilum*
Ab	antibody
Ag	antigen
AI	artificial insemination
APM	abortion and perinatal mortality
APM_{PR}	APM proportion
B. abortus	*Brucella abortus*
B. melitensis	*Brucella melitensis*
B. suis	*Brucella suis*
BCS	body condition score
BHV-1	bovine herpesvirus type 1
BTv	bluetongue virus
BVDv	bovine viral diarrhea virus
C. abortus	*Chlamydia abortus*
C. burnetii	*Coxiella burnetii*
C. pecorum	*Chlamydia pecorum*
C. psittaci	*Chlamydia psittaci*
C. suis	*Chlamydia suis*
CFT	complement fixation test
CI	confidence interval
CLO	*Chlamydia*-like organisms
CRL	crown-rump length
DGZ Vlaanderen	Animal Health Service Flanders
E. coli	*Escherichia coli*
EB	elementary body
EF	early fetal
EFL	early fetal loss
EGA	equine granulocytic anaplasmosis
ELISA	enzyme linked immunosorbent assay
EU	European Union
FASFC	Federal Agency for the Safety of the Food Chain
GA	gestational age
HE	haematoxylin and eosin
HGA	human granulocytic anaplasmosis
I. ricinus	*Ixodes ricinus*
IBR	infectious bovine rhinotracheitis
IgG	immunoglobulin G
IgM	immunoglobulin M
IPI	immunotolerant persistently infected calf

KOH	potassium hydroxide
L. interrogans serovar Hardjo	*Leptospira interrogans* serovar Hardjo
L. ivanovii	*Listeria ivanovii*
L. monocytogenes	*Listeria monocytogenes*
LE	late embryonic
LFL	late fetal loss
LSM	least-squares means
Msp	major surface protein
N. caninum	*Neospora caninum*
OR	odds ratio
P. acanthamoebae	*Parachlamydia acanthamoebae*
PB	persistent body
PCR	polymerase chain reaction
PI	persistently infected
RB	reticulate body
RBT	rose bengal plate test
RT-PCR	reverse transcription polymerase chain reaction
RVFv	Rift Valley Fever virus
SAT	serum agglutination test
SBv	Schmallenbergvirus
SEM	standard error of the mean
T. pyogenes	*Trueperella pyogenes*
TBF	tick-borne fever
VIF	variation inflation factor
WOAH	World Organisation of Animal Health
ZN	Ziehl-Neelsen

Preface

To ensure the economic sustainability of the dairy and beef cattle industry, reproductive efficiency plays a crucial role. In dairy herds, reproduction is vital for producing the next generation of females and initiating milk production after calving. In beef herds, profitability can be enhanced by maximizing the number of cows that produce marketable calves annually. Consequently, occurrences such as abortion and perinatal mortality, which disrupt pregnancy or result in an unsuccessful birth of a viable calf, have a significant economic impact on both milk and meat production. Various factors, including infectious and non-infectious ones, have been linked to bovine abortion and perinatal mortality, but their prevalence may vary depending on the region. Some of these infectious causes may pose a zoonotic risk (e.g., *Brucella abortus*, *Anaplasma phagocytophilum*, *Chlamydiae*), emphasizing the importance of reporting cases and regionally monitoring infectious causes of APM for the benefit of both animal and human health, and to safeguard international trade.

Chapter 1

General Introduction

Background of the cattle industry in Flanders

The oldest evidence for human consumption of animal tissues and meat dates from more than 3 million years ago (McPherron et al., 2010). At least 10,000 years ago, humans began drinking raw milk from other species (Spiteri et al., 2016). Nowadays, meat and milk are contested since they are associated with the production of greenhouse gases, fueling global warming (Givens, 2010; Gerber et al., 2013). Additionally, there is considerable uncertainty about whether these foods may contribute or protect to various human health problems, e.g., vascular disease and some cancers (Givens, 2010; Thorning et al., 2016). Nevertheless, both meat and milk are still considered important sources of necessary dietary nutrients and contribute to meet nutrient recommendations (Górska-Warsewicz et al., 2019). In Europe, the vast majority of produced and consumed milk is from bovine origin (Eurostat, 2022a), while production and consumption of meat is dominated by pig and poultry, followed by cattle industry (Eurostat, 2022b).

In 2022, the northern part of Belgium, Flanders, had a total of 1.25 million cattle (339,580 dairy cows (> 90% Holstein Friesian), 122,514 beef cows (> 90% Belgian Blue), and 786,101 other cattle). The cattle population had been on a declining trend since 2005 but experienced an increase between 2014 and 2016, in particular in dairy cattle. This surge is likely attributed to the abolishment of the milk quota system in 2015. In the most recent years, there has been a slight decrease again. Over the entire period from 2005 to 2022, the dairy cattle population has grown by 16%, while the beef cattle population has shrunk by 30%. The density of cattle in Flanders is shown in **Figure 1.1.**, and compared with other European countries in **Figure 1.2**.

In 2022, the average number of dairy cattle per farm was 77, compared to only 34 in the year 2000. For beef cattle, no clear data are available due to the presence of numerous small backyard farms where a small number of animals are kept as a hobby.

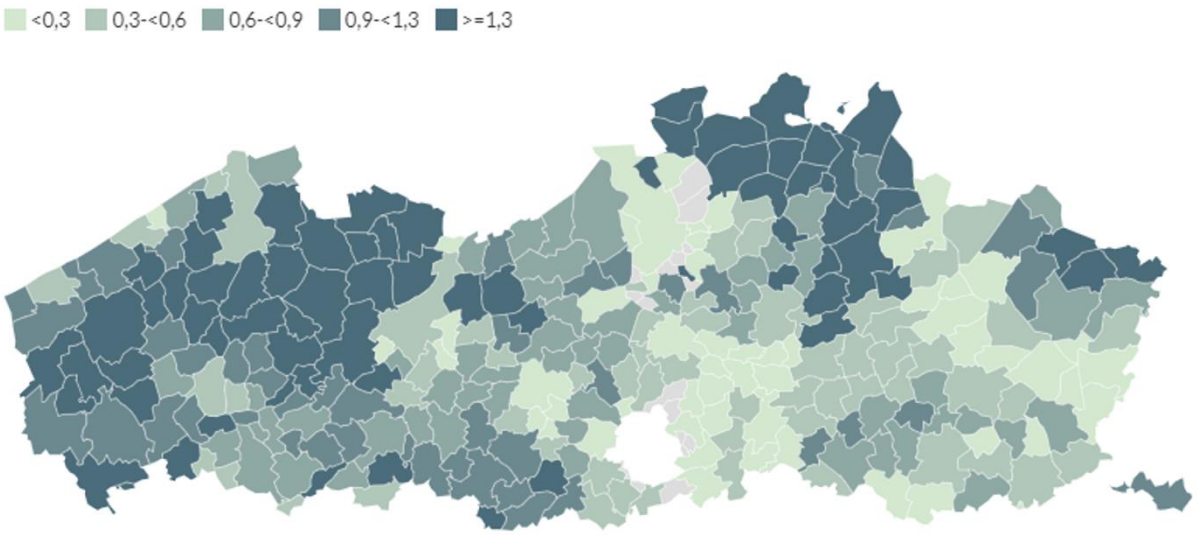

Figure 1.1. The number of cattle per hectare in the Flemish municipalities in 2022. (Source: Statistiek Vlaanderen)

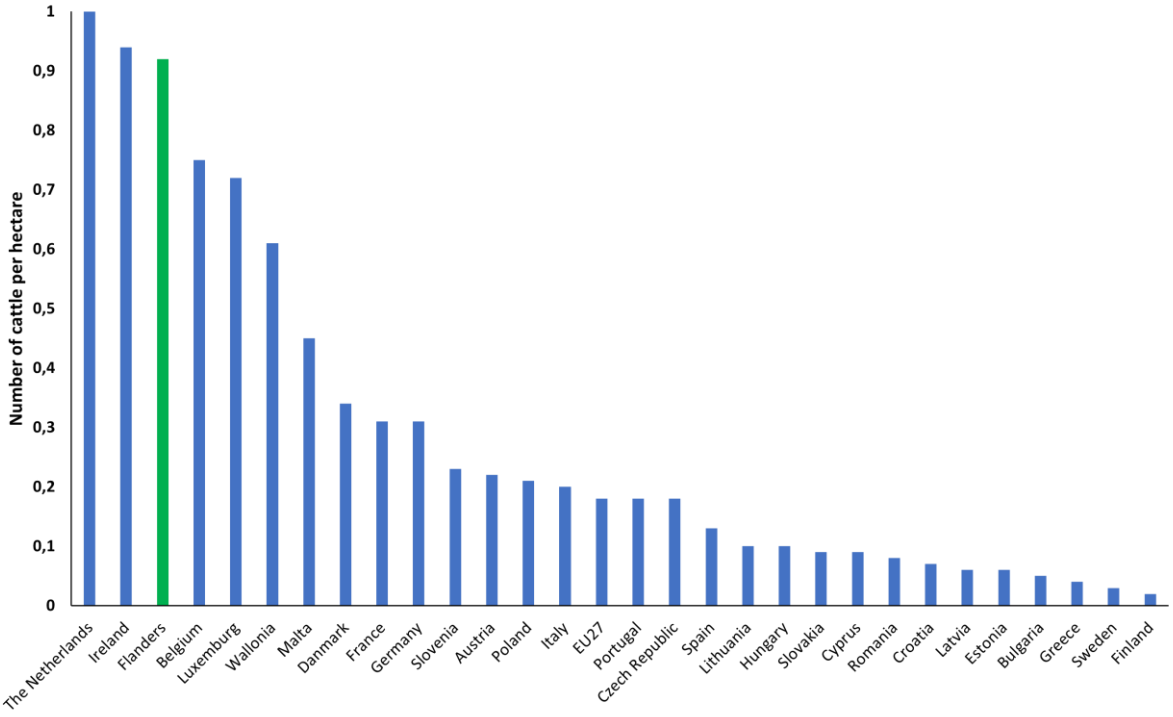

Figure 1.2. The number of cattle per hectare in the EU in 2022. (Adapted from Statistiek Vlaanderen)

In general, dairy cattle in Flanders are typically fed a balanced and nutritious diet that includes a mix of forages (such as grass and corn silage), grains, and supplements (including grain based concentrates, protein supplements, vitamins, minerals). Diets are often formulated to meet the nutritional needs of lactating cows, dry cows, and heifers. The management

practices for dairy cattle in Flanders can vary among farms, and both pasture-based and zero-grazing systems are utilized. However, it is important to note that the trend towards zero-grazing is more present in some regions. Larger farms may find it more practical to implement zero-grazing systems, especially if they can efficiently provide a well-balanced diet within barn facilities. Belgian Blue beef cattle are more common raised on forage based diets, with access to pasture. To meet the nutritional requirements for growth and development, they are commonly supplemented with concentrates. These may include grains, protein supplements, and mineral mixes. Pasture grazing between April and October is typically applied for reproductive Belgian Blue cows, often with a bull. However, female youngstock often is housed in barns until their first calving to monitor and control their diet. This allows optimal growth and muscle development. Indoor housing is also applied during the finishing stage.

Selective breeding is common in dairy herds in Flanders to improve milk production, reproductive efficiency, and overall cow health. Artificial insemination is widely used in 90% of the cows, while natural mating in only 10%. In beef herds, the focus is often on traits such as growth rate, feed efficiency, and carcass quality. The use of artificial insemination and natural mating are both applied in 50% of the cows.

Disease control measures, including vaccination, may vary among individual farms. Cattle farms in Flanders often implement vaccination programs against important diseases such as infectious bovine rhinotracheitis (IBR), bovine viral diarrhea (BVDv), and common respiratory pathogens like *Mannheimia haemolytica*, *Histophilus somni*, bovine respiratory syncytial virus, and para-influenza virus. Several eradication and monitoring programs for diseases such as brucellosis, IBR, BVDv and paratuberculosis are in place in the Belgian cattle industry. In 2006, a monitoring program for paratuberculosis was implemented in the Belgian dairy cattle industry. Participation became obligatory for dairy herds to qualify for delivering milk under a specific milk quality label. The program involves annual testing and culling of infected lactating cows. The IBR eradication program, initiated in 2007 and made mandatory since 2012, involves yearly vaccination of cattle with a gE-deleted vaccine. This vaccination strategy is complemented by testing and culling of infected animals, with the goal of reducing the number of IBR carrier animals. Similarly, a mandatory eradication program for BVDv has been in force since January 2015. The cornerstone of this program is the obligatory analysis of each newborn calf through ear notching to detect BVDv-specific antigen. The primary objective is to identify and eliminate immunotolerant persistently infected newborn calves.

Economic value of pregnancy in cattle

To ensure the economical viability of the bovine beef and dairy industry, reproductive efficiency is a major key factor (Plaizier et al., 1997; Meadows et al., 2005). In dairy herds, reproduction provides the next generation of females and initiates milk production after calving, while in beef herds, profitability can be optimized by maximizing the number of cows that yearly produce a marketable calf. Consequently, events that interrupt the pregnancy or lead to an unsuccessful birth of a viable calf have a major economic impact on both bovine milk and meat production. The lost calf and the loss of milk production are responsible for the direct costs of pregnancy loss, while the indirect economic effects continue in the form of longer calving interval, reduced availability of potential replacement heifers, decreased milk yield, cost of feed, increased number of inseminations, veterinary and labor costs, and higher culling costs (Cabrera, 2012). Additionally, hidden costs as reduced value of breeding stock infected with a specific pathogen should be taken into account. For instance, cows infected with *Neospora caninum* (*N. caninum*), one of the most important abortifacient agents in the world, mostly remain infected for life with a great potential of infecting their progeny. This pattern continues as the infected offspring, in turn, passes the infection to their progeny (Anderson et al., 1997). Therefore, it is advisable not to retain these animals as replacements in the breeding herd. However, selling *N. caninum* positive animals to another farm is unethical. Moreover, according to the Belgian legislation, neosporosis in female cattle is a disease that gives ground to annulment of sale. Culling these animals would result in economic repercussions. Consequently, such a scenario holds substantial implications for the value of infected animals. These considerations hold even more weight in herds possessing high genetic merit, wherein the sale of breeding heifers constitutes a substantial source of income (Trees et al., 1999).

Precisely estimating the cost of bovine pregnancy loss in different regions is difficult. The monetary value of cattle themselves, a replacement heifer, a kilogram of meat, or a litre of milk varies from country to country. Moreover, the cost of a pregnancy loss in dairy cattle mainly depends on the parity of the cow, and the gestational stage at which pregnancy is lost (Groenendaal et al., 2004; Cabrera, 2012; Cantón et al., 2022). Pregnancy losses during the embryonic period are more frequent, but, the economic impact of pregnancy losses during the fetal period is usually greater (Cabrera, 2012). Fetal loss results in extended calving intervals, and it may be too late to rebreed when the loss occurs late in pregnancy, potentially followed by early culling of productive cows. It was estimated that the cost of dairy pregnancy loss ranges from 90 to 1,900 US dollars, with an average of 555 US dollars (De Vries, 2006; Cabrera,

2014). In beef cattle, pregnancy loss probably represents an even more important economic factor because a significant part of the herd income is determined by the number of calves sold (Santos et al., 2004). Nevertheless, there is a lack of literature that precisely describes the financial consequences of failed pregnancies in beef cattle. In a recent study, it has been estimated that an abortion in a beef herd costs the farmer 440 US dollars (Cantón et al., 2022). However, the latter study was performed in Argentina, while, to the best of our knowledge, no data on the cost of abortion and perinatal mortality (APM) in Belgian Blue beef cattle, the most common beef breed in Belgium, are available. Belgian Blue calves have a higher economic value compared to most other beef cattle breeds, especially when compared to dairy breeds. The price of Belgian Blue beef calves is about a nine-fold higher than that of dairy calves. Consequently, the economic costs of APM estimated for other breeds, may not be extrapolated to Belgian Blue beef animals.

Pregnancy loss in cattle

Loss of pregnancy may occur at different stages of gestation. However, in literature, there is some overlap in the periods, making it difficult to find consistency in reported prevalence of pregnancy loss for each gestational period. Interestingly, the risk of pregnancy loss decreases as pregnancy progresses, and is much lower beyond day 60 of gestation (Diskin and Morris, 2008; Diskin et al., 2016). Pregnancy loss before 24 days of gestation is classified as early embryonic loss, while late embryonic loss is defined as loss of pregnancy between 24 and 42 to 50 days of gestation. It has been estimated that about 75% of pregnancy loss occurs in the embryonic stage (Inskeep and Dailey, 2005Diskin et al., 2012). Losses of pregnancy between 42 to 50 days and 265 days are considered fetal losses (Santos et al., 2004). During the fetal period, the risk of pregnancy loss decreases over time, with a slight increase in the last month of gestation (Augustine, 2000). The term “abortion” typically refers to pregnancy loss in the fetal stage, between 42 and 260 days of gestation, while the loss of a non viable fetus beyond day 260 of gestation, and deaths of full-term calves up to 48 h of age have been defined as perinatal mortality (Mee, 2013). The estimated pregnancy losses at different stages of pregnancy in dairy and beef cattle are depicted in **Figure 1.3.**

During the first half of gestation, its continuation requires the presence of a corpus luteum, which is sustained by the presence of the fetoplacental unit (Caldow and Gray, 2004). Destruction of the corpus luteum at that stage will usually result in the termination of pregnancy,

and the expulsion of a fresh fetus with minimal degeneration. Luteolysis in pregnant cows may occur due to various factors, such as an excess of prostaglandins (administered externally or as a result of stress) or Gram-negative bacterial septicemia (Foley, 1996). If fetal death occurs before luteolysis, the death fetus may persist in the uterine cavity for a while, which may lead to the expulsion of an autolyzed fetus, resorption, maceration, or mummification. To ensure the maintenance of pregnancy in later stages, both the fetus and placenta play crucial roles. However, during this phase, significant fetal stress can trigger endocrine ecbolic mechanisms, followed by parturition or APM. Consequently, chronically diseased fetuses can initiate their own premature delivery (Caldow and Gray, 2004).

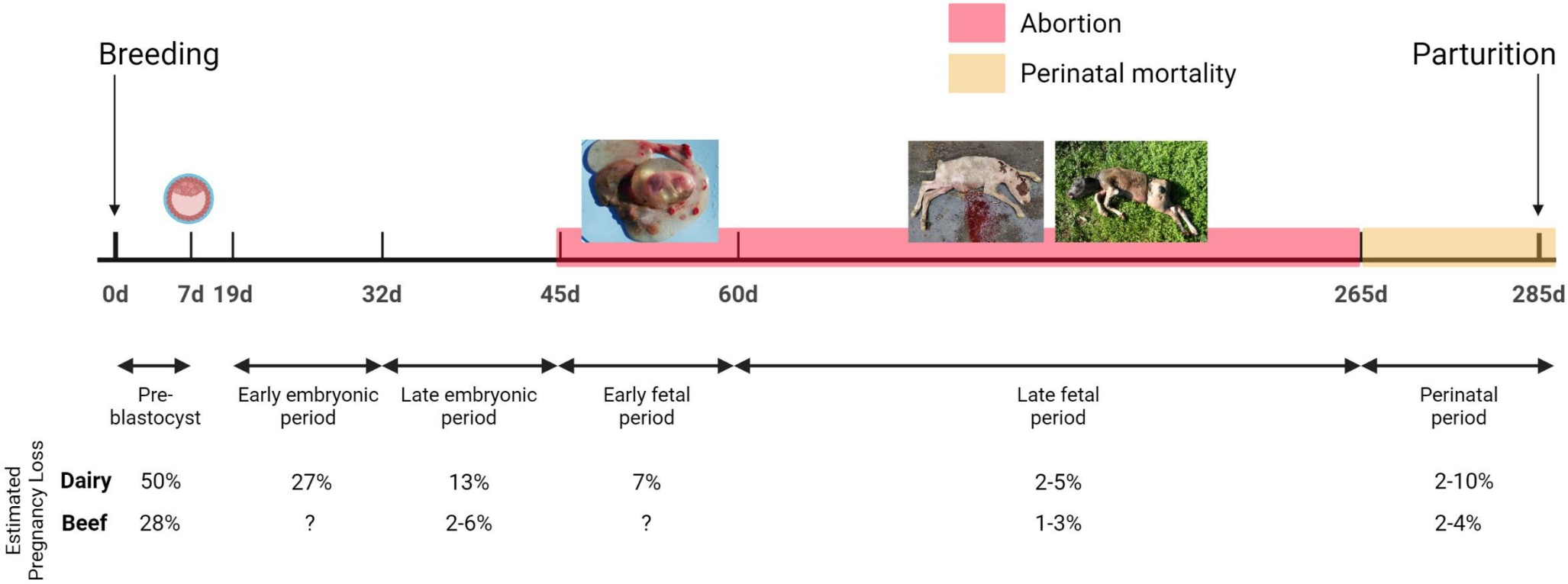

Figure 1.3. Estimated pregnancy losses at different stages of gestation in dairy and beef cattle (adapted from Albaaj et al., 2023).

Pre-blastocyst failure of pregnancy

In cattle, the pre-blastocyst period is defined as the first week after breeding. Failure of embryo development during this period results in restart of the estrous cycle as if there was no pregnancy (Wiltbank et al., 2016). Pre-blastocyst failure includes non-successful fertilization, and death of the early embryo before 7 days of gestation. In high yielding dairy cattle, pre-blastocyst failure may average 50%, with a range from 10 to 82% (Sartori et al., 2002; Wiltbank et al., 2016). Pre-blastocyst failure in beef cattle averages 28%, ranging from 3 to 44% (Reese et al., 2020). Many factors that lead to fertilization failure are described for both dairy and beef cattle. Improper heat detection and artificial insemination (AI) technique (López-Gatius, 2000; Diskin, 2018), presence of polymorphonuclear cells in the uterus (Carvalho et al., 2013; Pascottini et al., 2016), small elevations in circulating progesterone near time of AI (Monteiro et al., 2015; Lonergan and Sánchez, 2020), but also poor quality of sperm or oocyte could increase fertilization failure (Wiltbank et al., 2016). One of the causes of reduced oocyte quality may be prolonged persistency of follicles (Ahmad et al., 1995; Monteiro et al., 2015). Besides reduction of oocyte quality, growth and ovulation of a persistent dominant follicle causes changes in oviductal secretory proteins and gene expression (Binelli et al., 1999; Gonella-Diaza et al., 2015), which may also alter early embryo development. Follicular and oocyte quality are also hampered by the energy status and nutrition during the early postpartum period. Alterations in body condition score (BCS) during the early postpartum period may contribute to the subsequent fertility. The degree of BCS loss between parturition and first AI is negatively correlated with the fertility of lactating dairy cows (López-Gatius et al., 2003; Santos et al., 2009; Carvalho et al., 2014; Pinedo et al., 2022). One possible factor behind this relationship is a potential reduction in embryo quality, and an increase in numbers of degenerated embryos within the first seven days after insemination. This becomes more relevant in cases with an excessive loss of body weight postpartum (Carvalho et al., 2014). In contrast, excessive energy intake may also lead to reduced embryo quality because of altered insulin (Adamiak et al., 2005), and progesterone blood concentrations (Sangsritavong et al., 2002; Vasconcelos et al., 2003), especially in animals with moderately high BCS (Adamiak et al., 2005). Animals with higher feed intake have reduced circulating progesterone concentrations associated with an increased liver blood flow (Sangsritavong et al., 2002; Vasconcelos et al., 2003), which may lead to increased luteinizing hormone pulses, possibly followed by premature resumption of meiosis and ovulation of a low-quality oocyte (Roberson et al., 1989; Revah and Butler, 1996). The quality of the bovine preimplantation embryo may also be influenced by protein, fatty acid,

as well as vitamin and mineral supply in the dam. Before attaching to the endometrial caruncles, the embryo is free-floating, and histotroph. Histotroph refers to the embryo's reliance on uterine secretions present in the uterine lumen. These secretions provide necessary energy and amino acids, which are crucial for the proper embryonic development (Wiltbank et al., 2014). Consequently, deficiencies or excesses of energy, protein, or specific amino acids may have a detrimental effect on oocyte and/or embryo development. Increases in blood urea nitrogen due to intake of diets with an excess of urea or rumen-degradable protein may reduce fertility (Sinclair et al., 2000; Melendez et al., 2003), potentially due to effects on the oocyte, fertilization, and changes in uterine pH. Finally, also early postpartum uterine and non-uterine diseases not only reduce fertilization, but also have a negative impact on embryo quality in lactating dairy cows (Ribeiro et al., 2016).

Early embryonic loss

The early embryonic stage encompasses the time of normal luteolysis and maternal recognition of pregnancy (Wiltbank et al., 2016). However, on the basis of the practical consideration of being able to diagnose pregnancy, the early embryonic stage is often extended until day 28, 30 or 32 of gestation (Albaaj et al., 2023). There are reports identifying the second week of gestation as the most important single period of pregnancy loss in dairy cattle. This is when the blastocyst is hatching, and the embryo starts to elongate (Lonergan et al., 2016; Reese et al., 2020). Based on a meta-analysis, an early embryonic loss of 27% between day 19 and day 32 of gestation is considered normal in dairy cattle, although the variability is high (Albaaj et al., 2023). Factors typically contributing to embryonic death are heat stress, uterine infections, trauma, genetic factors, energy imbalances, maternal illness, aged oocytes from persistent follicles, twinning, and abnormal progesterone levels (Inskeep and Dailey, 2005; BonDurant, 2007).

Late embryonic and early fetal losses

Late embryonic (LE) and early fetal (EF) mortalities are generally defined as loss of pregnancy beyond day 24 of gestation (Diskin and Morris, 2008). For practical reasons (ability of pregnancy diagnosis), LE stage is often defined between day 30 and day 45 (the end of the differentiation stage) of pregnancy, while the EF period is situated between 45 and 60 days (Albaaj et al., 2023). Although the basis of the bovine synepitheliochorial placenta is created at around 19-21 days of gestation, placentation is not completed until day 60 of gestation. Based on previous studies, it seems that in dairy cattle, most EF mortalities occur during the final

process of placentation (between 42 and 60 days of gestation), when placental and fetal development are competing with the nutrient demands of lactation (López-Gatius et al., 2004; Santos et al., 2004). Based on the results of a meta-analysis, a LE loss (30 to 45 days) incidence of 13%, and an EF loss (day 45 to 60 of gestation) incidence of 7% may be expected in dairy cattle (Albaaj et al., 2023). In beef cattle, LE losses are estimated between 2 and 6% (Inskeep, 2004; Reese et al., 2020), which is significantly lesser compared to dairy cattle.

Loss of pregnancy during the LE and EF periods may be the result of placental insufficiency or embryonic deficiencies, but there are no studies available clearly distinguishing between these two alternatives (Wiltbank et al., 2016). Embryonic or fetal death may result in resorption, mummification, maceration, or abortion. A wide variety of factors ranging from an aberrant health status to adverse metabolic and nutritional conditions and hormonal imbalances are related to LE and EF losses. The risk of LE and EF mortality increases under conditions of high milk production and inflammatory diseases (Chebel et al., 2004; Grimard et al., 2006). Late embryonic and EF loss are also related to parity, although effects of parity are conflicting in literature. Abortion rates were reported as significantly higher in lactating dairy (Santos et al., 2004; Lopez-Gatius and Szenci, 2021; Sigdel et al., 2022) and suckler cows (Reese et al., 2020) compared to nulliparous heifers. Additionally, abortion rates were higher in older (>3rd parity) compared to younger cows (Thomsen et al., 2020; Nadri et al., 2021). However, conflicting findings exist, with some studies (Rafati et al., 2010; Norman et al., 2012; Gädicke and Monti, 2013) reporting higher abortion rates in first-parity animals than in older cows. In contrast, Silke et al. (2002) mentioned no difference in late embryonic/early fetal mortality between heifers and cows. However, it was suggested that metabolic stress associated with lactation could compromise fetal survival (López-Gatius et al., 2009). Other risk factors for LE and EF losses are the bull (some bulls have a higher risk for LE/EF mortality) (Franco et al., 2020), previous postpartum metabolic and uterine disorders (excessive loss in BCS during the first month post partum, retained placenta and (endo)metritis in the preceding postpartum period, and injuries to the uterine mucosa) (López-Gatius et al., 2002; Ribeiro et al., 2013), other clinical diseases (e.g., mastitis, and lameness) (Chebel et al., 2004; Santos et al., 2004), season (heat stress) (López-Gatius et al., 2004), and twinning (López-Gatius and Hunter, 2005; López-Gatius et al., 2009; Garcia-Ispierto and López-Gatius, 2019). Chromosomal defects are also known to be involved in LE and EF mortality (Shanks and Robinson, 1989; Charlier et al., 2014).

Numerous bacterial, viral, protozoan, and fungal pathogens have been associated with LE and EF loss in cattle. As many of these pathogens may also be involved in late fetal losses, they are briefly described in the section "*Causes of bovine abortion and perinatal mortality*".

Late fetal loss

Late fetal loss, which is defined as pregnancy loss between 60 and 260 days of gestation, is estimated at 2% in dairy cattle, ranging from 1 to 3% (Wiltbank et al., 2016; Albaaj et al., 2023). Beyond 120 days, a fetal loss rate threshold of 2 to 5% is considered normal (Mee, 2020), although opinions in this area tend to vary. In beef cattle, no clear abortion thresholds are known, although Waldner (2014) mentioned an abortion risk of 1.4 to 2.6% in cow-calf beef herds in Western Canada, with a median of 1.7%.

Beyond 60 days of gestation, the risk of fetal mortality is reduced (Ball, 1997). Fetal losses between day 60 and 130 are characterised by incomplete lysis of the corpus luteum of pregnancy, and related anoestrus, resorption of the amniotic and allantoic fluids, and dehydration of fetal tissues (mummification, **Figure 1.4.**). Mummified fetuses remain in utero, often for several weeks or even months. In less than half of the cases, progression to a macerated fetus may be present (Murray, 2015).

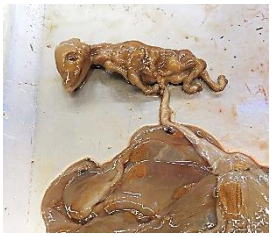

Figure 1.4. Mummified fetus with placenta. (Courtesy of Evelien Forrez, DVM, DGZ Vlaanderen)

Perinatal mortality

Perinatal mortality is defined as the death of full-term calves (following a gestation length of more than 260-270 days) at parturition and up to 24 or 48 h after birth (Gulliksen et al., 2009; Mee et al., 2021). In dairy cattle, perinatal mortality rates vary between 2.4 and 9.7%, with a median of 6.6% when the endpoint is set at 48 h after birth (Cuttance and Laven, 2019). In beef cattle, information on perinatal mortality rates is limited, but according to Waldner et al. (2019), the incidence of calf death from birth to 24 hours is reported as 2.1% in cows and 3.6% in nulliparous heifers in Canadian beef herds.

Causes of bovine abortion and perinatal mortality

Numerous infectious and non-infectious causes of bovine APM have been reported in literature (Anderson, 2007; Givens and Marley, 2008; Clothier and Anderson, 2016; Norquay et al., 2020).

Infectious causes

Possible transmission routes of infectious causes of APM are migration from the abdominal cavity through the fallopian tubes (which is rather uncommon), hematogenous via the placenta (considered the most important in cattle), or passage from the vagina through the cervix (Miller, 1977; Kirkbride, 1993; Jawor et al., 2021). Furthermore, intrauterine persistence prior to pregnancy of specific pathogens like *Trueperella pyogenes* (*T. pyogenes*) or *Campylobacter fetus* may be involved in fetal infection (Miller, 1977).

At its most basic level, infectious causes play a straightforward role in APM by leading to the death of the fetus/calf, but it is important to recognize that infection of the pregnant uterus does not necessarily causes fetal death. However, sub-lethal infections can give rise to various other issues that indirectly increase the risk of mortality. For instance, they may lead to shortened gestation or intrauterine growth retardation (Jawor et al., 2017). In some cases, these infections can result in the birth of live but non-viable calves (Smyth et al., 1999). Additionally, infections can produce live and viable calves that are either transiently or persistently infected, e.g., in cases of bovine viral diarrhea virus (BVDv) or *N. caninum* infection (Lanyon et al., 2014; Lindsay and Dubey, 2020). Additionally, isolation of an agent in fetal or placental tissues does not necessarily mean that this agent has caused the death of the fetus, as many agents appear to pass through the fetal-placental unit, and cause little or no damage (Miller, 1977). Moreover, some maternal infections result in systemic disturbances (e.g., fever, toxemia, circulatory failure, hypoxia, and endotoxemia) which indirectly may affect pregnancy, resulting in APM without infection of the placenta or fetus. In addition, many cases of APM are associated with fetal hypoxia, which may derive from maternal hypoxia, maternal circulatory system failure, or interference with oxygen transfer through the placental interface, most often associated with placentitis or premature placental separation (Holler, 2012). In such a case, the fetus tries to employ compensatory mechanisms to shunt and secure blood flow towards vital organs in order to maintain normal levels of oxygen. As a result, the fetus increases its respiration in an effort to compensate for hypoxia, often followed by strained breathing resulting in the aspiration of amniotic fluid. In cases where the placenta is compromised due to slow-growing opportunistic bacteria or fungi, and the fetus is not immediately overwhelmed by the infection, gradual damage to the placenta will eventually result in suffocation of the fetus due to oxygen deprivation or lack of nutrient transfer across the fetal-maternal interface. If the fetus is not yet viable, it will result in an abortion; if the fetus is already viable but weakened by hypoxia, inadequate nutrient transfer, and the possible detrimental effects of chronic

infection, the outcome is often a stillborn or weak calf (Holler, 2012). The pathogen's ability to injure the conceptus (embryo or fetus) is influenced by multiple factors, including the general health and previous exposure of the dam, the virulence of the infectious agent, and the stage of fetal development. Fetal development encompasses a continuous process of organogenesis, physiological, and immunity development. Consequently, during the early stages of gestation, the fetus is more susceptible to infections and severe diseases due to its underdeveloped immunological capabilities. As the fetus approaches the time of birth, it becomes stronger and more capable of defending itself against pathogens. Consequently, infections occurring at different stages of fetal development will result in varying outcomes for the fetus (Baumgartner, 2015).

Depending on the methodologies used to analyse a case of APM, and on the country or region, the prevalence of detected pathogens may vary, highlighting the importance of regional monitoring of APM. However, analysing each APM case for each known abortifacient pathogen is not possible due to the associated costs, which may cause an underestimation of the prevalence of specific pathogens. When investigating cases of APM, in most laboratories the primary emphasis is placed on searching for infectious agents that are most relevant and prevalent, and most easy to detect with routine methods, in that particular region. However, it is important to consider the possibility of emerging or re-emerging abortifacients being involved as well. For instance, in Belgium, many infectious causes of bovine APM (e.g., *N. caninum*, BVDv, bluetongue virus (BTv), bovine herpesvirus type 1 (BHV-1), *Coxiella burnetii* (*C. burnetii*), etc.) were identified in previous studies performed in the region (Callens et al., 2009; Ribbens et al., 2009; Vangeel et al., 2012), but also other less analysed pathogens like *Anaplasma phagocytophilum* (*A. phagocytophilum*) or *Chlamydiae* might be associated with cases of APM. However, to the best of our knowledge, up to now no APM cases positive for *A. phagocytophilum* and *Chlamydiae* have been described in Flanders.

In about 40% (ranging between 32 and 52%) of abortions, an infectious cause is detected. The remaining 60% includes both non-diagnosed infectious and non-infectious causes (Mee, 2023). In approximately 15% (ranging between 5 and 35%) of perinatal mortalities, an infectious cause is involved (Mee et al., 2021). The most common infectious causes associated with APM worldwide are shown in **Table 1.1.**

Table 1.1. The most commonly diagnosed infectious causes of bovine abortion (and perinatal mortality) internationally (2009-2023); top 5 pathogens (% of overall diagnoses) (adapted from Mee, 2023)

Country	Cases (n)	Dairy Beef	1	2	3	4	5	Reference
Argentina	1,163[a]	D/B	*C. foetus* (9)	*N. caninum* (9)	*Leptospira* spp. (5)	*B. abortus* (5)	BVDv (2)	Canton et al. (2022)
Belgium	100[b]	D/B	Aerobic bacteria (16)	*N. caninum* (10)	*A. fumigatus* (9)	BVDv (4)	*L. monocytogenes* (4)	Callens (2009)
Canada	236[b]	D/B	*N. caninum* (18)	*T. pyogenes* (4)	BHV-1 (3)	*Leptospira* spp. (1)	*Streptococcus* spp. (1)	Wilson et al. (2016)
Chile	398[a]	D/B	*Leptospira* spp. (15)	*N. caninum* (8)	BHV-1 (8)	*B. abortus* (7)	BVDv (6)	Paredes et al. (2010)
Denmark	162[b]	D/B	*N. caninum* (19)	*T. pyogenes* (3)	*S. aureus* (2)	*E. coli* (2)	*L. monocytogenes* (1)	Wolf-Jäckel et al. (2020)
Gr. Britain	476[b]	D/B	*N. caninum* (19)	*S.* Dublin (13)	*B. licheniformis* (12)	*T. pyogenes* (9)	Fungi (7)	APHA (2016)
Ireland	1,665[b]	D/B	*T. pyogenes* (10)	*S.* Dublin (6)	*B. licheniformis* (5)	*L. monocytogenes* (3)	*Aspergillus* spp. (1)	Hayes (2021)
Italy	4,562[a]	D	*N. caninum* (22)	*T. pyogenes* (6)	BVDv (6)	*C. burnetii* (5)	Other spp. (4)	Coin et al. (2022)
N. Ireland	323[b]	D/B	*B. licheniformis* (8)	*T. pyogenes* (8)	*Salmonella* spp. (7)	*N. caninum* (6)	*E. coli* (6)	Sheridan (2021)
Scotland	1,549[b]	D/B	*B. licheniformis* (18)	*T. pyogenes* (17)	*S.* Dublin (10)	Fungi (n.r.)	*N. caninum* (n.r.)	SRUC (2022)
Spain	44[a]	D	*C. burnetii* (21)	*N. caninum* (9)	*E. coli* (9)	*Leptospira* spp. (2)	Other spp. (n.r.)	Gonzales-Warleta et al. (2016)
Tanzania	71[a]	D/B	RVFv (20)	*N. caninum* (13)	BHV-1 (4)	Pestiviruses (3)	*B. abortus* (1)	Thomas et al. (2022)
Tunisia	150[a]	D	*Brucella* spp. (31)	*Chlamydia* spp. (5)	*W. chondrophila* (8)	*Parachlamydia* spp. (5)	*L. monocytogenes* (5)	Barkallah et al. (2014)
Uruguay	102[a]	D	*N. caninum* (29)	*C. burnetii* (6)	*C. foetus* (2)	Other spp. (n.r.)	Other spp. (n.r.)	Macias-Rioseco et al. (2020)
USA	709[a]	D/B	*P. abortibovis* (16)	*N. caninum* (9)	BHV-1 (4)	*T. pyogenes* (3)	*E. coli* (2)	Clothier et al. (2016)
Total	19,070[b]	D/B	*N. caninum* (17)	Opportunistic b. (13)	*Chlamydia* spp. (7)	BHV-1 (6)	BVDv (5)	Hecker et al. (2023)

[a] abortions; [b] abortions and perinatal mortalities; D = dairy, B = beef; n.r. = not recorded; RVFv = Rift Valley Fever virus; opportunistic b. = opportunistic bacteria
Note: not all laboratories test/report results for all pathogens

The most important focus points regarding common infectious causes of APM in Belgium are briefly addressed below and summarized in **Table 1.2**.

1. Bluetongue virus

   Bluetongue virus (BTv) is an *Orbivirus*, that is transmitted mainly by *Culicoides* midges (Ali et al., 2012). More than 20 serotypes of BTv are described, but in cattle the most severe disease is induced by serotype 8. Bovine fetuses infected within the first 100 days of gestation either get reabsorbed or experience abortion. Infections occurring between 70 and 130 days of gestation may lead to stillbirth, the birth of weak calves, or calves with cerebral abnormalities. However, infections occurring after 150 days of gestation typically do not have detrimental effects on the fetus (Maclachlan et al., 2000). Prolonged viremia up to 3 years has been described in cattle, suggesting the potential role of cattle as reservoir host of BTv (Bonneau et al., 2002). In June 2023, Belgium became officially free from BTv, but since October 2023 a newly introduced BTv serotype 3 strain is reported in the country after an initial outbreak in ruminants in the Netherlands.

2. Bovine herpesvirus type 1

   Bovine herpesvirus type 1 (BHV-1) is an *Alphaherpesvirus* known to cause respiratory and genital infections, as well as abortion (Miller and Van der Maaten, 1986; Muylkens et al., 2007). Transmission primarily occurs through contact with the upper respiratory tract (infectious bovine rhinotracheitis, IBR), the conjunctiva, or genital mucous membranes and aborted fetuses, but venereal transmission is also possible. Clinical signs in cows may include fever, anorexia, red nasal mucosa, coughing, and conjunctivitis, followed by abortion within a period of 15 to 64 days (Muylkens et al., 2007; Ali et al., 2012), although subclinical infections are common. Typically, abortions occur between 4 and 8 months of gestation. Bovine herpesvirus type 1 infection can also lead to early embryonic death (Miller and Van der Maaten, 1986). Additionally, BHV-1 establishes latent infections in the trigeminal and sacral ganglia, from where the virus can reactivate under conditions of immunosuppression. Consequently, infected animals serve as a source of infection for unexposed cattle. Infected fetuses show typical hepatic lesions, indicating the haematogenous transmission of the virus to the fetus via the umbilical vein (Smith, 1997). In Belgium, an eradication program was implemented in 2007, which involved blood sampling and vaccination. From January 2012, it became mandatory for

the entire national cattle industry to participate in this program. As a result, prevalence of BHV-1 at herd level decreased from 78% in 2012 to 4% in 2021 in Flanders (De Graef et al., 2022).

3. Bovine viral diarrhea virus

   Bovine viral diarrhea virus (BVDv) is a *Pestivirus* that is transmitted transplacentally or by inhalation or ingestion of material contaminated with infected secretions (Newcomer, 2021). Infection may be subclinical, but clinical signs in animals with an acute BVDv infection are numerous and include fever, nasal discharge, diarrhea, mucosal ulcerations, and immunosuppression as a result of leukopenia. Reproductive losses are the most economically important result of a BVDv infection (Evermann and Ridpath, 2002; Grooms, 2004). Pregnant animals infected within the first 45 days of gestation may experience reduced fertilization rates and embryonic death. Infections occurring between 45 and 175 days of gestation may cause abortion or mummification. However, fetuses that survive infection with a noncytopathic strain of BVDv between 40 and 150 days of gestation usually become persistently infected (PI). These PI animals shed large quantities of BVDv and generally do not produce antibodies against the virus. These animals may exhibit growth retardation or may appear completely healthy. Fetal infection between 100 and 150 days of gestation may result in congenital abnormalities, including cerebellar hypoplasia, microencephalopathy, cataracts, micro-ophthalmia, and thymic aplasia. Fetuses infected between 150 and 285 days of gestation typically clear the virus, but despite sometimes being more susceptible to infections (Barber et al., 1985; Muñoz-Zanzi et al., 2003), develop normally, and show precolostral neutralizing antibodies to BVDv (Ali et al., 2012). In Belgium, a mandatory eradication program is implemented by the government since January 2015, which relies on ear notch analyses of newborn calves in order to detect and cull PI animals. Since then, the prevalence of newborn PI animals in Flanders decreased from 0.55% in 2015 to 0.01% in 2021. Also, the prevalence of BVDv positive APM cases decreased from 4% in 2011 to only 0.06% in 2021 (Deschuytere et al., 2016; De Graef et al., 2022).

4. Schmallenbergvirus

Schmallenbergvirus (SBv) is an *Orthobunyavirus* that was first reported in 2011 in Northern Europe. The virus is transmitted by *Culicoides* midges. Acute infections are often subclinical in ruminants, but clinical disease has been described in cattle, and includes diarrhea, fever, and milk drop. Vertical transmission of SBV from infected dam to fetus occurs during the first and early-second trimester of gestation, which may result in abortion, stillbirth, or the birth of a malformed newborn (Wernike et al., 2014; König et al., 2019). Common musculoskeletal deformities in fetuses arisen from transplacental infection between 60 and 150 days of gestation are arthrogryposis (**Figure 1.5.**), lordosis, scoliosis, torticollis, and brachygnathia inferior (Wernike et al., 2014; König et al., 2019). In the central nervous system, hydranencephaly (**Figure 1.5.**), porencephaly, lissencephaly, hydrocephalus, cerebellar and cerebral hypoplasia and micromyelia may be observed (Endalew et al., 2019). In Belgium, the disease emerged in 2011 (Méroc et al., 2013; Van Loo et al., 2013), with several new outbreaks in the following years (Delooz et al., 2017; Sohier et al., 2017).

Figure 1.5. Arthrogryposis-hydranencephaly syndrome as a result of an intra-uterine infection with schmallenbergvirus in cattle.

5. *Coxiella burnetii*

Coxiella burnetii (*C. burnetii*) is a widespread bacterium, transmitted through inhalation, mucous membrane contact, ticks, and potentially semen (Guatteo et al., 2011; Keshavamurthy et al., 2020). Infected animals are usually asymptomatic. Infection commonly occurs through inhalation of infected aerosols, but can spread also through infected equipment, manure and ticks (Baumgartner, 2015). The organisms are shed in large numbers in feces, milk, urine, fetal fluids, and fetal membranes (Kelly, 2004). Pregnant cows may experience placentitis, and occasionally abort in the later stages of

gestation, although abortion is less frequent compared to small ruminants and does. In cattle, *C. burnetii* prevalence is considered as high worldwide (Guatteo et al., 2011); in Flanders seroprevalence in bulk milk samples was reported to be greater than 80% (DGZ Vlaanderen, 2019).

The bacterium is zoonotic, as it is the cause of Q fever in humans. Clinical manifestations of the infection in humans usually occur as a flu-like syndrome, and may include prolonged illness, undulant fever, atypical pneumonia, hepatitis, myalgia, or endocarditis. Q fever can also lead to abortion or stillbirth in pregnant women (Angelakis and Raoult, 2010). It needs to be mentioned that the public health risk is likely linked to specific genomic groups, mostly found in small ruminant strains (Tomaiuolo et al., 2021).

6. *Leptospira* spp.

   Many of about 300 known serovars of *Leptospira* spp. are non-pathogenic. Among the pathogenic ones, cattle are the maintenance hosts of strains in the Sejroe serogroup, mainly serovar Hardjo, which consists of two species: *Leptospira interrogans* serovar Hardjoprajitno, and *Leptospira borgpetersenii* serovar Hardjobovis (Loureiro and Lilenbaum, 2020). Transmission between maintenance hosts occurs through contact with infected urine, milk, placental fluid, and furthermore by transplacental or venereal transmission. In contrast, transmission to an incidental host occurs via contact with an environment contaminated by infected urine. The bacteria enter the host through the mucous membranes of the eyes, nose, vagina, or through damaged skin. Many leptospiral infections are subclinical, and so the infection is processing unobserved, although reproductive failure and decreased milk production are described (Dhaliwal et al., 1996; Loureiro and Lilenbaum, 2020). In pregnant animals, infection may result in usually late-term abortion, stillbirth, or the birth of weak calves (Grooms and Bolin, 2005). In July 2014, an outbreak of icteric abortions in the southern region of Belgium was associated with incidental strains of *Leptospira* spp. (Grippotyphosa and Australis) (Delooz et al., 2018). *Leptospira* spp. are potential zoonotic bacteria. Symptoms of human leptospirosis in the early stage are flu-like, but in severe cases with specific species, jaundice, bleeding, renal failure and death are possible (Toyokawa et al., 2011). In Flanders the seroprevalence of *Leptospira* Hardjo in bulk milk samples was lower than 10% (DGZ Vlaanderen, 2019).

7. *Listeria* species

   Listeria monocytogenes (*L. monocytogenes*) and *L. ivanovii* are associated with several diseases in cattle, which manifest as meningoencephalitis, neonatal septicemia, and sporadic abortion (Schneider, 2004). Although *Listeria* spp. are ubiquitous in the environment and can be isolated from the feces and nasal excretions of healthy animals, the occurrence of disease is often associated with the intake of contaminated silage (Baumgartner, 2015). The infection spreads hematogenous to the placenta within 5-12 days after ingestion, leading to subsequent fetal sepsis. *Listeria* spp. abortion typically takes place in the third trimester of gestation, although it may also occur in the second (Baumgartner, 2015). If infection arises early in the last trimester, rapid fetal septicemia and subsequent death occurs. Abortion follows a few days later, with partial masking of lesions due to autolysis (Schlafer and Foster, 2016). Infections near term are associated with metritis and septicemia in the dam, often leading to dystocia and retained membranes. In these cases, autolysis may be less extensive, allowing better visualization of fetal lesions (Schlafer and Foster, 2016), characterized by white foci of liver necrosis (Anderson, 2007). In humans, the disease is mainly known as a food-borne disease, although infections by direct contact with infected abortion tissues are also described (Regan et al., 2005; Laureyns et al., 2008). Human infection may result to fever, gastroenteritis, dermatitis, meningitis, encephalitis, endocarditis, arthritis, peritonitis, hepatitis, cholecystitis, and arteritis, as well as abortion and premature birth in pregnant women.

8. *Trueperella pyogenes*

   Trueperella pyogenes (*T. pyogenes*) is a commensal organism in the respiratory, gastrointestinal, and urogenital tracts of healthy cattle (Baumgartner, 2015). Tissue invasion may lead to abscessation (Kirkbride, 1993). The bacterium is often isolated from aborted tissues and is considered to be a primary pathogen in cattle abortion (Schlafer and Foster, 2016). Uterine infection can occur hematogenously or through the cervix. During pregnancy, this may lead to abortion, which is possible at any time throughout gestation, but mainly in the second half of gestation (Baumgartner, 2015).

9. Other bacterial pathogens

   Many species of bacteria, that are part of the normal microbiome or present in the environment, are associated with APM. These bacteria might include *Streptococcus* spp., *Staphylococcus* spp., *Histophilus somni*, *Mycoplasma* spp., *Ureaplasma diversum*,

Bacillus spp., *Escherichia coli*, *Pasteurella* spp., *Pseudomonas* spp., *Salmonella* spp., and many others. These opportunistic bacteria are usually associated with sporadic cases of APM, unless specific risk factors give a particular organism the chance to affect multiple animals (Holler, 2012). Some of these bacteria may gain entry to the pregnant uterus through the cervix, while others are able to spread hematogenously to the fetoplacental unit, and cause APM, usually in late second to third trimester of gestation (Caldow and Gray, 2004; Givens and Marley, 2008). Most of the cases are not associated with any clinical disease in the dam, although clinical signs may be present prior to or at the moment of APM (e.g., septicemia in *Salmonella* Dublin). Some of these bacterial pathogens also pose a zoonotic risk, highlighting the importance of handling aborted tissues with caution.

10. Mycotic pathogens

Mycotic abortions may be caused by various molds and yeasts (Knudtson and Kirkbride, 1992; Barr and Anderson, 1993). Prevalence of fungal types and commonality of fungal APM varies geographically. *Aspergillus fumigatus* (*A. fumigatus*) and other *Aspergillus* spp. are reported as the most common causes of mycotic abortion (Knudtson and Kirkbride, 1992). Additional pathogens include *Absidia* spp., *Morteriella wolfii*, *Rhizomucor pusillus*, *Rhizopus arrhizus*, *Pseudallescheria boydii*, species of *Penicillium*, *Candida*, and *Torulopsis*, but most are considered as opportunist pathogens (Murray, 2015). This condition typically occurs sporadically, often observed when animals are housed indoors or in confined spaces, and fed poorly preserved hay or ensiled feed that has undergone aerobic fermentation (Murray, 2015). Fungal pathogens can enter the respiratory or gastrointestinal tract, subsequently gaining access to the systemic circulation and spreading to the placentomes and amniotic fluid, from where the fetal skin (**Figure 1.6.**), respiratory system and gastrointestinal tract may be infected.

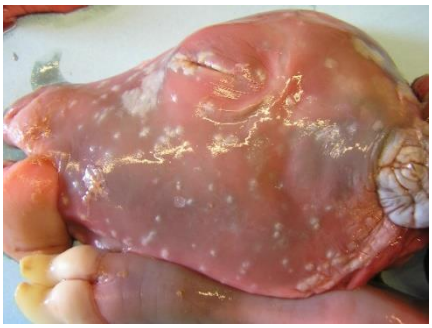

Figure 1.6. Mycotic dermatitis in a bovine fetus. Scattered throughout the skin are numerous multifocal to coalescing circular, raised epidermal plaques. These lesions are characteristic of in utero infections with fungal organisms. (Courtesy of Evelien Forrez, DVM, DGZ Vlaanderen)

Abortion typically takes place between 6 and 8 months of gestation, frequently associated with placentitis and retained placenta. Generally, no other clinical signs are

observed. However, cows infected with *Morteriella wolfii* may experience postabortion pneumonia and succumb to death within 72 hours following abortion (Givens and Marley, 2008).

11. <u>*Neospora caninum*</u>

Neospora caninum (*N. caninum*) is an apicomplexan protozoan. It is estimated that this parasite is responsible for 20% of bovine abortions worldwide (Dubey et al., 2007). Both domesticated and wild canids can serve as definitive hosts, while many other species (e.g., ruminants, deer, horses, chickens, dogs) are described as intermediate hosts. In cattle, transmission of *N. caninum* may occur both horizontally (after ingestion of sporulated oocysts via feed, water or soil contaminated with infected canine feces) and vertically (transplacentally from dam to offspring), vertical transmission being the primary source of infection. The life cycle of *N. caninum* is depicted in **Figure 1.7.** Possible outcomes include fetal death followed by resorption, mummification, abortion with autolysis, stillbirth, live birth with clinical disease (e.g., underweight, unable to rise, neurologic signs, flexed or hyperextended limbs, ataxia), and clinically normal birth of persistently infected offspring. Abortion and perinatal mortality may occur from three months of gestation to term, but typically occur at five to six months of gestation. A seroprevalence study revealed a prevalence of *N. caninum* in Belgium of 10% in purchased cows, and 20% in aborted dams (Roelandt et al., 2015). More recent data exhibited a general seroprevalence of about 10% in Flanders (De Graef et al., 2022).

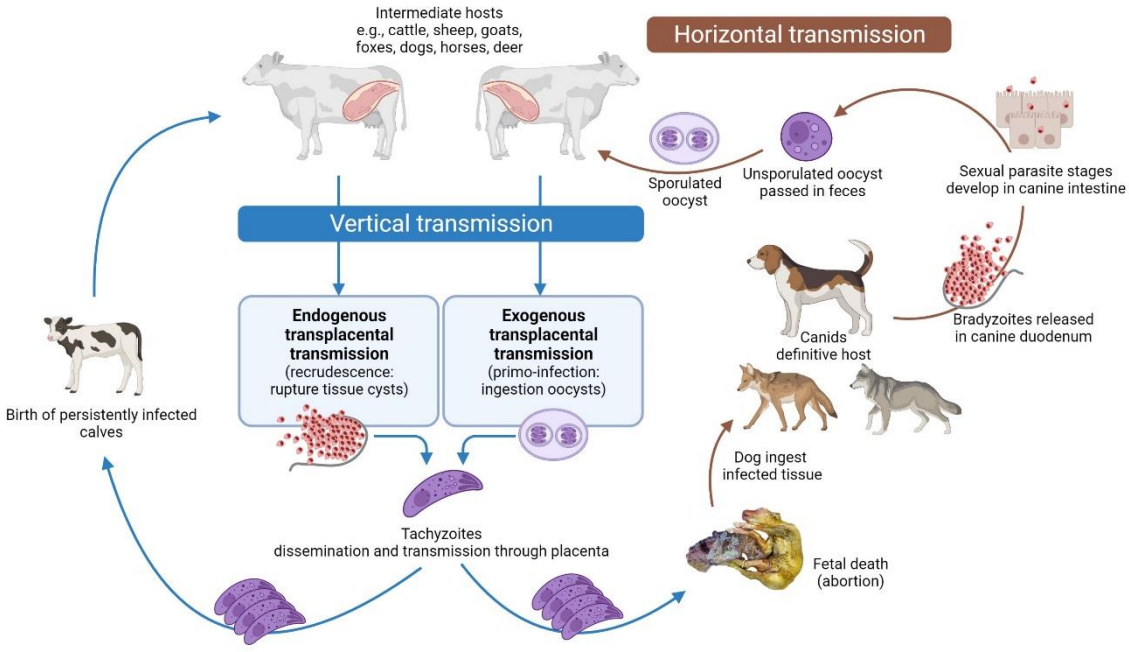

Figure 1.7. Schematic life cycle of *Neospora caninum* (adapted from Benavides et al., 2014)

Table 1.2. Common infectious causes of abortion and perinatal mortality (APM in cattle in Belgium, and corresponding clinical signs in aborting dams, main gestation length at APM, fetal/placental gross lesions, diagnostic tests available, and required test samples.

Pathogen	Clinical signs in aborting dams	Age at APM	Fetal/Placental gross lesions	Diagnostic tests available	Test samples
Viral					
Bluetongue virus	Usually no other clinical signs, but may be associated with fever, rhinitis, ulcerations and crusts in or around the muzzle, oral mucosae, and teats, salivation, epiphora, conjunctivitis, limb stiffness, coronitis	Any gestational age	Hydranencephaly, mummification, arthrogryposis	PCR Virus isolation Fetal serology	Fetal brain, spleen Fetal fluid/serum
Bovine Herpes virus type 1	Usually no other clinical signs, but may be associated with previous or intercurrent respiratory disease (high fever, drop in milk yield,	Any gestational age; mostly beyond 4 months of gestation	Fetus: none, autolyzed, foci of hepatic necrosis Placenta: possible edema	Virus isolation PCR Immunohistochemistry Fluorescent antibody test Virus isolation Histopathology	Fetal kidney, lung, liver, adrenal gland, spleen, placenta

	salivation, mucopurulent nasal discharge, coughing)			Fetal antibody titer	Fetal fluid/serum
Bovine viral diarrhea virus	Usually no other clinical signs, but any form of clinical disease is possible	Any gestational age; mostly in first half of gestation	None; mummification; fetal congenital abnormalities (cerebellar hypoplasia, hydrocephalus, ocular deformities, alopecia, thymic hypoplasia)	Virus isolation PCR Immunohistochemistry	Fetal kidney, lung, spleen, skin, placenta
				Gross pathology	Congenital deformities
				Histopathology	Fetal brain, eye, spleen, kidney, lung
				Fetal antibody titer	Fetal fluid/serum
Schmallenbergvirus	Usually no other clinical signs, but may be associated with fever, diarrhea and milk drop at the moment of initial contact	Any gestational age	None; congenital abnormalities (arthrogryposis-hydranencephalia syndrome)	Virus isolation PCR	Fetal brain, placenta, spleen
				Fetal serology	Fetal fluid/serum

Bacterial					
Anaplasma phagocytophilum	Usually no other clinical signs, but clinical disease (fever, anorexia, milk drop, coughing, limb edema, stiffness) may be seen at the moment of initial contact	Last trimester of gestation	Unknown	Giemsa-stained blood smears (morulae) PCR Serology	Maternal blood sample Fetal samples - unknown
Brucella abortus	No other clinical signs, except retained placenta	Third trimester	Placenta thickened with edema, necrosis of cotyledons and leathery. Fetuses are usually fresh, may be with bronchopneumonia	Bacterial isolation Acid-fast staining PCR Serology Histopathology	Placenta; fetal abomasal fluid, lung; uterus; milk; maternal lymph nodes Maternal serum, milk; fetal fluid/serum Placenta; major fetal organs

Campylobacter fetus subsp. *fetus*	Infertility (because of early embryonic loss), metritis	Primarily between 4-8 months of gestation	Fetus is fresh, autolyzed, or mummified. Possibly with fibrinous pleuritis, peritonitis, pericarditis, bronchopneumonia. Placenta thickened with surface exudate	Bacterial isolation Dark field microscopy Immunohistochemistry PCR Histopathology	Preputial smegma, vaginal secretions; placenta; fetal abomasal fluid, lung Placenta; fetal lung, liver
Chlamydia and *Chlamydia*-like organisms	Usually no signs; retention of the placenta	Typically in last trimester of gestation	Necrotizing placentitis, with or without vasculitis. Fetus subcutaneous edema, ascites, thymic and subcuticular petechiae, and pleuritis/peritonitis	PCR Immunohistochemistry Fluorescent antibody test Acid-fast staining	Placenta
Coxiella burnetii	Usually no signs	Typically in last trimester of gestation	Thick leathery appearance of placenta, with multifocal mineralization and pericotyledonary necrosis	PCR Immunohistochemistry Acid-fast staining	Placenta Fetal abomasal content
Leptospira interrogans serovar *hardjo*	Usually no signs, but agalactia and mastitis possible; retention of the placenta; infertility	At any stage, but mostly in last trimester of gestation	Fetus autolyzed, with no gross lesions. Avascular placenta, atonic yellow-brown	Bacterial isolation Fluorescent antibody test Immunohistochemistry	Fetal kidney, urine, aqueous humor, fluids

			cotyledons, brown gelatinous edema between allantois and amnion	PCR Serology	 Maternal serum, fetal fluid/serum
Listeria spp.	Usually no signs, but may be preceded by meningoencephalitis; metritis; retained placenta; septicemia	Mostly in last trimester, may occur in second	White foci of fetal liver necrosis, abomasal erosions, serositis, pneumonia, polyarthritis. Multifocal cotyledonary necrosis and intercotyledonary placentitis	Bacterial isolation	Fetal fluids or tissues
Trueperella pyogenes	Usually no signs, but occasionally preceded by systemic illness or mastitis; retained placenta	At any stage, but mostly in last trimester of gestation	Suppurative placentitis. Often associated with fetal bronchopneumonia	Bacterial isolation Histopathology	Placenta; fetal abomasal fluid, lung, liver
Opportunistic bacteria	Usually no signs	At any stage, but mostly in last half of gestation	Primarily placentitis and bronchopneumonia Fetus may be autolyzed	Bacterial isolation Histopathology	Placenta; fetal abomasal fluid, lung, liver

Mycotic (e.g., *Aspergillus* spp.)	Usually no clinical signs; placenta may be retained	Usually in second half of gestation	Fetus is usually fresh. Possible focal dermatitis, and bronchopneumonia. Marked placentitis with leathery scarred foci	Fungal isolation KOH wet mount Histopathology	Placental or fetal lesions; fetal abomasal fluid Skin lesions Thymus, lymph nodes, brain
Parasitic					
Neospora caninum	No clinical signs	At any stage, mostly between 5-7 months of gestation	No gross lesions Fetus may be autolyzed or mummified. Non-suppurative encephalitis and myocarditis are common	PCR Immunohistochemistry Histopathology	Fetal brain, muscle, lung, liver, kidney, placenta
Tritrichomonas fetus	Infertility (because of early embryonic loss), metritis, pyometra	Primarily in the first 5 months of gestation	Fetus is autolyzed with no gross lesions Placenta possible edematous	Protozoal isolation PCR Direct wet mount examination Histopathology	Preputial smegma, cervical mucus Placenta; fetal lung

Non-infectious causes

Non-infectious causes and risk factors of APM are diverse and their potential involvement is often overlooked, although most of the cases of perinatal mortality are associated with a non-infectious cause. More than 50% of perinatal mortality cases has been attributed to dystocia and asphyxia (Chassagne et al., 1999; Mee et al., 2021). Non-infectious causes of APM are traditionally classified into physical causes, stress, malnutrition and nutritional imbalances, environmental toxins, teratogenic compounds, hormone imbalances, and genetic causes. Physical causes of APM may be trauma, re-insemination of a pregnant animal, hyperthermia, and twinning. In dairy cattle, the twinning rate has increased alongside milk yield over the last decades, most likely because of the increased double ovulation rate in high milk yielding animals (Fricke and Wiltbank, 1999). The risk of pregnancy loss is three to nine times higher in cows carrying twins, compared to cows carrying singletons, with the highest risk for unilateral twins (Garcia-Ispierto and López-Gatius, 2019; López-Gatius et al., 2021). The mechanical stress due to a lack of space in unilateral twins may be involved in the APM process. The presence of multiple fetuses takes up more space in the uterus, which may restrict their proper growth and development. Moreover, nutritional inadequacy and hormonal imbalances may lead to inadequate fetal twin development, resulting in abortion. Other, rather uncommon physical causes of bovine APM are umbilical cord wrapping around parts of the fetus, and umbilical cord torsion. Nutritional imbalances, deficiencies, and toxicities may also play a role in causing APM (Cabell, 2007). Iodine, selenium, vitamin E, and vitamin A (or its precursor β-carotene) deficiencies have been associated with APM and weak neonatal calves (Hostetler et al., 2003; Khodakaram-Tafti and Ikede, 2005; Ensley, 2020). Cases of bovine APM due to iodine deficiency are usually seen in late gestation (Cabell, 2007). Many reproductive toxicants have been shown to be capable of causing infertility, subfertility, early embryonic death, fetal development deformities (teratogens), fetal death, abortion, and stillbirth in cattle (Baughman, 2015). These reproductive toxicants include nitrate/nitrite, several plant toxins, mycotoxins (ergot alkaloids, ergots, aflatoxins, and zearalenone), phytoestrogens, trace minerals and heavy metals (arsenic, cadmium, lead, and selenium), but also vitamin A, endocrine-disrupting compounds (pesticides, herbicides, fungicides, plasticizers, polystyrenes, polybrominated biphenyls, polychlorinated biphenyls, polychlorinated dibenzodioxins, and alkylphenolic compounds), and some pharmaceuticals (e.g., dexamethasone) (Baughman, 2015).

In the following paragraphs, we will provide a more detailed exploration of *A. phagocytophilum*, *Chlamydia* spp., *Chlamydia*-like organisms (CLO), and brucellosis. Notably, brucellosis is one of the most important infectious causes of APM in cattle on a global scale. This disease is an important zoonosis, and it is also the main driver for APM monitoring in Belgium since 1978. Furthermore, *A. phagocytophilum*, *Chlamydia* spp. and CLO are also described in literature as potential causes of bovine APM, and all of these bacteria possess zoonotic potential, making them particularly relevant subjects for further comprehensive research.

Anaplasma phagocytophilum

The bacterium

Anaplasma phagocytophilum is a tick-born organism with an important impact on both human and animal health (ruminants, dogs, cats, and horses). The organism is the causative agent of tick-borne fever (TBF) in sheep, goats and cattle. The granulocytic bacterium was described for the first time in 1940, 8 years after the disease was first recognized in Scotland (Gordon et al., 1940). This bacterium was first named *Rickettsia* (after the pathologist Howard Taylor Ricketts) *phagocytophilum*. Then, it was renamed *Cytoecetes* (for its morphological similarities to the *Cytoecetes microti*) *phagocytophilum*. Subsequently, it was included in the tribe *Ehrlichieae* of the order *Rickettsiales*, and it was named *Ehrlichia* (after Paul Ehrlich, in whose laboratory the first representatives of the genus *Ehrlichia* were discovered) *phagocytophilum*. In 2001, the granulocytic *Ehrlichieae* affecting ruminants, horses and humans were reclassified as variants of the same species, namely *Anaplasma phagocytophilum* (Woldehiwet, 2010). The new classification was based on the genetic analysis of the 16S rDNA gene, and this genetic marker has been used for detection and phylogenetic analysis (Jaarsma et al., 2019; Remesar et al., 2020; Cafiso et al., 2021). In addition, the genes groEL, ankA, major surface protein (Msp) 2 and Msp4 are frequently used for identifying *A. phagocytophilum* strains (Jahfari et al., 2014; Stigum et al., 2019).

Anaplasma phagocytophilum is a Gram-negative obligate intracellular bacterium (Woldehiwet, 2010; Adjadj et al., 2023). After infection and during the period of bacteremia, the organism is present in neutrophils, eosinophils and monocytes (Woldehiwet, 1987). Within infected granulocytes, the bacterium multiplies in intracytoplasmic vacuoles as microcolonies (morulae) (Woldehiwet, 2010). The pathogen has been found to display significant antigenic

and genetic diversities, which has important implications for disease diagnosis, treatment and prevention. Antigenic diversity refers to the variation in the surface antigens of the bacterium that can stimulate immune responses in an infected host. Several highly variable surface proteins like Msp2 and Msp4 have been detected in *A. phagocytophilum* (Lin et al., 2004; Woldehiwet, 2010). Several strains of the bacterium with different surface proteins have been detected, which enables strain-specific immune responses. Genetic diversity refers to the variability in the bacterium's DNA sequence, which has an impact on its virulence, transmission, and ability to evade host immune responses. Multiple strains with a different genetic makeup have been detected, resulting in variations in pathogenicity and host tropism (Atif, 2015; Rar et al., 2021). *Anaplasma phagocytophilum* has been classified into four distinct ecotypes present in the environment based on the groEL gene (Jahfari et al., 2014; Jaarsma et al., 2019) (**Table 1.3.**). Among these ecotypes, ecotypes III and IV exhibit specific host associations, with ecotype III linked to rodents and ecotype IV to birds. In contrast, variants I and II have a broader range of hosts: ecotype I is associated with goats, hedgehogs, roe deer, and humans, while ecotype II is linked to ruminants (Jahfari et al., 2014; Jouglin et al., 2017). Further classification of *A. phagocytophilum* strains can be achieved through the analysis of the ankA gene (**Table 1.3.**). This method allowed the identification of five distinct clusters, each correlated with specific host species (Scharf et al., 2011; Majazki et al., 2013). Cluster I encompasses strains associated with humans, companion animals, and farm animals. Cluster II and III are associated with roe deer, while cluster IV is connected to various ruminants such as sheep, European bison, and cattle. Cluster V is predominantly found in rodents (Scharf et al., 2011; Majazki et al., 2013; Jouglin et al., 2017).

Table 1.3. The relationship between *A. phagocytophilum* ecotypes (determined by the sequence of the groEL gene), clusters (identified by the sequence of the ankA gene), and their predominant hosts.

groEL gene ecotypes		**ankA gene clusters**	
Ecotype	**Hosts**	**Cluster**	**Hosts**
Ecotype I	Humans, ruminants, hedgehogs, roe deer	Cluster I	Humans, dogs, cats, horses, ruminants
Ecotype II	Ruminants	Cluster II	Roe deer
Ecotype III	Rodents	Cluster III	Roe deer
Ecotype IV	Birds	Cluster IV	Ruminants
		Cluster V	Rodents

The importance of different host species as reservoirs for various ecotypes or clusters of *A. phagocytophilum* is diverse and includes multiple key points. Genetic differences can have implications for the bacterium's virulence, host specificity, and adaptation to different environmental conditions. Different host species may harbor different ecotypes or clusters, contributing to the overall genetic diversity of the bacterium. Some ecotypes/clusters are more likely to infect and cause disease in humans than others. Understanding which host species serve as reservoirs for these zoonotic ecotypes is crucial for identifying the potential sources of human infection and for developing strategies for disease control and prevention. The choice of host species can affect the transmission dynamics of *A. phagocytophilum*. Some hosts may be more competent at maintaining and transmitting the bacterium to ticks, facilitating its life cycle. Understanding the reservoir competence of different host species is important for studying the epidemiology of the disease. Understanding the role of different host species in maintaining the bacterium can have implications for both veterinary medicine and public health, guiding strategies for disease surveillance, prevention, and control in human, livestock and companion animals.

Dugat et al. (2017) described specific genetic differences in *A. phagocytophilum* strains from cattle that had aborted compared to strains from cattle that had not aborted. Additionally, specific strains possess more zoonotic potential than others, which highlights the importance of characterization and monitoring the presence of the pathogen. However, the attribution of zoonotic potential to specific strains is not completely elucidated yet (Bianchessi et al., 2023), and subpopulations within the species are still under discussion (Stuen et al., 2013).

Transmission, hosts, reservoirs and vectors of Anaplasma phagocytophilum

Anaplasma phagocytophilum is transmitted by ticks belonging to the *Ixodes persulcatus* complex, which are mainly found in the northern hemisphere. Many species of *Ixodes* ticks (*I. scapularis*, *I. pacificus*, *I. spinipalpis*, *I. ricinus*, *I. persulcatus*, *I. ovatus*) are involved in the transmission of *A. phagocytophilum*, but in Europe, *Ixodes ricinus* (*I. ricinus*) (**Figure 1.8.**) is considered the main vector (Woldehiwet, 2010; Stuen et al., 2013), although the pathogen has been identified in other tick species too (Gandy et al., 2022).

Figure 1.8. *Ixodes ricinus*, the main vector-tick of *A. phagocytophilum* in Europe (*Source*: European Centre for Disease Prevention and Control)

Ticks acquire *A. phagocytophilum* from infected vertebrate hosts through a blood meal. The bacterium survives in the salivary glands and hindgut cells of infected ticks, which may transmit the bacterium to a new vertebrate host during subsequent blood meals (Bianchessi et al., 2023). Transstadial transmission of *A. phagocytophilum* by *I. ricinus* is demonstrated, which facilitates the pathogen persistence in the ecosystem, and reduces the dependence on the presence of suitable reservoir hosts (Hauck et al., 2020).

Anaplasma phagocytophilum has been isolated from multiple species, but fatal cases have only been reported in sheep, cattle, horses, reindeer, roe deer, moose, dogs and humans (Franzén et al., 2009; Stuen et al., 2013). Several mammals may also serve as reservoirs of infection. A reservoir host of a tick-borne disease is a host that 1) may be fed upon by infected vector ticks, 2) must take up a critical number of the infectious agent, 3) must allow the pathogen to multiply and survive for a period, and 4) must allow the pathogen to find its way into other feeding ticks (Kahl et al., 2002). *Anaplasma phagocytophilum* has been detected in wild ruminant species worldwide, such as white-tailed deer and roe deer. In Europe, roe deer are considered the major feeding reservoir hosts for adult ticks and are regarded as contributing to the increased prevalence of ticks (Medlock et al., 2013), while rodents are the major feeding hosts for larvae and nymphs (**Figure 1.9.**).

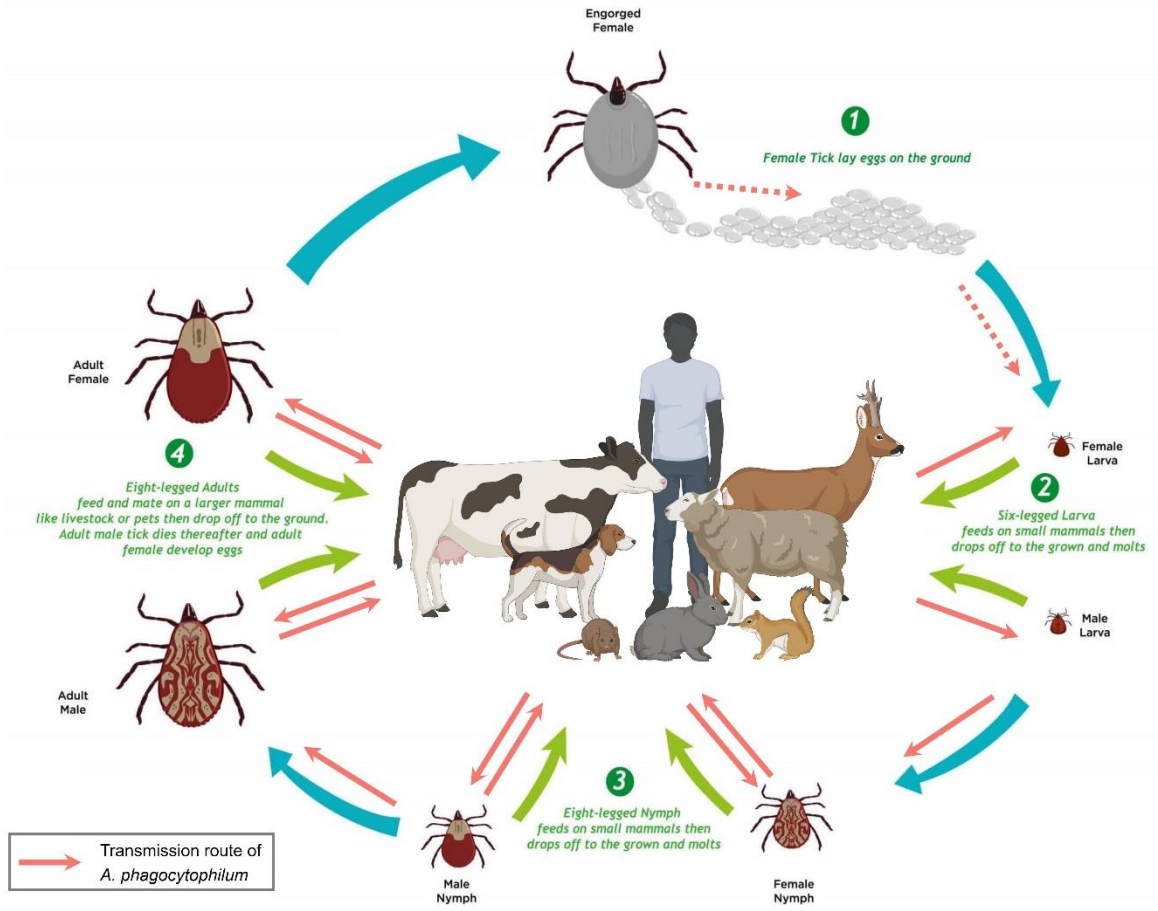

Figure 1.9. Developmental cycle of *Ixodes* tick and transmission routes of *A. phagocytophilum* (adapted from Jackson County Vector Control District).

A high density of roe deer was also positively correlated with the *A. phagocytophilum* prevalence in ticks (Rosef et al., 2009). Other European deer species (red deer, fallow deer, sika deer), small mammals such as rodents and insectivores also appear to be efficient reservoir hosts for the pathogen (Stuen et al., 2013; Dugat et al., 2014). In Europe, red deer are currently suspected of carrying strains infecting cattle (Ebani et al., 2008; Lagrée et al., 2018). Species such as wild boars, foxes, bears, birds, and reptiles have also been found to be hosts or reservoirs competent for *A. phagocytophilum* (Stuen et al., 2013). Several domestic animals (dogs, cats, horses, and ruminants) are considered potential hosts for *A. phagocytophilum* (Stuen et al., 2013). Interestingly, depending on the involved strains, *A. phagocytophilum* may persist for several months in hosts like sheep, dogs, cattle, horses, and red deer (Franzén et al., 2009; Stuen et al., 2013; Lagrée et al., 2018). Consequently, it is suggested that cattle could play the role of reservoir for strains with bovine host tropism (Lagrée et al., 2018).

Both infected ticks and hosts may spread the infection between different geographic regions (Stuen et al., 2013). Many deer carry large numbers of ticks (Vor et al., 2010), and move over

long distances, which may add to the spread of tick-borne diseases like *A. phagocytophilum* infection in Europe. Moreover, landscape changes (e.g., reforestation) and climate change may lead to an expansion of tick habitats (woodlands and forests), numbers of ticks and their corresponding wildlife hosts (Medlock et al., 2013; Stuen et al., 2013). Infected domestic animals may spread the infection when transported to other geographic areas (Stuen et al., 2013).

Clinical signs and pathogenesis of APM by Anaplasma phagocytophilum

Anaplasma phagocytophilum may cause clinical signs in multiple mammalian species, including humans. In domestic animals, *A. phagocytophilum* is the causative agent of TBF in ruminants, equine granulocytic anaplasmosis (EGA) in horses, and granulocytic anaplasmosis in dogs and cats. Tick borne fever is mainly seen in young ruminants, notably in individuals purchased from tick-free areas and introduced into tick-infested pastures for the first time (Stuen et al., 2013). Clinical signs usually occur 1-2 weeks after the start of pasturing (Pusterla et al., 1997). Cattle may become infected with variable severity of illness, including dullness, anorexia, sudden milk yield drop in dairy cattle, and respiratory distress such as coughing, polypnea, and nasal discharge (Pusterla et al., 1997). Other symptoms include stiff walking, limb edema, lymph node hypertrophy, and abortion/stillbirth in the last 2 months of gestation (Tuomi, 1967; Nieder et al., 2012; Atif, 2015). The abortifacient potential of *A. phagocytophilum* in cattle has never been experimentally proven, but several studies have reported abortions in domestic ruminants infected with the pathogen (Dugat et al., 2017). Abortion storms may occur, particularly when pregnant cows are moved to tick-infested pastures during the last months of gestation (Wilson et al., 1964).

The pathophysiology of bovine APM due to *A. phagocytophilum* remains obscure. Previous studies have suggested that the increase in body temperature, which typically lasts for approximately 7 days, may play a role (Wilson et al., 1964; Woldehiwet, 2010). However, evidence of transplacental fetal infection with *A. phagocytophilum* is reported in sheep, goats and cattle (Pusterla et al., 1997; Reppert et al., 2013; Chochlakis et al., 2020), suggesting that the pathogen is able to injure the conceptus (fetus and/or placenta). In infected ovine fetuses, histopathological examination of brain tissue has revealed leukomalacia in the cerebral and cerebellar white matter following transplacental infection (Chianini et al., 2004). Leukomalacia, as described by Loeliger et al. (2003), may be associated with ischemia and/or hypoxia in late pregnancy, which may be a result of placental dysfunction (Chianini et al., 2004). Lambs born after transplacental *A. phagocytophilum* infection exhibited lesions

primarily in the lymphoid system, characterized by splenomegaly and lymphomegaly (Reppert et al., 2013). Currently, there is limited additional information available regarding the pathophysiology of APM caused by *A. phagocytophilum* infection in cattle, and further research is warranted.

Diagnosis

The presence of high fever, sometimes in combination with other clinical signs such as respiratory signs, lower limb edema, stiff walking and a significant milk drop, in cattle that have recently been moved into tick-infested pastures, is one of the first indications of TBF (Woldehiwet, 2006; Silaghi et al., 2018). Confirmation of TBF relies on laboratorial analyses. The severe leukopenia, in particular the prolonged neutropenia, and thrombocytopenia can be used as non-specific indicators of infection (Woldehiwet, 2010). *Anaplasma phagocytophilum* is Gram-negative, but the bacteria do not stain well with Gram staining (Woldehiwet, 2010). During the acute phase of TBF, typical inclusions (morulae) may be seen in granulocytes of peripheral blood-stained smears from 5-8 days post infection (Pusterla et al., 1997; Pusterla et al., 1998). More sensitive techniques such as PCR tests were developed to detect specific nucleic acids of *A. phagocytophilum* in blood and tissue samples (Courtney et al., 2004). Furthermore, serological techniques can be used to demonstrate rising antibody titers, or to establish infection (Atif, 2015).

Treatment, prevention and control measures

Tetracyclines are currently used for treatment of clinical TBF in ruminants (Stuen and Bergström, 2001). The existing control strategies focus on minimizing tick infestations (vector control) when cattle are released into pastures. The use of synthetic pyrethroids through pour-on application is applied to reduce tick populations and minimize losses (Woldehiwet, 2006; Silaghi et al., 2018). Vector control also implies cleaning vegetation, habitat modification, and control of wild deer population (Atif, 2015). It is crucial to note that pregnant cattle should never be relocated from tick-free areas to pastures infested with ticks to prevent potential abortion outbreaks. The proposition of using long-acting oxytetracycline as a preventive measure for infection has been made (Atif, 2015). However, it is no longer advisable given current measures against antibiotic resistance.

Numerous antigens have been proposed as potential vaccine candidates. However, the primary challenge in developing an effective vaccine lies in the presence of diverse variants, the selection of appropriate conserved antigens, the lack of cross-protection studies, and the

occurrence of antigenic variation across various genotypes (Stuen et al., 2013). Up until today, there is no commercial vaccine against *A. phagocytophilum* available.

Prevalence in cattle

In recent years, increases in tick activity and incidence of tick-borne diseases have been observed in many European countries. Investigations indicate that *A. phagocytophilum* is the most widespread tick-borne infection among animals in Europe (Stuen et al., 2013). In Belgium, the first case of bovine TBF was detected in 2005 in the province of Liège (Guyot et al., 2011). A recent study revealed an overall seroprevalence in the country of *A. phagocytophilum* in cattle of 34%, with a higher seroprevalence in Wallonia (49%) compared to Flanders (24%) (Adjadj et al., 2023). Tavernier et al. (2015) also found a high *A. phagocytophilum* seroprevalence of 46% in roe deer in Flanders. Delooz et al. (2018) reported an average yearly incidence of APM cases positive for *A. phagocytophilum* ranging from 3.6 to 7.6% for the years 2014 till 2017. In that study, samples were collected during periods of high tick activity (April-December) in the southern part of Belgium (Wallonia), and analyzed by PCR on a pool of abortion samples, including placenta, and fetal spleen, kidney, and liver tissue samples. However, to the best of our knowledge, no data about the involvement and prevalence of the pathogen in bovine APM cases in the northern part of Belgium (Flanders) are available.

Zoonotic importance

In human, the incubation period for *A. phagocytophilum* is 5-21 days following the infecting tick bite (Bakken and Dumler, 2015; Hing et al., 2018). Asymptomatic infections are frequent, but when clinical signs of human granulocytic anaplasmosis are present, they may range from mild self-limiting febrile illness to fatal infections. The major clinical signs are non-specific flu-like disease with fever, headache, myalgias, and malaise (Bakken et al., 1994; Bakken and Dumler, 2015). More severe cases develop prolonged fever, septic shock-like illness, respiratory distress, acute renal failure, gastrointestinal tract bleeding, rhabdomyolysis, and secondary opportunistic infections (Bakken and Dumler, 2015). Due to the challenges and delays in diagnosing human granulocytic anaplasmosis (HGA), which stem from its non-specific clinical manifestations, non-discriminatory alterations in routine blood chemistry and hematological tests, and a lack of awareness among general practice doctors and the general public, numerous cases are likely to be undiagnosed or misdiagnosed. The strains of *A. phagocytophilum* identified in Europe seem to be more virulent for wild and farm animals,

particularly for cattle and ruminants, than for humans (Scharf et al., 2011; Myczka et al., 2021), which is different from the USA.

In Belgium, human anaplasmosis is monitored by the national reference laboratory Sciensano. The first report of *A. phagocytophilum* infections in humans in Belgium dates back to 1995 (Piérard et al., 1995). Reported cases exhibit seasonal variations, with 25% occurring in spring and 50% in summer. Nevertheless, the overall number of reported cases remains low (**Figure 1.10.**) and likely underestimates the actual incidence, as the disease is often underrecognized, or patients do not consult a doctor due to mild symptoms.

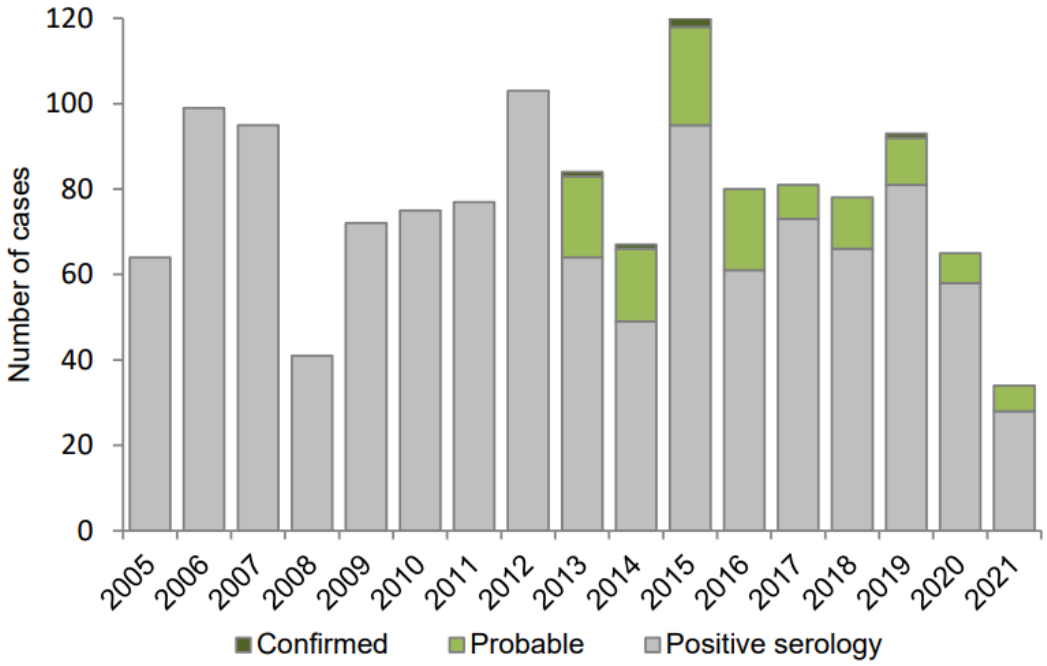

Figure 1.10. Number of reported cases of human anaplasmosis per year in Belgium between 2005 and 2021 (*Source:* Lernout et al., 2022b)

Chlamydia and Chlamydia-like organisms

The bacterium

Chlamydiae are obligate intracellular bacteria. Chlamydial organisms were first described in 1907. Initially, it was thought that these organisms were protozoa, and they were named "chlamydozoa" after the Greek word "chlamys" for mantle, because the reddish elementary bodies (EBs) appeared to be embedded in a blue matrix or mantle. Between 1929 and 1930, a pandemic of human psittacosis resulted from the shipment of exotic birds from Argentina to Europe and North America, but in that period, the causative organisms were classified as

viruses. The first infection in domestic mammals was reported in 1936 following abortions in sheep, and no earlier than 1966 *Chlamydiae* have been classified as bacteria, based on several characteristics such as its susceptibility to antibiotics (Longbottom et al., 2003). The genus *Chlamydia* within the family *Chlamydiaceae* and the order *Chlamydiales* was introduced by Page (1966). The family *Chlamydiaceae* comprises a group of obligate intracellular Gram-negative bacteria, responsible for a wide range of infections in multiple species (Longbottom et al., 2003). Since 1999, the family has undergone a number of taxonomic reclassifications at the genus and species levels based on biological differences, and on sequence analysis of the 16S and 23S rRNA genes (Sachse et al., 2015). Current classification within this family recognizes a single genus, *Chlamydia*, and 14 species, namely *Chlamydia abortus*, *Chlamydia avium*, *Chlamydia buteonis*, *Chlamydia caviae*, *Chlamydia felis*, *Chlamydia gallinacea*, *Chlamydia muridarum*, *Chlamydia pecorum*, *Chlamydia pneumoniae*, *Chlamydia poikilothermis*, *Chlamydia psittaci*, *Chlamydia serpentis*, *Chlamydia suis* and *Chlamydia trachomatis* (Sachse et al., 2015; Staub et al., 2018; Laroucau et al., 2019), plus four Candidatus species (Ca. Chlamydia ibidis, Ca. Chlamydia corallus, Ca. Chlamydia sanzinia and Ca. Chlamydia testudinis) (Vorimore et al., 2013; Taylor-Brown et al., 2016, 2017; Laroucau et al., 2020). Furthermore, within the order of *Chlamydiales*, three new families are recognized, namely *Parachlamydiaceae*, *Waddliaceae*, and *Simkaniaceae* (Everett et al., 1999).

Chlamydiae have a unique biphasic developmental cycle (**Figure 1.11.**) characterized by two distinct morphological forms, the small extracellular infectious EB (0.3 μm in diameter) and the intracellular non-infectious, metabolically active reticulate body (RB) (0.5-1.6 μm in size). The cycle starts with the attachment of EBs to eukaryotic cells, followed by internalization within tight, endocytic vesicles (inclusions), after which the EBs differentiate into larger RBs residing within the intracytoplasmic inclusions. The newly formed inclusions efficiently avoid fusion with cellular lysosomes and subsequent acidification. Reticulate bodies migrate towards the periphery of the inclusion, to start replication through binary fission (asexual reproduction by a separation of the body into two new bodies) from 8 h post infection on, followed by rapid filling and expansion of the inclusion. After 24-48 hours, depending on the species, RBs transform back into metabolically inactive, infectious EBs, which are then stored in the lumen of the inclusion. Depending on the species, EBs and some non-differentiated RBs are released upon host cell rupture (lysis or reverse endocytosis) at 24 to 72 h post infection, and go on to invade neighboring cells. The central replication phase of the cycle resembles that of other organotrophic bacteria, except that with *Chlamydiae* the growth and cell

division are restricted to an intracellular environment. The EBs demonstrate resistance to physical and chemical factors in the extracellular environment due to their rigid and osmotically stable cell envelope, as well as their reduced surface area compared to the RBs. This adaptation allows the EB to survive outside the natural host for extended periods, potentially lasting several months (Longbottom et al., 2003). The RB can also enter a state of quiescence (persistent bodies; PBs) if exposed to critical environmental conditions, e.g., the depletion of essential growth substances or the presence of antibiotics (Turin et al., 2022). Once the stress-inducing factor is removed, PBs revert to normal RBs, complete the developmental cycle and generate infectious EBs. *Chlamydiae* are capable of producing minimally or non-toxic substances and adjust their growth according to the availability of nutrients in the host's cytoplasm. These characteristics contribute to the low cytotoxicity of *Chlamydiae*. Consequently, disease typically arises when an ineffective immune response allows for persistent infection in the host (Wang et al., 2009).

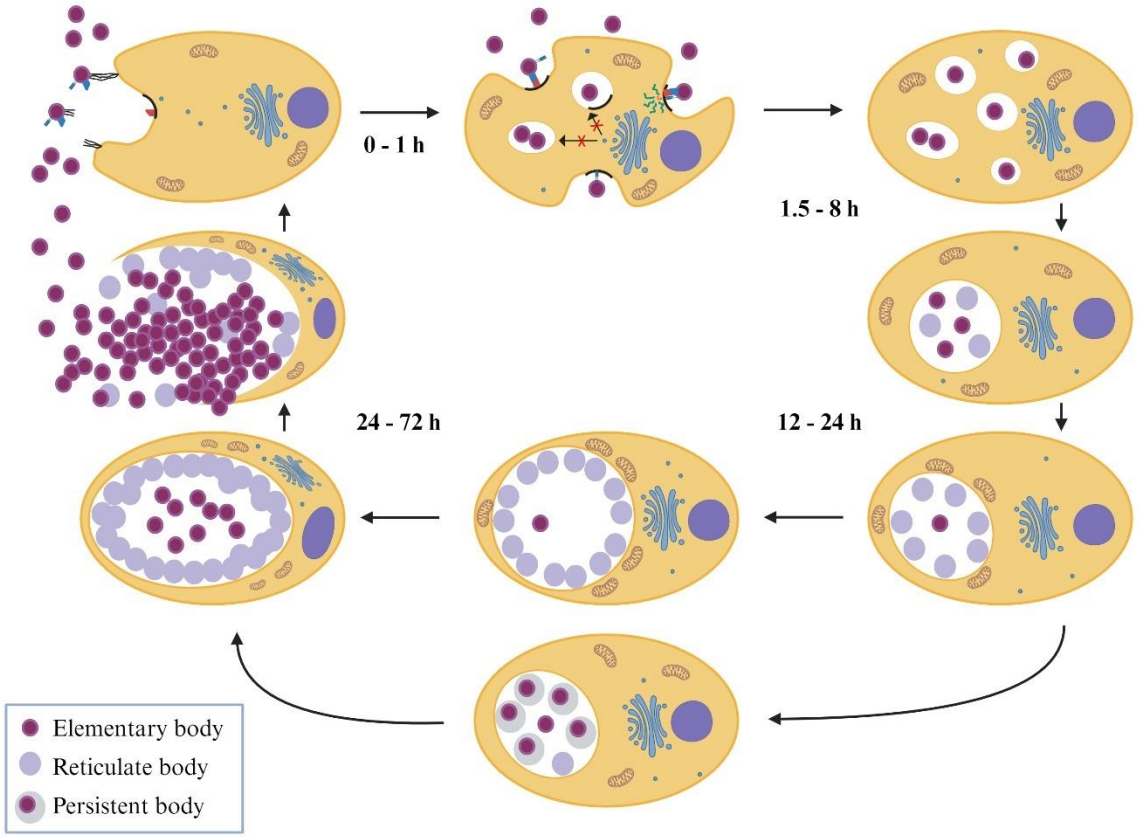

Figure 1.11. Schematic presentation of *Chlamydiae* developmental cycle (adapted from Beeckman, 2009).

Transmission, hosts, reservoirs and vectors of Chlamydiae

In *Chlamydia*-positive herds, carrier cows are the most likely source of infection for other animals (Reinhold et al., 2011). The pathogen may be detected in the respiratory, intestinal and reproductive tracts of clinically healthy animals, which suggests these organ systems are potential reservoirs of infection. The main route of infection is via oronasal transmission, after ingestion of the placenta, or contaminated bedding, or via aerosol inhalation of infectious EBs released in the environment by infected animals. In general, infectious EBs can be shed in feces, nasal, ocular or vaginal discharges, uterine fluids, urine, or semen, depending on the particular syndrome (Borel et al., 2006). Once the EBs enter the host, they colonize the tonsils and/or the nasopharyngeal lymphatic tissues, and from there disseminate to other organs via blood and lymph (Gutierrez et al., 2011; Turin et al., 2022). Additionally, *Chlamydiae* have been detected in the semen of clinically normal bulls (Kauffold et al., 2007), but also in the semen of bulls with seminal vesiculitis (Storz et al., 1968), suggesting the possibility of venereal transmission. Cows contaminated with infected semen, in particular through natural breeding, may develop genital chlamydiosis (Kemmerling et al., 2009). Furthermore, naive bulls may become infected during mating, and subsequently may function as a vector for infection (Reinhold et al., 2011).

Clinical signs and pathogenesis of APM by Chlamydiae in cattle

Many chlamydial infections in cattle are subclinical, although the potential pathogenic effects of these organisms on animal health and production are described (Reinhold et al., 2011). *Chlamydiae* have the ability to chronically infect multiple organs. This, in combination with their ubiquitous distribution, facilitates long-standing and low-level infections of the udder, and the respiratory, reproductive, and intestinal tract. Conjunctively, these capabilities may negatively affect milk production, udder health, fertility, respiratory health, and growth rate. *Chlamydiae* are also associated with other clinical syndromes like arthritis, encephalomyelitis, conjunctivitis, and hepatitis (Reinhold et al., 2011; Walker et al., 2015). Next to the potential involvement of *Chlamydiae* as primary pathogens, a possible role for these organisms as part of multi-factorial disease processes must be considered (Reinhold et al., 2011). Bovine associated chlamydial species are *C. pecorum*, *C. psittaci*, *C. abortus*, and rarely *C. suis*. *Chlamydia abortus*, *C. pecorum*, and *C. psittaci* may affect placental function in cattle, which may result in abortion, premature calving, or perinatal mortality (Cavirani et al., 2001; Wang et al., 2001; Pospischil et al., 2002; Kemmerling et al., 2009), although the exact pathophysiology is not clearly described for cattle in literature. *Chlamydia abortus* is the cause of enzootic abortion in sheep. If a pregnant ewe is infected around 5-6 weeks before lambing,

the most common outcome is a subclinical infection, resulting in clinical disease (abortion, or dead/weak lambs) during the last 2-3 weeks of gestation. If the infection takes place when the ewe is not pregnant or not within the final 5-6 weeks of gestation (Aitken and Longbottom, 2007), no clinical disease or abortion will occur. However, a latent form of infection is established, potentially within the lymphoid tissue. The infected ewe will seem healthy until the subsequent pregnancy, when abortion may happen (Brown and Entrican, 1996; Longbottom et al., 2013). The establishment of latency is believed to be connected to unfavorable conditions encountered by the bacterium during the initial infection of the host. The pathogen then remains in latent until the later stages of sheep pregnancy. At this point, the bacterium reactivates from latency (Brown and Entrican, 1996), likely due to hormonal changes related to pregnancy that disrupt the balance of the immune response. An aborting ewe infected with *C. abortus* releases numerous organisms via the uterine fluids, the aborted fetus, and the placenta, which may lead to the infection of other ewes (Shewen and Mary, 1980). The pathogen is an uncommonly diagnosed cause of abortion in cattle (Livingstone and Longbottom, 2006; Anderson, 2012). Fetal loss is more sporadic than in sheep, but abortion rates of up to 20% may occur (Livingstone and Longbottom, 2006). It is speculated that transmission in cattle follows a similar pattern, but this hypothesis has yet to be confirmed (Borel et al., 2006). Bovine abortion due to *C. abortus* occurs mainly between the 6th and 8th month of gestation (Ruhl et al., 2009). *Chlamydiae* multiply primarily in the cotyledons, causing severe inflammation and necrosis, resulting in a reduction of the efficiency of the fetal-maternal exchanges, which may lead to APM (Ruhl et al., 2009; Sammin et al., 2009). Purulent and/or necrotizing placentitis with or without vasculitis is the most consistent pathological lesion in bovine abortions associated with *Chlamydiae* (Borel et al., 2006). Placental tissues have leathery, reddish, opaque intercotyledonary patches, and multifocal cotyledonary necrosis. Fetuses may have pink/red subcutaneous edema, ascites, thymic and subcuticular petechiae, and serofibrinous pleuritis/peritonitis (Anderson, 2004).

Chlamydia-like organisms have also been described as being involved in bovine abortions (Ruhl et al., 2009; Wheelhouse et al., 2010, 2012). Amongst these *Chlamydia*-like organisms (CLO), *Waddlia chondrophila*, *Parachlamydia acanthamoebae*, *Rhabdochlamydia* and *Neochlamydia* species were detected in fetal and placental tissue (Dilbeck et al., 1990; Wheelhouse et al., 2010). *Parachlamydia* and *Waddlia* associated abortions also exhibit necrotizing placentitis, but vasculitis is rather uncommon (Blumer et al., 2011). However, CLO are also detected in nasal and rectal samples of healthy cattle (Wheelhouse et al., 2022), which

makes it unclear whether these CLO possess abortifacient potential, and whether they should be classified as primary or secondary causes of APM.

The relevant *Chlamydiae* and CLO in cattle and their clinical significance are shown in **Table 1.4**.

Table 1.4. *Chlamydiae* and *Chlamydia*-like organisms in cattle and their clinical significance

Species	Hosts	Clinical significance in cattle
Chlamydia abortus	Ruminants, swine, horses, birds, human	Reproductive disorders, (sub)clinical mastitis, chronic pulmonary inflammation, growth retardation
Chlamydia pecorum	Ruminants, other mammals, human	Polyarthritis, enteritis, pneumonia, keratoconjunctivitis, urogenital infections, encephalomyelitis, chronic pulmonary inflammation, growth retardation
Chlamydia psittaci	Birds, poultry, cattle, swine, horses, dogs, rats	Reproductive disorders, airway inflammation
Chlamydia suis	Swine, cattle (rarely), goats, sheep, horses	No reports
Waddlia chondrophila	Cattle, human	Reproductive disorders?
Parachlamydia acanthamoebae	Human, cattle	Reproductive disorders?

Diagnosis and treatment

The diagnosis of *Chlamydia* spp. or CLO infections in cattle necessitates laboratory tests since their signs are not specific. Serological diagnosis, which relies on detecting antibody responses against the MOMP peptide, is generally suitable for identifying recent infections due to the robust antibody responses typically induced by most chlamydial infections, in particular in chlamydial abortion cases in ruminants (Reinhold et al., 2011). Serology of a single blood sample is of questionable worth, although a rise in titer is suggestive of significant infection as delivery of an infected fetus leads to a rise in antibodies two to three weeks post parturition (Yaeger and Holler, 2007). Presence of *Chlamydia*-specific antibodies in fetal serum or fluids is considered as confirmative. As culturing *Chlamydia* spp. and CLO can be challenging, more sensitive techniques such as PCR tests have been developed (Kaltenboeck et al., 2005; Ruhl et

al., 2009; Pantchev et al., 2010). Nowadays, real-time PCR (qPCR) is available for routine diagnostic use, with target genes such as ompA or 23S rRNA, although the ompA PCR may have lower sensitivity. Commercial PCR test kits (real-time PCR) are available, suitable for use in ruminants, including cattle. Specifically, commercial kits for *C. pecorum* and *C. abortus* are available. However, there are currently no commercial kits for ruminants for *C. psittaci* and *Chlamydia*-like organisms. However, as already mentioned, infections with *Chlamydia* spp. and CLO can often be subclinical (Kemmerling et al., 2009; Reinhold et al., 2011; Turin et al., 2022), which makes it challenging to establish causality solely based on the detection of the organisms. Therefore, histopathology or immunohistochemistry are vital diagnostic tools that may help to establish a definitive diagnosis (Borel et al., 2006; Ruhl et al., 2009). To diagnose chlamydial abortion, examination of the placenta is essential, because the organism multiplies in cotyledons (Borel et al., 2006). The presence of placentitis with identification of the organisms within lesions by immunohistochemistry as well as PCR is diagnostic (Baumgartner, 2015). Placental smears stained with Giemsa, Gimenez, or modified Ziehl-Nielsen acid-fast methods are useful for diagnosis. Staining methods exhibit low sensitivity and numerous cross-reactions with other acid-fast bacteria (e.g., *Coxiella*, *Brucella*).

Growing evidence indicates the presence of tetracycline resistance in *C. suis* in pigs, likely linked to feed supplemented with tetracyclines, such as doxycycline. Additionally, resistance to sulfadiazine has been observed in this context. However, there is limited information on antibiotic resistance in *C. abortus* or *C. psittaci*, and no resistance has been reported in cattle or other species for *C. pecorum*. Most *Chlamydia*-like organisms (CLOs) exhibit resistance to quinolones and β-lactams, with *Parachlamydia* and *Neochlamydia* spp. also displaying phenotypic resistance to amoxicillin, ceftriaxone, and imipenem. Beyond antibiotic resistance, the phenomenon of antibiotic-induced persistence poses an additional challenge. The confirmation of infection and antibiotic resistance in laboratories is a complex issue. PCR is commonly used, but routine antibiotic resistance determination is not part of standard procedures (Bommana and Polkinghorne, 2019).

Prevalence and risk factors in cattle

In ruminants, infections with *C. abortus*, *C. pecorum*, *C. psittaci* and rarely *C. suis* have been described (Reinhold et al., 2011). In cattle, *C. psittaci* is reported as the most prevalent representative of *Chlamydia* spp., followed by *C. abortus* and *C. pecorum*. A higher prevalence of *Chlamydiae* was observed in herds with poor hygiene and nutritional management, which indicates that appropriate herd management can reduce the risk of infection (Kemmerling et al.,

2009; Reinhold et al., 2011). Other main risk factors for *Chlamydia* spp. infections in dairy cattle include the introduction of animals from outside sources, the use of a breeding bull, and the absence of separate calving pens (Kemmerling et al., 2009).

In the USA, Asia, Africa and Europe, herd-level prevalence of *Chlamydiae* ranges from 45 to 100%, depending on the selection of the herds (randomly or pre-selected) (Barkallah et al., 2018; Reinhold et al., 2011). A German study identified *Chlamydia* spp. by PCR in 61% of randomly selected dairy farms, equating to a 13.5% seroprevalence at the animal level (Kemmerling et al., 2009). In Belgium, epidemiological data on *Chlamydia* spp. and CLO in cattle are scant. In an epidemiological study, a *C. abortus* cow-level seroprevalence of 1.7% and a herd-level seroprevalence of 14.7% were reported (Yin et al., 2014). One case report described the detection of *C. psittaci* in Belgian dairy cattle with signs of respiratory disease and milk drop syndrome (Van Loo et al., 2014). However, no other data are available about the presence of *Chlamydia* spp. and CLO in the Belgian bovine population.

Zoonotic importance

Both *Chlamydia* spp. and CLO may have zoonotic implications and have been isolated from human cases of respiratory disease, as well as from fetal loss or premature delivery (Longbottom et al., 2003; Baud et al., 2008). From the *Chlamydia* spp. found in cattle, mainly *C. psittaci* and *C. abortus* are involved in human chlamydial infections. *Chlamydia psittaci* mainly affects birds but it may also cause human psittacosis when contaminated aerosols from infected birds are inhaled. The pathogen can be found in several tissues including blood and fecal material of infected birds. Consequently, individuals having contact with birds are more at risk to become infected with *C. psittaci* (Dai et al., 2022). In contrast to *C. psittaci* infections, transmission of *C. abortus* from livestock to men is relatively rare, and mainly related to direct exposure to infected small ruminants, especially during lambing season through contact with infected sheep or goats, or secondary contaminated environments (Longbottom et al., 2003; Turin et al., 2022). For *Parachlamydia* spp., interaction with farm animals is described as a risk factor for seropositivity in human, and the pathogen may be involved in fetal loss in pregnant women (Baud et al., 2009; Ammerdorffer et al., 2017).

In Belgium, human psittacosis is monitored by the national reference laboratory Sciensano. The number of reported cases of human psittacosis shows annual fluctuations but remains generally low and likely underestimates the actual incidence, as the disease is often not recognized, or patients do not consult a doctor due to mild symptoms, although there is an

upward trend in reported cases (**Figure 1.12.**). Data on prevalence of CLO in humans in Belgium is not available.

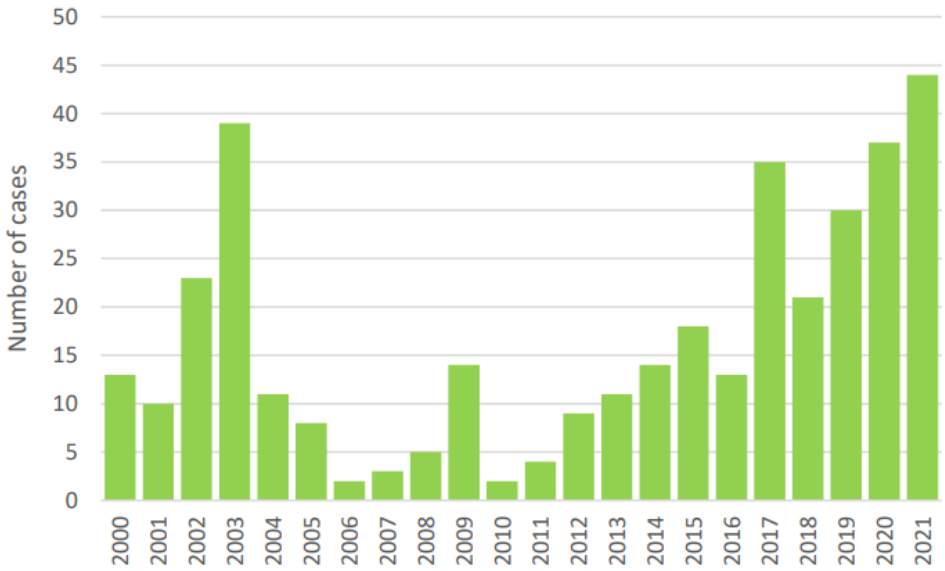

Figure 1.12. Number of reported cases of human psittacosis per year in Belgium between 2000 and 2021 (*Source:* Lernout et al., 2022)

Bovine brucellosis

The disease

Brucella spp. are the etiologic agents of brucellosis. These Gram-negative, facultative intracellular bacteria are able to infect a wide range of mammalian species, including humans. According to the World Health Organization, brucellosis is the world's most widespread zoonosis. Humans might get infected mainly by ingestion (e.g., raw or unpasteurized milk or dairy products), inhalation, contact with mucosae, or puncture wounds (Franco et al., 2007; Dhanashekar et al., 2012). The most common symptoms of human brucellosis are flue like (fever, anorexia, fatigue, arthralgia, headaches, myalgia, etc.) and may remain for several months. Some cases may result in more severe syndromes like hepatitis, encephalitis, meningitis, endocarditis, osteomyelitis, bursitis, tenosynovitis and/or arthritis. In men, infertility may be observed due to orchitis, epididymitis and prostatitis, while abortion during first and second trimester of pregnancy is reported in women, although rather uncommon (Khurana et al., 2021). The transmission cycle of *Brucella abortus* (*B. abortus*) is depicted in **Figure 1.13**.

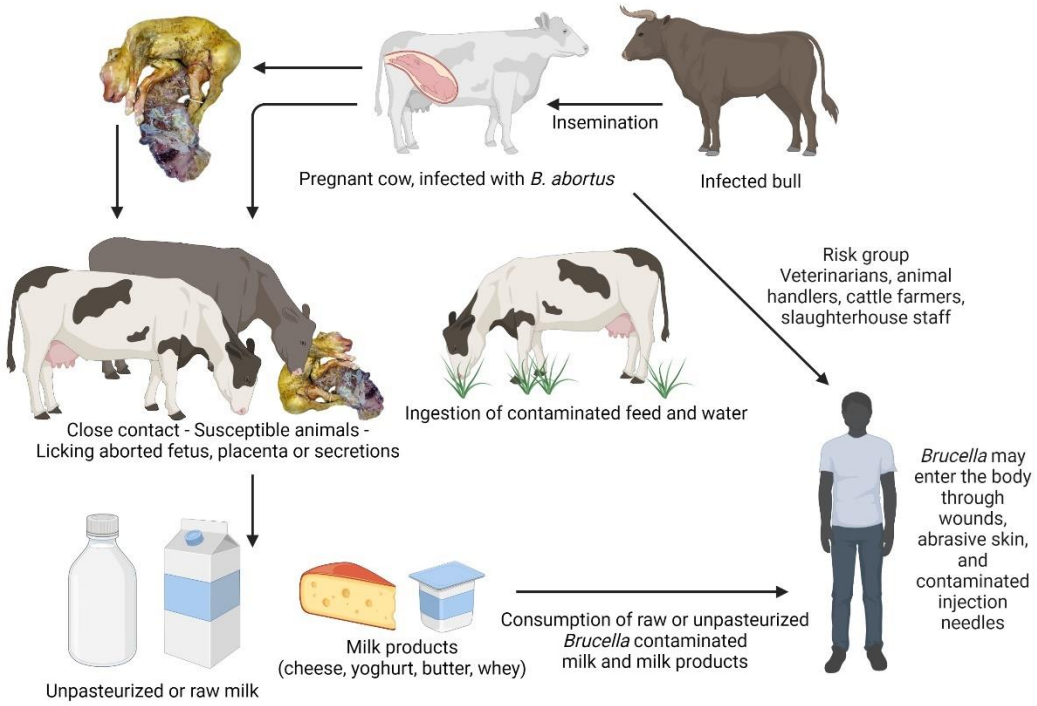

Figure 1.13. Transmission cycle of *Brucella abortus*. (Adapted from Khurana et al., 2021)

Twelve named *Brucella* spp., and four unnamed isolates are known (Hull and Schumaker, 2018). However, in cattle, brucellosis is caused by *B. abortus*, and occasionally by *B. melitensis* (Álvarez et al., 2011) or *B. suis* (Szulowski et al., 2013). *Brucella* bacteria are highly resistant, and may survive for several months in the environment, especially under favourable conditions like high humidity and cold weather (Khurana et al., 2021). Infection and transmission of *Brucella* spp. is possible via horizontal (through ingestion, inhalation, via conjunctiva, or through skin lesions) or vertical route (from dam to offspring) (Hull and Schumaker, 2018; Khurana et al., 2021). Cattle mainly get infected by contact with fetal and placental tissues and associated fluids expelled after calving or APM events. Newborn calves may also get infected by ingestion of raw milk from infected cows. Infected bulls are potential sources of infection by natural service or artificial insemination (Givens, 2018). The clinical signs in cattle are mainly related to the reproductive tract of both male and female animals. In female cattle, the disease is manifested by late term abortions, birth of weak calves, placental retention, reduced fertility, endometritis, and decreased milk yield. In contrast to human brucellosis, abortion is the hallmark of infection in cattle (Kiros et al., 2016). This difference between human and bovine brucellosis is the result of the presence of anti-*Brucella* activity in human amniotic fluid, and the absence of erythritol production in human placenta (Jankowski et al., 1977). Erythritol

is a carbohydrate which plays a significant role in the clinical presentation of brucellosis in ruminants, and also in pigs. *Brucella* spp. use erythritol as a growth-stimulating factor; they prefer erythritol as a carbon and energy source over glucose (Samartino and Enright, 1996). From the fifth month of gestation, bovine placenta contains high concentrations of erythritol, which has been suggested as the basis for the predilection of *B. abortus* for the pregnant bovine uterus (Anderson and Smith, 1965). Initially after infection, *B. abortus* is located in lymphoid tissue, but during pregnancy, when erythritol is released from the placenta, the bacterium is translocated to reproductive tissues. This translocation results in invasion of the chorioallantoic villi, extending into the cotyledons on the fetal side of the placenta. At this level, the bacterium replicates, induces infiltration of inflammatory cells and necrosis of trophoblasts, and leads to vasculitis and ulceration. Finally, this placentitis causes a compromised fetal-maternal metabolism, resulting in fetal loss (Neta et al., 2010). Abortion rates after a *B. abortus* infection may be high in naïve cattle herds, and may vary from 30 to 80% (Kiros et al., 2016). The mammary gland and associated lymph nodes may also be infected, and the organism may be excreted in colostrum and milk. In bulls, orchitis is the most significant lesion, often associated with seminal vesiculitis and epididymitis, frequently followed by a permanent infertility (Khurana et al., 2021).

Diagnosis

To diagnose brucellosis, laboratory confirmation is necessary. Laboratory tests include direct diagnostic tests to detect the pathogen or serological assays (WOAH, 2022). A presumptive diagnosis of brucellosis is possible by detection of *Brucella*-like acid-fast organisms using modified acid-fast staining (Khurana et al., 2021). Since staining methods have a low sensitivity, and other acid-fast abortifacient agents (e.g., *Chlamydia* spp., and *C. burnetii*) may be difficult to differentiate from *Brucella* organisms, isolation of *Brucella* spp. should be performed to confirm the disease, and to determine the *Brucella* species involved. Bacteriological analysis following identification of suspicious colonies still remains the gold standard diagnostic technique (Khurana et al., 2021). *Brucella* organisms need special media with carboxyphilic environment, and are relatively slow growing (Kiros et al., 2016), which results in a long time period (usually two weeks) before definitive identification (Khurana et al., 2021). Polymerase chain reaction methods are also available for the detection of *Brucella* specific DNA. However, in countries where vaccination against *B. abortus* is allowed, PCR-based methods have been reported to fail to distinguish the field strain from the vaccine strain (Khurana et al., 2021). Required samples to detect the pathogen are uterine or vaginal discharge,

udder secretions or, post-mortem, spleen, lymph nodes (iliac, mammary, and prefemoral) and male or female reproductive organs. The most reliable sources for isolating the pathogen from infected animals are uterine discharges, placental tissue, and aborted fetuses. When collecting samples from aborted fetuses, the preferred specimens include abomasal contents, spleen, liver, lungs, and lymph nodes (Yagupsky, 1999).

Serological tests play a crucial role in worldwide monitoring, surveillance, control, and eradication programs of bovine brucellosis. Following infection with *Brucella* spp., antibodies typically begin to appear in the blood after approximately one week. The initial antibodies detected are immunoglobulin (Ig) M, followed by the subsequent appearance of IgG. Various serological tests, including rose bengal plate test (RBT), serum agglutination test (SAT), immune capture agglutination, complement fixation test (CFT), milk ring test, Coombs test, enzyme linked immunosorbent assay (ELISA), and lateral flow assay, are frequently utilized to diagnose brucellosis (Khurana et al., 2021). In monitoring programs, the use of SAT and indirect ELISA in parallel or in series is often applied. The SAT is the most popular diagnostic tool used worldwide, due to its simplicity and low cost. One of the limitations of the test is the possible cross-reaction with IgM against some other bacteria, e.g., *Escherichia coli*, *Salmonella* spp., *Francisella tularensis* and *Yersinia enterocolitica* (Khurana et al., 2021).

Treatment and prevention

The efficacy of antibiotic treatment for brucellosis is frequently compromised by the intracellular survival of *Brucella* bacteria and their ability to adept within macrophages (Seleem et al., 2010). In Belgium, as in most European countries, *Brucella* infected animals are mandatory culled, making treatment an impractical choice.

Effectively managing brucellosis is dependent on implementing a range of strategies. These include robust surveillance mechanisms to identify and cull infected animals, prevention of spread from infected animals and herds to non-infected herds, eliminating sources of infection, and taking preventive measures to prevent disease reintroduction. Although not allowed in Belgium, vaccination of susceptible animal hosts may be part of an eradication program, especially in endemic regions (Beauvais et al., 2016). Common *Brucella* strains such as 19, RB51 and Rev1 are widely used as vaccines against both infection and associated health issues in livestock (Khurana et al., 2021). For large-scale vaccination, RB51 is the only available option due to its minimal interference with standard serological tests (RBT and/or CFT). The RB51 vaccine has been used in cattle with success within some EU regions. This vaccine holds potential as a tool for eradicating bovine brucellosis from well-controlled epidemiological units,

provided that it is applied massively and consistent over a sufficient time period, in conjunction with rigorous test-and-cull strategies.

Prevalence of brucellosis in the European Union and Belgium

Brucellosis is a priority disease for many European member states, and it is listed as a category B disease in the European Union (EU), which means that the disease must be controlled in all member states with the goal of eradicating it. Even though many countries have developed strategies to eradicate brucellosis in ruminants, and to prevent human infection, brucellosis is still present in some European countries (WOAH, 2022). However, many years of intensive testing to detect infected animals, followed by their removal from affected herds, animal movement restrictions to prevent disease spread, vaccination to protect against the disease, disinfection to eliminate the pathogen from the environment, etc. contributed to the eradication of *Brucella* in cattle, sheep, and goat herds in major parts of the EU. In **Figure 1.14.**, the actual situation on bovine brucellosis in the EU is depicted.

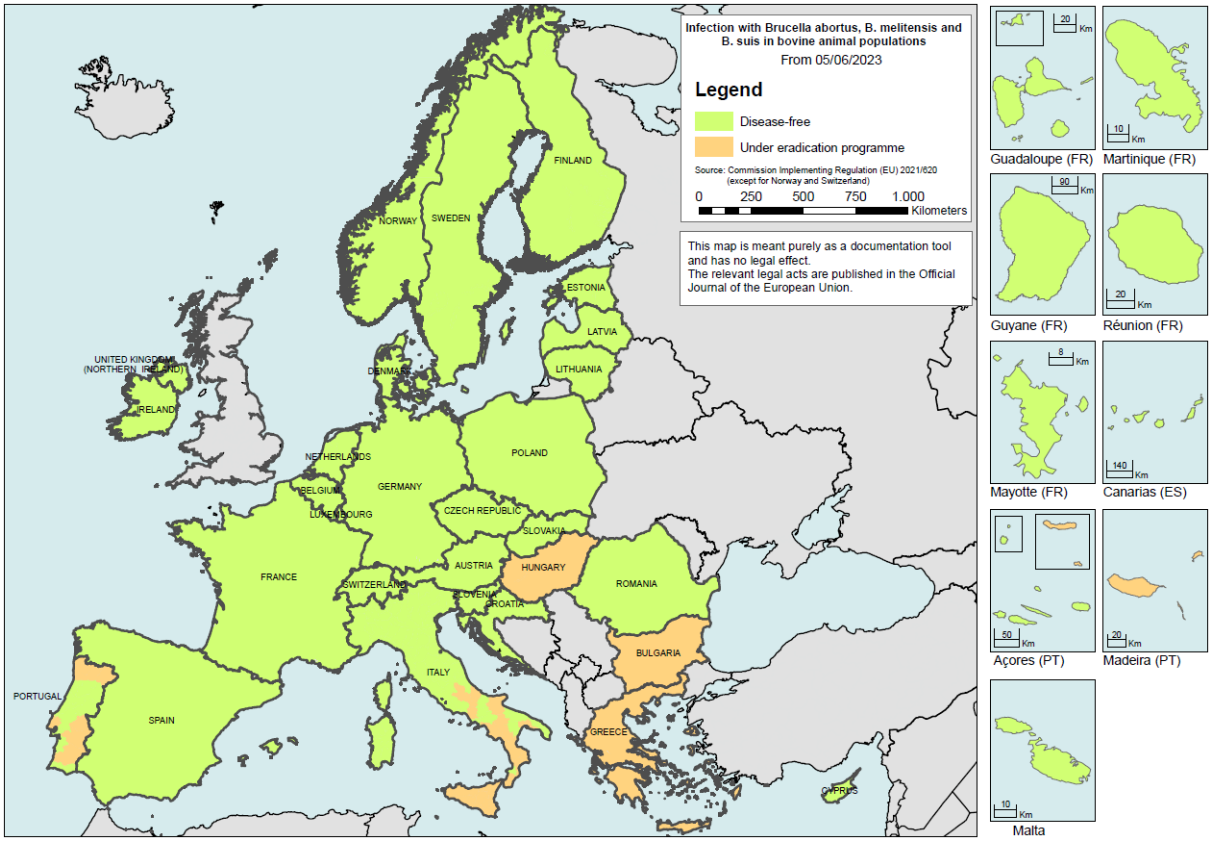

Figure 1.14. Map of countries/areas with *Brucella* status within the European Union. The map (except as regards Iceland, Norway and Switzerland) is based on Annexes I to VII to Commission Implementing Regulation (EU) 2021/620 laying down lists of Member States and zones thereof which have *Brucella* free status or approved eradication programmes for *Brucella*.

Belgium was highly affected by brucellosis until the late 1980s. However, the prevalence of bovine brucellosis in Belgium decreased over the years, to the point that the last diagnosed case before obtaining the official brucellosis-free status was reported in 2000. In 2003, the European Commission declared Belgium officially free from brucellosis (COMMISSION DECISION of 23 June 2003 establishing the official tuberculosis, brucellosis, and enzootic-bovine-leukosis-free status of certain Member States and regions of Member States as regards bovine herds, 2003). The "officially free" status allows for the unrestricted trade of animals between EU countries and other third countries in international markets. Nevertheless, between 2010 and 2013 a re-emergence of *Brucella* outbreaks was detected in the country. In late November 2010, a brucellosis outbreak was identified in the province of Liège, after detection of *B. abortus* in an aborted fetus that was analyzed within the mandatory abortion monitoring program. Despite this relapse case, the official brucellosis-free status was maintained after Belgium implemented measures in response to the outbreak. The origin of this outbreak has not yet been determined, despite extensive epidemiological investigations. A second outbreak was detected in March 2012 in the province of Namur, also after the analysis of an aborted fetus. During the next month, brucellosis was confirmed in 3 additional farms. All these farms were already placed under surveillance because of contact with the farm where the second outbreak had taken place. In April 2012, the Federal Agency for the Safety of the Food Chain (FASFC) conducted a systematic brucellosis screening on bulk tank milk samples from 9,013 dairy farms in the country. As a result of this screening, one herd in the province of Namur was found positive for *Brucella suis* biovar 2, which is rather uncommon in cattle (Fretin et al., 2013). Since this last outbreak, no other *Brucella* infections were detected in Belgium.

European and Belgian legislation on brucellosis

Brucellosis is a regulated, notifiable disease and is listed in the World Organisation of Animal Health (WOAH) Terrestrial Animal Health Code. The EU imposes a compulsory surveillance program in order to eradicate the disease, with the objective to obtain and maintain the official freedom of disease status in each member state, but also to allow trade of animals (2016/429, 2016). In the past, like in many other countries, the Belgian cattle industry suffered from the consequences of this disease. The initial Belgian brucellosis surveillance program consisted of active and passive surveillance components. This surveillance program was installed since 1978 onwards and laid down in a royal decree. The active component of this surveillance program consisted of antibody detection in serum and milk samples. The serological screening on blood samples was performed on imported cattle from both officially

free and non-officially free countries. Also, cattle traded in Belgium were analysed via serum sampling, and serology was performed on a serum sample of 33% of all Belgian beef cattle farms during winter, ensuring that the whole beef population was tested every 3 years. Additionally, all imported female cattle older than 24 months of age and originating from non-officially free countries were serologically tested during winter for three consecutive years after introduction in the herd. On dairy herds, analysis of bulk tank milk was performed at least 4 times a year with an ELISA milk ring test. The passive component of the brucellosis surveillance program consisted of clinical surveillance and reporting of cases of APM by farmers to their corresponding accredited regional sanitary veterinarian, who is responsible for the epidemiological surveillance of the herd. This veterinarian was responsible for collecting a blood sample from the dam. This blood sample was sent to the accredited regional laboratory along with the associated fetus and/or placenta sample for *Brucella* specific analyses (serology on the maternal blood sample, and detection of *Brucella* spp. via staining and culture). Inclusion criteria for this abortion monitoring program were abortions (gestation length of 42-260 days) and perinatal mortalities of (pre)mature calves following a gestation length of more than 260 days, that died within 48h after birth. As already mentioned, Belgium obtained the officially free status for bovine brucellosis in 2003. As determined by council directive 64/432/EC, EU member states with an officially free status for bovine brucellosis for 5 consecutive years are allowed to re-evaluate the national surveillance for the disease in relation to frequency of sampling and sample size. As a result, since October 2009, serological analysis of cattle imported from officially free EU member states to Belgium was excluded, as well the serology of cattle traded in the country. On dairy herds, bulk tank milk analysis was reduced to twice a year. The main pillar of this new brucellosis surveillance program was the obligatory notification and testing of each case of APM. Additionally, a random selection of a number of herds where no APM was reported over the last 3 years, and where reproductive females are present, but yet are suspected to have had an abortion (based on previous annual birth rates, submission of low weight carcasses sent to rendering plant, as well as diagnostics lab results) was executed. On these herds, a maximum of 20 female animals older than 24 months are randomly selected for serological analysis. Blood samples are serially tested for brucellosis by SAT and an indirect ELISA test by the regional laboratories. In case of a positive test result, a concurrent ELISA confirmation test is performed at the national reference laboratory (Sciensano). In case of a positive ELISA confirmation test, the animal is suspected of being infected with *Brucella*. Cattle with a serological or epidemiological suspicion are mandatory

culled, and bacteriological examination for *Brucella* spp. is performed. When *Brucella* has been isolated, the affected animal is classified as brucellosis infected.

Diagnosing bovine abortion and perinatal mortality

Investigating cases of APM can be used to identify pathogens that entered or that are circulating in the herd, and, consequently, to put in place the appropriate measures to prevent further spread. Additionally, monitoring APM cases is important for the surveillance of specific (zoonotic) diseases. Unfortunately, the diagnostic rate of bovine APM cases is generally low, ranging from 23 to 47% (Kirkbride, 1993; Jamaluddin et al., 1996; Khodakaram-Tafti and Ikede, 2005; Murray and Patch, 2011; Wolf-Jäckel et al., 2020), depending on the capabilities and experience of the diagnostician, and the available laboratory support. Another limitation while studying the potentially underlying cause of infectious APM, is the time-lag between the moment of infection, the moment of fetal death, and the moment the abortion/calf death took place, which may occur days or even weeks later (Kennedy and Richards, 1964; Daniel L. Grooms and Bolin, 2005; Dubey et al., 2006). Therefore, at the moment of the laboratory analysis, the agent responsible for the death of the fetus or calf may no longer be present (McClurkin et al., 1984; Baumgartner, 2015). Another plausible reason for non-diagnosis is an eventually poor quality of the sample(s) due to autolysis, mummification, or maceration. Autolysis occurs rapidly, prior to fetal expulsion, and can have a significant deleterious effect on the sensitivity of diagnostic tests (Cabell, 2007). Furthermore, tissues from many APM cases exhibit little if any recognizable changes of significance. This may be the result of intrauterine autolysis, masking subtle lesions. Besides, the relative ease and rapidity by which fetuses die, allows only a brief window for development of gross lesions. Furthermore, there is often only a rudimentary inflammatory response from the fetus to an infection or injury (Baumgartner, 2015).

According to Borel et al. (2014) and Mee et al. (2021), a stepwise investigative approach to diagnose bovine APM at the farm and at diagnostic laboratories is recommended to optimize the diagnostic rate. Unfortunately, the focus in this approach is mainly on diagnosing infectious causes only, which implies that most of the non-infectious causes of APM are missing.

1. Start by the collection of pertinent case history information, such as differentiating between early and late-term abortions, distinguishing between mummified and fresh fetuses, determining the frequency of abortions relative to the number of pregnant

animals, noting the time interval from the first abortion to the first case submission along with the corresponding number of abortions, documenting any clinical signs in the dams, and considering any changes in husbandry and management practices. Collection of herd health history data such as details of most recent vaccinations against (abortifacient) agents, recent cattle purchases, introduction of heifers that were raised on contract-rearing farms, and bulk milk test results is essential.

2. Collect blood samples from affected and unaffected animals from the same group for serological analysis. Differences in exposure (presence/absence, prevalence) between affected and unaffected dams may be determined by sero-diagnosis. This step is particularly valuable in diagnosing non-endemic infections and/or identifying infections against which the animals have not been vaccinated.
3. Perform a clinical examination of the dam(s) that aborted. A blood sample of the dam may be useful as a proxy sample for fetal material. However, the results of single blood samples from aborted cows are of very limited diagnostic value and must be interpreted in context with management data of the corresponding herd (Taylor and Njaa, 2012; Clothier and Anderson, 2016). For some diseases, e.g., *N. caninum*, a maternal seronegative result may be used to rule out the pathogen as the cause of APM (Pereira-Bueno et al., 2000; Mee, 2020). A seropositive result for an infectious abortifacient agent indicates recent exposure that may or may not be associated with the abortion, an endemic condition in the population, or previous vaccination (Taylor and Njaa, 2012; Clothier and Anderson, 2016). Paired acute (at the time of abortion) and convalescent (2 weeks after abortion) serum samples may demonstrate an increasing titer to a particular pathogen and may provide stronger evidence of association (Anderson, 2007; Clothier and Anderson, 2016) for some infectious causes of abortion (e.g., *Salmonella* Dublin (Sánchez-Miguel et al., 2018)). However, for most of the abortifacient pathogens, maternal seroconversion may have occurred already before the abortion due to the lag phase between infection and the expulsion of the dead fetus (Taylor and Njaa, 2012).
4. Conduct a macroscopic examination of the placenta and fetus. External examination of the fetus by measuring crown-rump length (CRL) and body weighing may detect fetal undersize, which may be a possible indicator of fetal infection or/and shortened gestation (Jawor et al., 2017). Several external fetal characteristics can be used to estimate fetal age (**Table 1.5.**), although some are less useful due to their gradual

appearance and subjectivity in assessment. However, measuring fetal CRL is the most useful method to estimate fetal age using formulae, e.g., gestational age (GA) in days = 68 + 2.25 × CRL (in cm), or GA = (CRL + 21) × 2.5, or GA = 53 + (2.3 × CRL). More complex multi-variable formulae are available (Krog et al., 2018), and also measuring of tibial length by radiology can be used (GA = 114 + (0.91 × tibial length (in mm) (Murray, 2015).

Tabel 1.5. Gestational age estimates of bovine fetuses.

Age (months)	**Relative size**	**Crown-rump length (cm)**	**Weight**	**External characteristics**
2	Mouse	6-8	8-30 g	Claw buds and scrotum present
3	Rat	13-17	200-400 g	Hair on lips, chin, and eyelids
4	Small cat	22-32	1-2 kg	Fine hair on eyebrows, claws developed
5	Large cat	30-45	3-4 kg	Hair on eyebrows and lips, testes in scrotum, teats developing
6	Small dog (beagle)	40-60	5-10 kg	Hair on inside of ear and around horn pits, tip of tail, and muzzle
7	Dog	55-75	8-18 kg	Hair of metatarsal, metacarpal, and phalangeal region of extremities and beginning on back, long hair on tail tip
8	Large dog	60-85	15-25 kg	Fine short hair all over body, incisor teeth not erupted

Source: Taylor and Njaa (2012)

During external examination of the fetus, common lesions like pneumonia, pleuritis (**Figure 1.15.**), peritonitis, pericarditis (**Figure 1.16.**), meningitis, hypopyon, intestinal rupture, hemorrhages and sepsis may be observed (Mee, 2020).

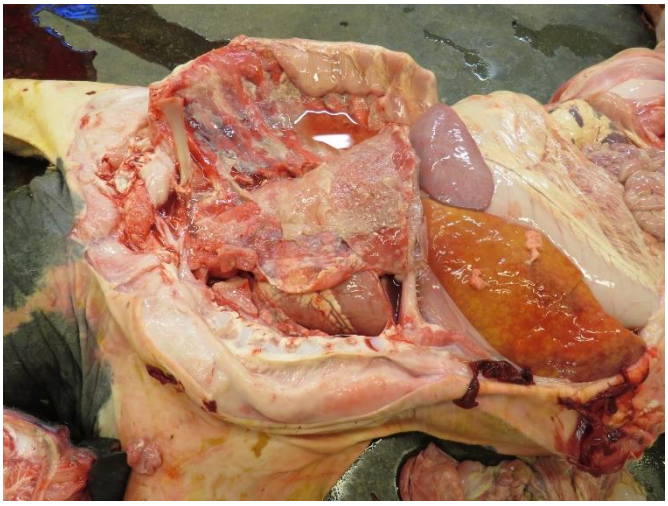

Figure 1.15. Aborted fetus, opened thorax and abdomen. Fibrin is present on the pleural surfaces pleuritis. (Courtesy of Evelien Forrez, DVM, DGZ Vlaanderen)

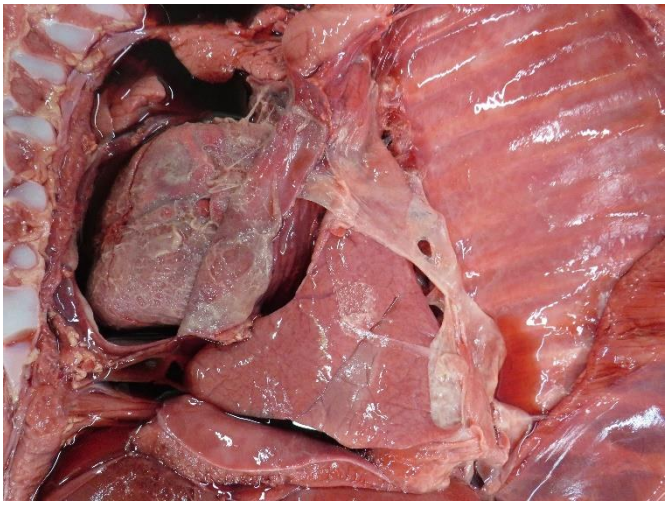

Figure 1.16. Aborted fetus, opened thorax. Fibrin is present in the pericard (pericarditis). (Courtesy of Evelien Forrez, DVM, DGZ Vlaanderen)

Macroscopic examination of the placenta may reveal lesions of placentitis, which grossly manifests as discolored, necrotic, exudative cotyledons, and opaque, erythematous, thickened, edematous intercotyledonary tissue (Mee, 2020) (**Figure 1.17.**). Gross placental lesions are characteristic of chronic bacterial and fungal infections, while viral infections generally produce no lesions (Schlafer et al., 2000). However, Botta et al. (2019) reported that histological lesions in the bovine placenta must be interpreted with caution, because lesions might not be necessarily linked to an infectious etiological cause, and can even be part of physiological processes during pregnancy (e.g., apoptotic processes, or regulation of cell proliferation and regression as key for placental maturation) or placental expulsion.

Figure 1.17. Discolored, opaque, erythematous, thickened, and edematous placental tissue during a cesarean section in a Belgian Blue cow.

5. Sample the placenta and fetal organs for microbiological, histopathological, and molecular analyses. Selection of samples and analyses is typically guided by the local laboratory standard operating procedures often based on eventual governmental monitoring programs for (zoonotic) pathogens. However, it is also influenced by factors such as the case anamnesis, cost considerations, and the quality of the available samples. Fetal abomasal contents are amongst the most crucial samples to collect as they provide a representative sample of the amniotic fluid, which surrounds the fetus throughout pregnancy (Mee, 2020). To avoid microbiological contamination, sampling of abomasal contents may be done after searing the abomasal serosa, e.g., with a heated scalpel blade, and aspirating into a plain vacutainer tube. Lung, liver or brain samples are suitable alternatives for abomasal content, however abomasal samples have a greater likelihood of bacterial agent recovery than lung tissues, even in cases with primary lung pathology (Clothier and Anderson, 2016).

   Serological sampling of fetal body fluids may aid in the diagnosis. Due to the synepitheliochorial placenta, there is normally no transfer of antibodies from cow to fetus during pregnancy (Ohmann, 1981). Hence, detection of antibodies in fetal fluids indicates fetal infection, although not necessarily fetopathy (Mee, 2020). The immunocompetence of the bovine fetus varies depending on the pathogen, and typically starts at 120 days of gestation. During the third trimester of pregnancy, the fetus develops the ability to produce antibodies against the majority of antigens (Banks and McGuire, 1989). In a previous study, the prevalence of perinatal mortality in cases with pathogen-specific antibodies was higher than in those with detection of pathogens

(Jawor et al., 2017). Based on this finding, it seems that focusing on the antibody response provides a higher chance to detect contact with pathogens during pregnancy, at least for perinatal mortalities. However, reliance on fetal serology alone may also grossly underestimate infection rates in specific cases of abortion (Mee et al., 2016). On the other hand, it needs to be mentioned that leakage of maternal antibodies to the fetus is also possible (Gabriël et al., 2005), which may result in false positive results. Yet, the probability of endogenous fetal antibody production is higher.

Sampling of the placenta is of particular importance in cases where infections with placental tropism, e.g., *C. burnetii* and *Chlamydia* spp., are suspected (Mee and Ley, 2021).

6. Perform routine bacteriological investigations, including direct smears, aerobic cultures, and the digestion of suspicious lesions with potassium hydroxide to reveal potential fungal organisms. The presence of pure growth of a pathogen with associated histopathologic lesions, in the absence of other causes of APM, is usually considered a diagnostic criterium (Mee et al., 2021). Nevertheless, recent studies on variations in pathogen virulence, indicate that in the future, it may be important to assess the presence of virulence factors as well (Rzewuska et al., 2019).
7. Undertake histopathological examinations of the placenta and/or fetal organs, as this is valuable in differentiating bacterial contamination from infection.
8. Utilize immunohistochemistry or *in situ* hybridization techniques to confirm the presence of pathogens within placental or fetal tissues.
9. Take samples for trace element analyses. By examining the fetal thyroid gland, one can identify the presence of absolute goitre, where the thyroid is enlarged beyond a specific threshold weight (e.g., >30 g at the end of gestation; **Figure 1.18.**), or relative goitre, where the thyroid is enlarged in relation to a threshold thyroid-to-body weight ratio of $\text{max to min range weight of normal fetal thyroid gland (g)} = \frac{\text{weight of fetus}}{2\,(\text{max}) \text{ or } 3\,(\text{min})}$ (Murray, 2015). Additionally, submitting a fresh lobe for iodine content analysis, and a formalin-fixed lobe for histopathological examination can help detect any imbalances in dietary iodine (Mee, 2020). If there is a suspicion of selenium deficiency (**Figure 1.19.**), potentially in conjunction with iodine deficiency, it is recommended to analyze a fetal liver sample, preferably, or the kidneys. Late term fetuses and early neonates have a higher normal range of selenium than adults. There is maternal movement of a significant amount of selenium to the fetus during gestation. The stored concentration

in the liver helps prevent deficiency during the time period of a predominantly milk diet. Fetuses or neonates with liver selenium near the low end of the normal range for adults likely suffer deficiency (Ensley, 2020). For adult cattle, proper levels of liver selenium range between 1.25 and 2.5 µg/g (wet weight), whereas for neonates, this level varies between 2.3 and 8 µg/g (Guyot and Rollin, 2007).

Figure 1.18. Thyroid glands of 2 stillborn calves. Left normal (16 grams) and right enlarged thyroid gland (89 grams). (Courtesy of Evelien Forrez, DVM, DGZ Vlaanderen)

Figure 1.19. Muscle tissue of a stillborn Belgian Blue calf. Muscular dystrophy (white muscle disease), indicative of selenium deficiency. (Courtesy of Evelien Forrez, DVM, DGZ Vlaanderen)

10. Promptly report any notifiable diseases (e.g., brucellosis) upon diagnosis.

Surveillance of bovine abortion

Attitude, barriers and motivators

Monitoring of diseases relies on reporting and submission/analysis of cases. However, even under mandatory circumstances, under-reporting of bovine APM is a major issue worldwide, particularly in early gestation when it is more difficult to observe APM cases (Bronner et al., 2014; Clothier et al., 2020). Forar et al. (1995) concluded in a review that only about 30% of abortions are visually detected, which may be one of the reasons of under-reporting of bovine APM. Aside from not observing APM, it was estimated in Canada and France that more than 60% of observed APM cases were not reported (Bronner et al., 2014; Denis-Robichaud et al.,

2019), and an even lower submission rate of only 5.5% was reported in New Zealand (Thobokwe and Heuer, 2011). Several potential reasons for this under-reporting are mentioned in literature (Bronner et al., 2014; Denis-Robichaud et al., 2019; Clothier et al., 2020). A possible barrier for reporting is the laboratory cost associated with analyzing APM cases (Denis-Robichaud et al., 2019), which is implicit with the traditional assumption that farmers are profit driven (Kristensen and Jakobsen, 2011). However, reducing decision-making on individual farms solely to financial gain is an oversimplification of reality. Merely viewing farmers as profit maximizers overlooks the significance of individual attitudes and motivators that shape their decision-making process (Willock et al., 1999a, 1999b). Attitudes are based on an individual's perception of truth, which can be influenced by beliefs, values, information, and knowledge (Willock et al., 1999a). Farmers hold varying views on what constitutes APM, particularly considering the diverse definitions of abortion already existing (Bronner et al., 2014; Clothier et al., 2020). Furthermore, farmers' knowledge and experience regarding cattle abortion may influence their attitudes towards its management (Clothier et al., 2020). The variability in attitudes among farmers may, in part, be attributed to their communication with their veterinarian (Clothier et al., 2020). If the topic of APM is not actively discussed, farmers may fail to fully comprehend the true impact of the issue, which limits motivation to investigate and address APM problems (Clothier et al., 2020). Garner et al. (2016) mentioned that educating farmers increases the likelihood to report signs of disease. Furthermore, fear of consequences in case of detection of a notifiable disease, or practical obstacles (e.g., catching a cow on pasture, and long distance to the laboratory) are other possible barriers (Bronner et al., 2014; Clothier et al., 2020). Moreover, differences in acceptable APM incidence thresholds may influence the motivation of farmers and their veterinarians to submit an APM case. For instance, French farmers have suggested an abortion threshold of 1.5-5%. Below the threshold of 1.5%, these farmers considered APM cases as sporadic, and did not seek veterinary assistance (Bronner et al., 2014). In the UK, dairy and beef farmers appear to have different thresholds. Dairy farmers set their threshold above 2%, while beef farmers considered any APM or an incidence of 1% as requiring attention. Additionally, veterinarians seem to tolerate a higher incidence of APM (4%) before recommending laboratory investigation (Clothier et al., 2020). Finally, it is also known that diseases that are not currently present in the country (e.g., brucellosis) are considered to be of negligible risk, whereas farmers are more likely to be aware of a disease and adopt more biosecurity measures in an outbreak situation (Ekboir, 1999;

Bronner et al., 2014). Consequently, all these factors may limit the number of submitted APM cases and hamper the early detection of APM.

Identifying the cause of APM was recognized as the main motivator for farmers to submit an APM case for analysis. Furthermore, an increased abortion incidence and the accompanying financial loss, reduced cost of investigation services, a high rate of diagnostic success, and ease of sample submission were previously recognized as motivators (Bronner et al., 2014; Clothier et al., 2020). One might suppose that the obligatory character of APM is another motivator for case submission, but farmers commonly hold a negative perception towards legislative requirements and interference from policy makers (Willock et al., 1999b). As a result, the obligation to submit APM cases is not strictly adhered. In France and the UK, only a low number of farmers (10%) seems to be convinced to submit APM cases for analysis by the legal requirement (Bronner et al., 2014; Clothier et al., 2020), probably also due to the lack of consequences when APM cases were not reported. For Belgium, no data about the number of APM submissions, and the factors which may influence the number of submitted cases are described.

The Belgian bovine abortion monitoring program since the end of 2009

To ensure freedom of brucellosis in Belgium with a 99% confidence level, Welby et al. (2009) calculated a minimum required number of 8,000 APM cases submitted per year [4,000 per region (Flanders and Wallonia)], in combination with the other components of the national brucellosis surveillance system. Therefore, to increase the reporting of bovine APM, a communication campaign to emphasize the importance of APM monitoring was initiated at the end of 2009. This communication campaign consisted of information leaflets on the relevance of reporting and submitting cases of APM. Additionally, two veterinarians were employed in the diagnostic regional accredited laboratories to visit cattle herds with an APM problem, and to educate farmers and their veterinarians during specific meetings around the topic. Concurrently, to monitor alternative relevant infectious causes of APM in Belgium, an extensive set of analyses was added to the APM monitoring program. Based on previous studies performed in the region, a set of analyses was composed to detect the most relevant abortifacient pathogens (Callens et al., 2009; Ribbens et al., 2009; Vangeel et al., 2012). Analyses for *N. caninum, C. burnetii*, BHV-1, BVDv, BTv, *L. interrogans* serovar Hardjo, *Listeria* spp., *Salmonella* spp. and opportunistic aerobic bacteria and yeasts/molds were included. In addition, daily collection of the samples (maternal blood sample, placenta, and fetus/calf) at the farm was organized by the diagnostic laboratories. As an incentive, the FASFC fully funded both the on-

farm collection of the samples, and the newly introduced laboratory analyses, in addition to *Brucella*-specific analyses that were already funded.

As mentioned earlier, many of the infectious agents responsible for bovine APM in Flanders can also affect humans, raising zoonotic concerns. Therefore, closely monitoring bovine APM is of utmost importance for both human and animal health. In this context, an effective surveillance program to investigate the factors contributing to bovine APM risks is essential and should be regularly assessed for its efficacy. Additionally, it is crucial to address emerging, re-emerging, and zoonotic causes of bovine APM that might have been overlooked in the past in routine laboratory analyses due to their inability to be detected by traditional diagnostic procedures (examples include *A. phagocytophilum*, *Chlamydia* spp., and CLO).

References

2016/429, E.R., 2016. REGULATION (EU) 2016/429 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 9 March 2016 on transmissible animal diseases and amending and repealing certain acts in the area of animal health ('Animal Health Law').

Adamiak, S.J., Mackie, K., Watt, R.G., Webb, R., Sinclair, K.D., 2005. Impact of Nutrition on Oocyte Quality: Cumulative Effects of Body Composition and Diet Leading to Hyperinsulinemia in Cattle 1. Biol Reprod 73, 918–926. https://doi.org/10.1095/biolreprod.105.041483

Adjadj, N.R., Cargnel, M., Ribbens, S., Quinet, C., Malandrin, L., Mignon, B., Mori, M., 2023. Prevalence of Anaplasma phagocytophilum, Borrelia burgdorferi sensu lato, Rickettsia spp. and Babesia spp. in cattle serum and questing ticks from Belgium. Ticks Tick Borne Dis 14, 102146. https://doi.org/10.1016/J.TTBDIS.2023.102146

Ahmad, N., Schrick, F.N., Butcher, R.L., Inskeep, E.K., 1995. Effect of Persistent Follicles on Early Embryonic Losses in Beef Cows. Biol Reprod 52, 1129–1135. https://doi.org/10.1095/BIOLREPROD52.5.1129

Aitken, I.D., Longbottom, D., 2007. Chlamydial abortion, in: Aitken, I.D. (Ed.), Diseases of Sheep. Wiley-Blackwell, Edinburgh, pp. 105–112.

Albaaj, A., Durocher, J., Leblanc, S.J., Dufour, S., 2023a. Meta-analysis of the incidence of pregnancy losses in dairy cows at different stages to 90 days of gestation. JDSC 4, 144–148. https://doi.org/10.3168/jdsc.2022-0278

Albaaj, A., Durocher, J., Leblanc, S.J., Dufour, S., 2023b. Meta-analysis of the incidence of pregnancy losses in dairy cows at different stages to 90 days of gestation. JDSC 4, 144–148. https://doi.org/10.3168/jdsc.2022-0278

Ali, H., Ali, A.A., Atta, M.S., Cepica, A., 2012. Common, Emerging, Vector-Borne and Infrequent Abortogenic Virus Infections of Cattle. Transbound Emerg Dis. https://doi.org/10.1111/j.1865-1682.2011.01240.x

Álvarez, J., Sáez, J.L., García, N., Serrat, C., Pérez-Sancho, M., González, S., Ortega, M.J., Gou, J., Carbajo, L., Garrido, F., Goyache, J., Domínguez, L., 2011. Management of an outbreak of brucellosis due to B. melitensis in dairy cattle in Spain. Res Vet Sci 90, 208–211. https://doi.org/10.1016/J.RVSC.2010.05.028

Ammerdorffer, A., Stojanov, M., Greub, G., Baud, D., 2017. Chlamydia trachomatis and Chlamydia-like bacteria: New enemies of human pregnancies. Curr Opin Infect Dis 30, 289–296. https://doi.org/10.1097/QCO.0000000000000369

Anderson, A., 2004. Chlamydiosis, in: Coetzer, J.A.W., Tustin, R.C. (Eds.), Infectious Diseases of Livestock. Oxford University Press, Cape Town, pp. 550–564.

Anderson, J.D., Smith, H., 1965. The Metabolism of Erythritol by Brucella abortus. J. gen. Microbiol 38, 109–124.

Anderson, M.L., 2012. Disorders of cattle, in: Njaa, B.L. (Ed.), Kirkbride's Diagnosis of Abortion and Neonatal Loss in Animals. Oxford: UK, pp. 13–48.

Anderson, M.L., 2007a. Infectious causes of bovine abortion during mid- to late-gestation. Theriogenology 68, 474–486. https://doi.org/10.1016/j.theriogenology.2007.04.001

Anderson, M.L., Reynolds, J.P., Rowe, J.D., Sverlow, K.W., Packham, A.E., Barr, B.C., Conrad, P.A., 1997. Evidence of vertical transmission of Neospora sp. infection in dairy cattle. J Am Vet Med Assoc 210, 1169–1172.

Angelakis, E., Raoult, D., 2010. Q fever. Vet Microbiol 140, 297–309. https://doi.org/10.1016/J.VETMIC.2009.07.016

APHA. 2016. Veterinary Investigation Surveillance Report. 2016, Cattle. 7.

Atif, F.A., 2015. Anaplasma marginale and Anaplasma phagocytophilum: Rickettsiales pathogens of veterinary and public health significance. Parasitology Research 2015 114:11 114, 3941–3957. https://doi.org/10.1007/S00436-015-4698-2

Augustine, P.T., 2000. Abortions in Dairy Cows: New Insights and Economic Impact. Advances in Dairy Technology 12, 233.

Bakken, J.S., Dumler, J.S., 2015. Human Granulocytic Anaplasmosis. Infect Dis Clin North Am 29, 341–355. https://doi.org/10.1016/J.IDC.2015.02.007

Bakken, J.S., Dumler, J.S., Chen, S.M., Eckman, M.R., Van Etta, L.L., Walker, D.H., 1994. Human Granulocytic Ehrlichiosis in the Upper Midwest United States: A New Species Emerging? JAMA 272, 212–218. https://doi.org/10.1001/JAMA.1994.03520030054028

Ball, P., 1997. Later embryo and early fetal mortality in the cow. Animal Breeding Abstracts 65, 167–175.

Banks, K.L., McGuire, T.C., 1989. Neonatal immunology, in: Saunders (Ed.), Veterinary Clinical Immunology. Philadelphia, pp. 193–204.

Barber, D.M., Nettleton, P.F., Herring, J.A., 1985. Disease in a dairy herd associated with the introduction and spread of bovine virus diarrhoea virus. Vet Rec 117, 459–464.

Barkallah, M., Gharbi, Y., Hassena, A. B., Slima, A. B., Mallek, Z., Gautier, M., Greub, G., Gdoura, R., Fendri, I., 2014. Survey of infectious etiologies of bovine abortion during mid to late gestation in dairy herds. PLoS One, 9, e91549.

Barkallah, M., Jribi, H., Ben Slima, A., Gharbi, Y., Mallek, Z., Gautier, M., Fendri, I., Gdoura, R., 2018. Molecular prevalence of Chlamydia and Chlamydia-like bacteria in Tunisian domestic ruminant farms and their influencing risk factors. Transbound Emerg Dis 65, e329–e338. https://doi.org/10.1111/TBED.12757

Barr, B.C., Anderson, M.L., 1993. Infectious diseases causing bovine abortion and fetal loss. Vet Clin North Am Food Anim Pract 9, 343–368.

Barr, B.C., Conrad, P.A., Dubey, J.P., Anderson, M.L., 1991. Neospora-like encephalomyelitis in a calf: pathology, ultrastructure, and immunoreactivity. J Vet Diagn Invest 3, 39–46. https://doi.org/10.1177/104063879100300109

Bartels, C.J.M., Wouda, W., Schukken, Y.H., 1999. Risk factors for Neospora caninum-associated abortion storms in dairy herds in the Netherlands (1995 to 1997). Theriogenology 52, 247–257. https://doi.org/10.1016/S0093-691X(99)00126-0

Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. lme4: Linear mixed-effects models using Eigen and S4. R package version 1:1-7 [WWW Document]. URL http://cran.r-project.org/package=lme4

Baud, D., Kebbi, C., Külling, J.P., Greub, G., 2009. Seroprevalence of different Chlamydia-like organisms in an asymptomatic population. Clin Microbiol Infect 15 Suppl 2, 213–215. https://doi.org/10.1111/J.1469-0691.2008.02134.X

Baud, D., Regan, L., Greub, G., 2008. Emerging role of Chlamydia and Chlamydia-like organisms in adverse pregnancy outcomes. Current Opinion in Infectious Diseases 21, 70–76.

Baughman, B., 2015. Bovine Abortifacient and Teratogenic Toxins, in: Hopper, R. (Ed.), Bovine Reproduction. Wiley-Blackwell, Oxford, UK, pp. 839–862.

Baumgartner, W., 2015. Fetal disease and abortion: diagnosis and causes, in: Hopper, R.M. (Ed.), Bovine Reproduction. Wiley-Blackwell, Oxford, UK, p. 496.

Beauvais, W., Musallam, I., Guitian, J., 2016. Vaccination control programs for multiple livestock host species: An age-stratified, seasonal transmission model for brucellosis control in endemic settings the LCNTDR Collection: Advances in scientific research for NTD control. Parasit Vectors 9. https://doi.org/10.1186/s13071-016-1327-6

Beeckman, DSA., 2009. Examining the Role of Type III Secretion in the Biology and Intracellular Pathogenesis of Chlamydophila psittaci. PhD thesis, Ghent University, Ghent, Belgium.

Benavides, J., Collantes-Fernández, E., Ferre, I., Pérez, V., Campero, C., Mota, R., Innes, E., Ortega-Mora, L.M., 2014. Experimental ruminant models for bovine neosporosis: What is known and what is needed, in: Parasitology. Cambridge University Press, pp. 1471–1488. https://doi.org/10.1017/S0031182014000638

Benjamini, Y., Hochberg, Y., 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society. Series B (Methodological) 57, 289–300.

Bianchessi, L., Rocchi, M.S., Maley, M., Allen, K., Ballingall, K., Turin, L., 2023. Presence of Anaplasma phagocytophilum ecotype I in UK ruminants and associated zoonotic risk. Pathogens 12, 216. https://doi.org/10.3390/PATHOGENS12020216/S1

Binelli, M., Hampton, J., Buhi, W.C., Thatcher, W.W., 1999. Persistent dominant follicle alters pattern of oviductal secretory proteins from cows at estrus. Biol Reprod 61, 127–134. https://doi.org/10.1095/BIOLREPROD61.1.127

Blumer, S., Greub, G., Waldvogel, A., Hässig, M., Thoma, R., Tschuor, A., Pospischil, A., Borel, N., 2011. Waddlia, Parachlamydia and Chlamydiaceae in bovine abortion. Vet Microbiol 152, 385–393. https://doi.org/10.1016/j.vetmic.2011.05.024

Bommana, S., Polkinghorne, A., 2019. Mini Review: Antimicrobial control of Chlamydial infections in animals: current practices and issues. Front Microbiol 10, 113.

BonDurant, R.H., 2007. Selected diseases and conditions associated with bovine conceptus loss in the first trimester. Theriogenology 68, 461–473. https://doi.org/10.1016/J.THERIOGENOLOGY.2007.04.022

Bonfini, B., Chiarenza, G., Paci, V., Sacchini, F., Salini, R., Vesco, G., Villari, S., Zilli, K., Tittarelli, M., 2018. Cross-reactivity in serological tests for brucellosis: a comparison of immune response of Escherichia coli O157:H7 and Yersinia enterocolitica O:9 vs Brucella spp. Vet Ital 54, 107–114. https://doi.org/10.12834/VetIt.1176.6539.2

Bonneau, K.R., Demaula, C.D., Mullens, B.A., Maclachlan, N.J., 2002. Duration of viraemia infectious to Culicoides sonorensis in bluetongue virus-infected cattle and sheep. Vet Microbiol 88, 115–125.

Borel, N., Frey, C.F., Gottstein, B., Hilbe, M., Pospischil, A., Franzoso, F.D., Waldvogel, A., 2014. Laboratory diagnosis of ruminant abortion in Europe. Vet J 200, 218–29. https://doi.org/10.1016/j.tvjl.2014.03.015

Borel, N., Kempf, E., Hotzel, H., Schubert, E., Torgerson, P., Slickers, P., Ehricht, R., Tasara, T., Pospischil, A., Sachse, K., 2008. Direct identification of chlamydiae from clinical

samples using a DNA microarray assay-A validation study. Mol Cell Probes 22, 55–64. https://doi.org/10.1016/J.MCP.2007.06.003

Borel, N., Polkinghorne, A., Pospischil, A., 2018. A Review on Chlamydial Diseases in Animals: Still a Challenge for Pathologists? Vet Pathol 55, 374–390. https://doi.org/10.1177/0300985817751218/FORMAT/EPUB

Borel, N., Thoma, R., Spaeni, P., Weilenmann, R., Teankum, K., Brugnera, E., Zimmermann, D.R., Vaughan, L., Pospischil, A., 2006. Chlamydia-related abortions in cattle from Graubunden, Switzerland. Vet Pathol 43, 702–708. https://doi.org/10.1354/VP.43-5-702

Botta, C., Pellegrini, G., Hässig, M., Pesch, T., Prähauser, B., Wunderlin, S., Guscetti, F., Schneeberger, M., Schmitt, S., Basso, W., Hilbe, M., Schuler, G., Borel, N., 2019. Bovine fetal placenta during pregnancy and the postpartum period. Vet Pathol 56, 248–258. https://doi.org/10.1177/0300985818806453

Brickell, J.S., Mcgowan, M.M., Wathes, D.C., 2010. Association between Neospora caninum seropositivity and perinatal mortality in dairy heifers at first calving. Veterinary Record 167, 82–85. https://doi.org/10.1136/vr.c3583

Bronner, A., Hénaux, V., Fortané, N., Hendrikx, P., Calavas, D., 2014. Why do farmers and veterinarians not report all bovine abortions, as requested by the clinical brucellosis surveillance system in France? BMC Vet Res 10, 93. https://doi.org/10.1186/1746-6148-10-93

Bronner, A., Hénaux, V., Vergne, T., Vinard, J.-L., Morignat, E., Hendrikx, P., Calavas, D., Gay, E., 2013. Assessing the mandatory bovine abortion notification system in France using unilist capture-recapture approach. PLoS One 8, e63246. https://doi.org/10.1371/journal.pone.0063246

Brown, J., Entrican, G., 1996. Interferon-y Mediates Long-term Persistent Chlamydia psittaci Infection In Vitro. J. Comp. Path 115, 383.

Cabell, E., 2007. Bovine abortion: aetiology and investigations. In Pract 29, 434–491.

Cabrera, V.E., 2014. Economics of fertility in high-yielding dairy cows on confined TMR systems. Animal 8, 211–221. https://doi.org/10.1017/S1751731114000512

Cabrera, V.E., 2012. A simple formulation and solution to the replacement problem: A practical tool to assess the economic cow value, the value of a new pregnancy, and the cost of a pregnancy loss. J Dairy Sci 95, 4683–4698. https://doi.org/10.3168/JDS.2011-5214

Cafiso, A., Bazzocchi, C., Cavagna, M., Lorenzo, E. Di, Serra, V., Rossi, R., Comazzi, S., 2021. Molecular Survey of Babesia spp. and Anaplasma phagocytophilum in Roe Deer from aWildlife Rescue Center in Italy. Animals 11.

Caldow, G., Gray, D., 2004. Fetal loss, in: Andrews, A.H., Blowey, R.W., Boyd, H., Eddy, R.G. (Eds.), Bovine Medicine: Diseases and Husbandry of Cattle. Blackwell Publishing, Oxford, pp. 577–593.

Callens, J., Debleecker, K., Stoop, S., Miry, C., 2009. Abortion in cattle: developing an optimal diagnostic approach, in: Espinasse, C. (Ed.), European Buiatrics Forum, 1st, Abstracts. French Buiatrics Association, Marseille, pp. 151–151.

Cantón, G.J., Moreno, F., Fiorentino, M.A., Hecker, Y.P., Spetter, M., Fiorani, F., Monterubbianesi, M.G., García, J.A., Altamiranda, E.G., Cirone, K.M., Louge Uriarte, E.L., Verna, A.E., Marin, M., Cheuquepán, F., Malena, R., Morsella, C., Paolicchi, F.A., Morrell, E.L., Moore, D.P., 2022. Spatial-temporal trends and economic losses associated with bovine abortifacients in central Argentina. Trop Anim Health Prod 54, 242. https://doi.org/10.1007/S11250-022-03237-0/FIGURES/3

Carpenter, T.E., Chrièl, M., Andersen, M.M., Wulfson, L., Jensen, A.M., Houe, H., Greiner, M., 2006. An epidemiologic study of late-term abortions in dairy cattle in Denmark, July 2000-August 2003. Prev Vet Med 77, 215–29. https://doi.org/10.1016/j.prevetmed.2006.07.005

Carvalho, P.D., Souza, A.H., Amundson, M.C., Hackbart, K.S., Fuenzalida, M.J., Herlihy, M.M., Ayres, H., Dresch, A.R., Vieira, L.M., Guenther, J.N., Grummer, R.R., Fricke, P.M., Shaver, R.D., Wiltbank, M.C., 2014. Relationships between fertility and postpartum changes in body condition and body weight in lactating dairy cows. J Dairy Sci 97, 3666–3683. https://doi.org/10.3168/JDS.2013-7809

Carvalho, P.D., Souza, A.H., Sartori, R., Hackbart, K.S., Dresch, A.R., Vieira, L.M., Baruselli, P.S., Guenther, J.N., Fricke, P.M., Shaver, R.D., Wiltbank, M.C., 2013. Effects of deep-horn AI on fertilization and embryo production in superovulated cows and heifers. Theriogenology 80, 1074–1081. https://doi.org/10.1016/J.THERIOGENOLOGY.2013.08.008

Cavirani, S., Cabassi, C.S., Donofrio, G., De Iaco, B., Taddei, S., Flammini, C.F., 2001. Association between Chlamydia psittaci seropositivity and abortion in italian dairy cows. Prev Vet Med 50, 145–151. https://doi.org/10.1016/S0167-5877(01)00197-0

Charlier, C., Li, W., Harland, C., Littlejohn, M., Creagh, F., Keehan, M., Druet, T., Coppieters, W., Spelman, R., Georges, M., 2014. NGS-based reverse genetic screen reveals loss-of-function variants compromising fertility in cattle, in: 10th World Congress of Genetics Applied to Livestock Production. pp. 17–22. https://doi.org/10.13140/2.1.2467.9360

Chassagne, M., Barnouin, J., Chacornac, J.P., 1999. Risk factors for stillbirth in holstein heifers under field conditions in France: A prospective survey. Theriogenology 51, 1477–1488. https://doi.org/10.1016/S0093-691X(99)00091-6

Chebel, R.C., Santos, J.E.P., Reynolds, J.P., Cerri, R.L.A., Juchem, S.O., Overton, M., 2004. Factors affecting conception rate after artificial insemination and pregnancy loss in lactating dairy cows. Anim Reprod Sci 84, 239–255. https://doi.org/10.1016/j.anireprosci.2003.12.012

Chianini, F., Adams, C., Buxton, D., 2004. Neuropathological changes in ovine fetuse caused by tickborne fever. Vet Rec 155, 805–806.

Chochlakis, D., Giadinis, N., Petridou, E., Filioussis, G., Tselentis, Y., Psaroulaki, A., Ioannidou, E., Papanikolopoulou, V., Karatzias, H., 2020. Molecular evidence of Anaplasma phagocytophilumin aborted goat fetuses and placenta. Vet Ital 56, 301–303. https://doi.org/10.12834/VETIT.1173.6516.2

Clothier, G., Wapenaar, W., Kenny, E., Windham, E., 2020. Farmers' and veterinary surgeons' knowledge, perceptions and attitudes towards cattle abortion investigations in the UK. Veterinary Record 187. https://doi.org/10.1136/vr.105921

Clothier, K., Anderson, M., 2016. Evaluation of bovine abortion cases and tissue suitability for identification of infectious agents in California diagnostic laboratory cases from 2007 to 2012. Theriogenology 85, 933–938. https://doi.org/10.1016/j.theriogenology.2015.11.001

Coin, P., Dall'Ava, B., Martignago, F., Tonellato, F., Schiavon, E., Capello, K., Versentini, G., Cornaggia, M., Cavirani, S., Barberio, A., 2022. Retrospective study on surveillance on dairy cows infective abortions in Northeastern Italy from 2006 to 2019. Proceedings of the 31st World Buiatrics Congress, Madrid, Spain, 116–117.

COMMISSION DECISION of 23 June 2003 establishing the official tuberculosis, brucellosis, and enzootic-bovine-leukosis-free status of certain Member States and regions of Member States as regards bovine herds, 2003. . Official Journal of the European Union.

Courtney, J.W., Kostelnik, L.M., Zeidner, N.S., Massung, R.F., 2004. Multiplex real-time PCR for detection of Anaplasma phagocytophilum and Borrelia burgdorferi. J Clin Microbiol 42, 3164–3168. https://doi.org/10.1128/JCM.42.7.3164-3168.2004/FORMAT/EPUB

Crilly, J., 2004. The epidemiology of bovine salmonellosis in Cork and Kerry. End of Project Report, Teagasc, Project MKDC-0506-4992.

Cuttance, E., Laven, R., 2019. Estimation of perinatal mortality in dairy calves: A review. Veterinary Journal. https://doi.org/10.1016/j.tvjl.2019.105356

Dai, N., Li, Q., Geng, J., Guo, W., Yan, W., 2022. Severe pneumonia caused by Chlamydia psittaci: report of two cases and literature review. J Infect Dev Ctries 16, 1101–1112. https://doi.org/10.3855/JIDC.16166

Daniel Givens, M., Marley, M.S.D., 2008. Infectious causes of embryonic and fetal mortality. Theriogenology 70, 270–285. https://doi.org/10.1016/J.THERIOGENOLOGY.2008.04.018

De Graef, E., Van Mael, E., Ryckaert, V., Van Praet, W., Lipkens, Z., De Bleecker, K., Ribbens, S., Brossé, C., Coysman, A., Gillis, M., Vanblaere, T., 2022. Activiteitenverslag 2021 - Diergeneeskundige begeleiding Herkauwers. Torhout.

De Meerschman, F., Speybroeck, N., Berkvens, D., Rettignera, C., Focant, C., Leclipteux, T., Cassart, D., Losson, B., 2002. Fetal infection with Neospora caninum in dairy and beef cattle in Belgium. Theriogenology 58, 933–45. https://doi.org/10.1016/s0093-691x(02)00934-2

De Vries, A., 2006. Economic value of pregnancy in dairy cattle. J Dairy Sci 89, 3876–3885. https://doi.org/10.3168/JDS.S0022-0302(06)72430-4

Delooz, L., Czaplicki, G., Gregoire, F., Dal Pozzo, F., Pez, F., Kodjo, A., Saegerman, C., 2018. Serogroups and genotypes of Leptospira spp. strains from bovine aborted foetuses. Transbound Emerg Dis 65, 158–165. https://doi.org/10.1111/TBED.12643

Delooz, L., Gregoire, F., Saegerman, C., 2018. Bovine abortion caused by Anaplasma phagocytophilum in southern Belgium, evidence of an outbreak in summer 2017, in: First PhD Student Day - Liège.

Delooz, L., Saegerman, C., Quinet, C., Petitjean, T., De Regge, N., Cay, B., 2017. Resurgence of Schmallenberg Virus in Belgium after 3 Years of Epidemiological Silence. Transbound Emerg Dis 64, 1641–1642. https://doi.org/10.1111/TBED.12552

Denis-Robichaud, J., Kelton, D.F., Bauman, C.A., Barkema, H.W., Keefe, G.P., Dubuc, J., 2019. Biosecurity and herd health management practices on Canadian dairy farms. J Dairy Sci 102, 9536–9547. https://doi.org/10.3168/jds.2018-15921

Deschuytere, H., Callens, J., De Bleecker, K., Pierré, E., Ribbens, S., Ryckaert, V., Van de Wouwer, E., Van Mael, E., Van Praet, W., Van Schoubroeck, L., 2016. Activiteitenverslag 2015 - Diergeneeskundige begeleiding Herkauwers. Torhout.

DGZ Vlaanderen, 2019. Activiteitenrapport Veepeiler Rund 2018. Torhout.

Dhaliwal, G.S., Murray, R.D., Dobson, H., Montgomery, J., Ellis, W.A., 1996. Effect of Leptospira interrogans serovar hardjo infection on milk yield in endemically infected dairy herds. Vet Rec 139, 319–320.

Dhanashekar, R., Akkinepalli, S., Nellutla, A., 2012. Milk-borne infections. an analysis of their potential effect on the milk industry. Germs 2, 101–109. https://doi.org/10.11599/germs.2012.1020

Dilbeck, P.M., Evermann, J.F., Crawford, T.B., Ward, A.C.S., Leathers, C.W., Holland, C.J., Mebus, C.A., Logan, L.L., Rurangirwa, F.R., McGuire, T.C., 1990. Isolation of a previously undescribed rickettsia from an aborted bovine fetus. J Clin Microbiol 28, 814–816. https://doi.org/10.1128/JCM.28.4.814-816.1990

Diskin, M.G., 2018. Review: Semen handling, time of insemination and insemination technique in cattle. Animal 12, s75–s84. https://doi.org/10.1017/S1751731118000952

Diskin, M.G., Morris, D.G., 2008. Embryonic and Early Foetal Losses in Cattle and Other Ruminants. Reproduction in Domestic Animals 43, 260–267. https://doi.org/10.1111/J.1439-0531.2008.01171.X

Diskin, M.G., Parr, M.H., Morris, D.G., 2012. Embryo death in cattle: An update. Reprod Fertil Dev 24, 244–251. https://doi.org/10.1071/RD11914

Diskin, M.G., Waters, S.M., Parr, M.H., Kenny, D.A., 2016. Pregnancy losses in cattle: Potential for improvement. Reprod Fertil Dev 28, 83–93. https://doi.org/10.1071/RD15366

Dubey, J.P., Buxton, D., Wouda, W., 2006. Pathogenesis of Bovine Neosporosis. J Comp Pathol. https://doi.org/10.1016/j.jcpa.2005.11.004

Dubey, J.P., Schares, G., Ortega-Mora, L.M., 2007. Epidemiology and control of neosporosis and Neospora caninum. Clin Microbiol Rev. https://doi.org/10.1128/CMR.00031-06

Dugat, T., Chastagner, A., Lagrée, A.C., Petit, E., Durand, B., Thierry, S., Corbière, F., Verheyden, H., Chabanne, L., Bailly, X., Leblond, A., Vourc'h, G., Boulouis, H.J.,

Maillard, R., Haddad, N., 2014. A new multiple-locus variable-number tandem repeat analysis reveals different clusters for Anaplasma phagocytophilum circulating in domestic and wild ruminants. Parasit Vectors 7, 1–11. https://doi.org/10.1186/1756-3305-7-439

Dugat, T., Haciane, D., Durand, B., Lagrée, A.C., Haddad, N., Boulouis, H.J., 2017. Short Report: Identification of a Potential Marker of Anaplasma phagocytophilum Associated with Cattle Abortion. Transbound Emerg Dis 64, e1–e3. https://doi.org/10.1111/TBED.12508

Ebani, V., Cerri, D., Fratini, F., Ampola, M., Andreani, E., 2008. Seroprevalence of Anaplasma phagocytophilum in domestic and wild animals from central Italy. New Microbiologica 31, 371–375.

Ekboir, J.M., 1999. The role of the public sector in the development and implementation of animal health policies. Prev Vet Med 40, 101–115. https://doi.org/10.1016/S0167-5877(99)00015-X

Elbers, A.R.W., Gorgievski-Duijvesteijn, M.J., Zarafshani, K., Koch, G., 2010. To report or not to report: A psychosocial investigation aimed at improving early detection of avian influenza outbreaks. OIE Revue Scientifique et Technique 29, 435–449. https://doi.org/10.20506/rst.29.3.1988

Endalew, A.D., Faburay, B., Wilson, W.C., Richt, J.A., 2019. Schmallenberg Disease-A Newly Emerged Culicoides-Borne Viral Disease of Ruminants. Viruses 11, 1065. https://doi.org/10.3390/v11111065

Ensley, S., 2020. Evaluating Mineral Status in Ruminant Livestock. Veterinary Clinics of North America - Food Animal Practice 36, 525–546. https://doi.org/10.1016/j.cvfa.2020.08.009

Eurostat, 2022a. Milk and milk product statistics - Statistics Explained [WWW Document]. URL https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Milk_and_milk_product_statistics (accessed 4.12.23).

Eurostat, 2022b. Agricultural production - livestock and meat - Statistics Explained [WWW Document]. URL https://ec.europa.eu/eurostat/statistics-explained/index.php?oldid=427096 (accessed 4.12.23).

Evans, C.A., Pinior, B., Larska, M., Graham, D., Schweizer, M., Guidarini, C., Decaro, N., Ridpath, J., Gates, M.C., 2019. Global knowledge gaps in the prevention and control of

bovine viral diarrhoea (BVD) virus. Transbound Emerg Dis. https://doi.org/10.1111/tbed.13068

Everett, K.D.E., Bush, R.M., Andersen, A.A., 1999. Emended description of the order Chlamydiales, proposal of Parachlamydiaceae fam. nov. and Simkaniaceae fam. nov., each containing one monotypic genus, revised taxonomy of the family Chlamydiaceae, including a new genus and five new species, and standards. Int J Syst Bacteriol 49, 415–440.

Evermann, J.F., Ridpath, J.F., 2002. Clinical and epidemiologic observations of bovine viral diarrhea virus in the northwestern United States. Vet Microbiol 89, 129–139. https://doi.org/10.1016/S0378-1135(02)00178-5

Foley, G.L., 1996. Pathology of the corpus luteum of cows. Theriogenology 45, 1413–1428.

Forar, A.L., Gay, J.M., Hancock, D.D., 1995. The frequency of endemic fetal loss in dairy cattle: A review. Theriogenology. https://doi.org/10.1016/0093-691X(95)00063-E

Forar, A.L., Gay, J.M., Hancock, D.D., Gay, C.C., 1996. Fetal loss frequency in ten Holstein dairy herds. Theriogenology 45, 1505–1513.

Franco, G., Reese, S., Poole, R., Rhinehart, J., Thompson, K., Cooke, R., Pohler, K., 2020. Sire contribution to pregnancy loss in different periods of embryonic and fetal development of beef cows. Theriogenology 154, 84–91. https://doi.org/10.1016/j.theriogenology.2020.05.021

Franco, M.P., Mulder, M., Gilman, R.H., Smits, H.L., 2007. Human brucellosis. Lancet Infect Dis 7, 775–786. https://doi.org/10.1016/S1473-3099(07)70286-4

Franzén, P., Aspan, A., Egenvall, A., Gunnarsson, A., Karlstam, E., Pringle, J., 2009. Molecular Evidence for Persistence of Anaplasma phagocytophilum in the Absence of Clinical Abnormalities in Horses after Recovery from Acute Experimental Infection. J Vet Intern Med 23, 636–642. https://doi.org/10.1111/J.1939-1676.2009.0317.X

Fredriksen, B., Sandvik, T., Løken, T., Ødegaard, S.A., 1999. Level and duration of serum antibodies in cattle infected experimentally and naturally with bovine virus diarrhoea virus. Veterinary Record 144, 111–114. https://doi.org/10.1136/vr.144.5.111

Fretin, D., Mori, M., Czaplicki, G., Quinet, C., Maquet, B., Godfroid, J., Saegerman, C., 2013. Unexpected Brucella suis biovar 2 Infection in a dairy cow, Belgium. Emerg Infect Dis 19, 2053–4. https://doi.org/10.3201/eid1912.130506

Fricke, P.M., Wiltbank, M.C., 1999. Effect of milk production on the incidence of double ovulation in dairy cows. Theriogenology 52, 1133–1143. https://doi.org/10.1016/S0093-691X(99)00205-8

Gabriël, S., Geldhof, P., Phiri, I.K., Cornillie, P., Goddeeris, B.M., Vercruysse, J., 2005. Placental transfer of immunoglobulins in cattle infected with Schistosoma mattheei. Vet Immunol Immunopathol 104, 265–272. https://doi.org/10.1016/j.vetimm.2004.12.010

Gädicke, P., Monti, G., 2013. Factors related to the level of occurrence of bovine abortion in Chilean dairy herds. Prev Vet Med 110, 183-189

Gandy, S., Hansford, K., McGinley, L., Cull, B., Smith, R., Semper, A., Brooks, T., Fonville, M., Sprong, H., Phipps, P., Johnson, N., Medlock, J.M., 2022. Prevalence of Anaplasma phagocytophilum in questing Ixodes ricinus nymphs across twenty recreational areas in England and Wales. Ticks Tick Borne Dis 13, 101965. https://doi.org/10.1016/j.ttbdis.2022.101965

Garcia-Ispierto, I., López-Gatius, F., 2019. Abortion in dairy cattle with advanced twin pregnancies: Incidence and timing. Reproduction in Domestic Animals 54, 50–53. https://doi.org/10.1111/RDA.13510

Garigliany, M., De Leeuw, I., Kleijnen, D., Vandenbussche, F., Callens, J., Van Loo, H., Lebrun, M., Saulmont, M., Desmecht, D., De Clercq, K., 2011. The presence of bluetongue virus serotype 8 RNA in Belgian cattle since 2008. Transbound Emerg Dis 58, 503–9. https://doi.org/10.1111/j.1865-1682.2011.01230.x

Garner, M.G., East, I.J., Kompas, T., Ha, P. V., Roche, S.E., Nguyen, H.T.M., 2016. Comparison of alternatives to passive surveillance to detect foot and mouth disease incursions in Victoria, Australia. Prev Vet Med 128, 78–86. https://doi.org/10.1016/J.PREVETMED.2016.04.009

Gates, M.C., Earl, L., Enticott, G., 2021. Factors influencing the performance of voluntary farmer disease reporting in passive surveillance systems: A scoping review. Prev Vet Med 196, 105487. https://doi.org/10.1016/J.PREVETMED.2021.105487

Gerber, PJ, Steinfeld, Henderson, Mottet, Opio, Dijkman, Falcucci, Tempio, 2013. A globAl Assessment of emissions And mitigAtion opportunities.

Gilbert, L., 2021. The Impacts of Climate Change on Ticks and Tick-Borne Disease Risk. https://doi.org/10.1146/annurev-ento-052720

Gilbert, W.H., Häsler, B.N., Rushton, J., 2014. Influences of farmer and veterinarian behaviour on emerging disease surveillance in England and Wales. Epidemiol Infect 142, 172–186. https://doi.org/10.1017/S0950268813000484

Givens, D.I., 2010. Milk and meat in our diet: Good or bad for health? Animal 4, 1941–1952. https://doi.org/10.1017/S1751731110001503

Givens, M.D., 2018. Review: Risks of disease transmission through semen in cattle. Animal. https://doi.org/10.1017/S1751731118000708

Givens, M.D., Marley, M.S.D., 2008. Infectious causes of embryonic and fetal mortality. Theriogenology 70, 270–85. https://doi.org/10.1016/j.theriogenology.2008.04.018

Glover, A.D., Rech, R.R., Howerth, E.W., 2011. Pathology in practice. Mycotic abortion. J Am Vet Med Assoc 239, 319–21. https://doi.org/10.2460/javma.239.3.319

Gonella-Diaza, A.M., Da Silva Andrade, S.C., Sponchiado, M., Pugliesi, G., Mesquita, F.S., Van Hoeck, V., De Francisco Strefezzi, R., Gasparin, G.R., Coutinho, L.L., Binelli, M., 2015. Size of the Ovulatory Follicle Dictates Spatial Differences in the Oviductal Transcriptome in Cattle. PLoS One 10, e0145321. https://doi.org/10.1371/JOURNAL.PONE.0145321

Gonzalez-Warleta, M., Ferreras, M., Calvo Santilla, C., Fernandez, M., Benavidas, J., Perez, V., Castro-Hermida, J., Mezo, M., 2016. Infectious agents as a cause of abortion in dairy farms from Galicia. Proceedings of the 21st Congreso Internacional Anembe de Medicina Bovina, Santiago de Compostela, Spain, 244-245.

Gordon, W.S., Brownlee, A., Wilson, D.R., 1940. Studies on louping ill, tick-borne fever and scrapie, in: 3rd International Congress of Microbiology. p. 362.

Górska-Warsewicz, H., Rejman, K., Laskowski, W., Czeczotko, M., 2019. Milk and dairy products and their nutritional contribution to the average polish diet. Nutrients 11. https://doi.org/10.3390/nu11081771

Graham, D.A., Beggs, N., Mawhinney, K., Calvert, V., Cunningham, B., Rowan-Layberry, L., Mclaren, I., 2009. Comparative evaluation of diagnostic techniques for bovine viral diarrhoea virus in aborted and stillborn fetuses. https://doi.org/10.1136/vr.164.2.56

Griffiths, P.C., Plater, J.M., Horigan, M.W., Rose, M.P.M., Venables, C., Dawson, M., 1996. Serological Diagnosis of Ovine Enzootic Abortion by Comparative Inclusion Immunofluorescence Assay, Recombinant Lipopolysaccharide Enzyme-Linked Immunosorbent Assay, and Complement Fixation Test. J Clin Microbiol 34, 1512–1518.

Grimard, B., Freret, S., Chevallier, A., Pinto, A., Ponsart, C., Humblot, P., 2006. Genetic and environmental factors influencing first service conception rate and late embryonic/foetal mortality in low fertility dairy herds. Anim Reprod Sci 91, 31–44. https://doi.org/10.1016/j.anireprosci.2005.03.003

Groenendaal, H., Galligan, D.T., Mulder, H.A., 2004. An economic spreadsheet model to determine optimal breeding and replacement decisions for dairy cattle. J Dairy Sci 87, 2146–2157. https://doi.org/10.3168/JDS.S0022-0302(04)70034-X

Grooms, D.L., 2004. Reproductive consequences of infection with bovine viral diarrhea virus. Veterinary Clinics of North America: Food Animal Practice 20, 5–19. https://doi.org/10.1016/J.CVFA.2003.11.006

Grooms, Daniel L, Bolin, C.A., 2005. Diagnosis of Fetal Loss Caused by Bovine Viral Diarrhea Virus and Leptospira spp. Vet Clin Food Anim 21, 463–472. https://doi.org/10.1016/j.cvfa.2005.02.010

Guatteo, R., Nicolet, P., LeDréan, E., Ninio, C., Joly, A., 2014. Diagnostic différentiel des causes infectieuses d'avortements chez les bovins intérêt d'une démarche harmonisée et d'une technique PCR multi-agents, in: Journées Nationales GTV.

Guatteo, R., Seegers, H., Taurel, A.F., Joly, A., Beaudeau, F., 2011. Prevalence of Coxiella burnetii infection in domestic ruminants: A critical review. Vet Microbiol 149, 1–16. https://doi.org/10.1016/J.VETMIC.2010.10.007

Guinat, C., Wall, B., Dixon, L., Pfeiffer, D.U., 2016. English Pig Farmers' Knowledge and Behaviour towards African Swine Fever Suspicion and Reporting. PLoS One 11. https://doi.org/10.1371/JOURNAL.PONE.0161431

Gulliksen, S.M., Lie, K.I., Løken, T., Østerås, O., 2009. Calf mortality in Norwegian dairy herds. J Dairy Sci 92, 2782–2795. https://doi.org/10.3168/jds.2008-1807

Gutierrez, J., Williams, E.J., O'donovan, J., Brady, C., Proctor, A.F., Marques, P.X., Worrall, S., Nally, J.E., Mcelroy, M., Bassett, H.F., Sammin, D.J., Markey, B.K., 2011. Monitoring clinical outcomes, pathological changes and shedding of Chlamydophila abortus following experimental challenge of periparturient ewes utilizing the natural route of infection. Vet Microbiol 147, 119–126. https://doi.org/10.1016/j.vetmic.2010.06.015

Guyot, H., Ramery, E., O'Grady, L., Sandersen, C., Rollin, F., 2011. Emergence of bovine ehrlichiosis in Belgian cattle herds. Ticks Tick Borne Dis 2, 116–118. https://doi.org/10.1016/J.TTBDIS.2010.12.001

Guyot, H., Rollin, F., 2007. Le diagnostic des carences en sélénium et iode chez les bovins. Ann Med Vet 151, 166–191.

Hall, G.A., Jones, P.W., 1977. A study of the pathogenesis of experimental Salmonella dublin abortion in cattle. J Comp Pathol 87, 53–65. https://doi.org/10.1016/0021-9975(77)90079-2

Hansen, T.R., Smirnova, N.P., Van Campen, H., Shoemaker, M.L., Ptitsyn, A.A., Bielefeldt-Ohmann, H., 2010. Maternal and fetal response to fetal persistent infection with bovine viral diarrhea virus. Am J Reprod Immunol 64, 295–306. https://doi.org/10.1111/j.1600-0897.2010.00904.x

Hauck, D., Jordan, D., Springer, A., Schunack, B., Pachnicke, S., Fingerle, V., Strube, C., 2020. Transovarial transmission of Borrelia spp., Rickettsia spp. And Anaplasma phagocytophilum in Ixodes ricinus under field conditions extrapolated from DNA detection in questing larvae. Parasit Vectors 13, 1–11. https://doi.org/10.1186/S13071-020-04049-7/TABLES/3

Hayes, C., (2021). Bovine abortion, stillbirth and perinatal mortality. All- Island Disease Surveillance Report 2020, DAFM/AFBI, 21-25.

Hecker, Y.P., González-Ortega, S., Cano, S., Ortega-Mora, L.M., Horcajo, P., 2023. Bovine infectious abortion : a systematic review and meta-analysis. Front Vet Sci 10:1249410 ecollection

Hemphill, A., Gottstein, B., 2000. A European perspective on Neospora caninum. Int J Parasitol. https://doi.org/10.1016/S0020-7519(00)00072-2

Henker, L.C., Lorenzett, M.P., Fagundes-Moreira, R., Dalto, A.G.C., Sonne, L., Driemeier, D., Soares, J.F., Pavarini, S.P., 2020. Bovine abortion, stillbirth and neonatal death associated with Babesia bovis and Anaplasma sp. infections in southern Brazil. Ticks Tick Borne Dis 11, 101443. https://doi.org/10.1016/j.ttbdis.2020.101443

Henniger, T., Henniger, P., Grossmann, T., Distl, O., Ganter, M., Von Loewenich, F.D., 2013. Congenital infection with Anaplasma phagocytophilum in a calf in northern Germany.

Hing, M., Van Den Bossche, D., Lernout, T., Cochez, C., Pirnay, J.-P., Heuninckx, W., 2018. Prevalence of Anaplasma phagocytophilum in humans in Belgium for the period 2013-2016. Acta Clin Belg 74, 280–285.

Holler, L.D., 2012. Ruminant Abortion Diagnostics. Veterinary Clinics of North America: Food Animal Practice 28, 407–418. https://doi.org/10.1016/J.CVFA.2012.07.007

Hostetler, C.E., Kincaid, R.L., Mirando, M.A., 2003. The role of essential trace elements in embryonic and fetal development in livestock. The Veterinary Journal 166, 125–139. https://doi.org/10.1016/S1090-0233(02)00310-6

Hovingh, E., 2009. Abortions in Dairy Cattle - II: Diagnosing and Preventing Abortion Problems | VCE Publications | Virginia Tech, Virginia Cooperative Extension.

Hull, N.C., Schumaker, B.A., 2018. Comparisons of brucellosis between human and veterinary medicine. https://doi.org/10.1080/20008686.2018.1500846

Inskeep, E.K., Dailey, R.A., 2005. Embryonic death in cattle. Veterinary Clinics of North America - Food Animal Practice 21, 437–461. https://doi.org/10.1016/J.CVFA.2005.02.002

Inskeep, K., 2004. Preovulatory, postovulatory, and postmaternal recognition effects of concentrations of progesterone on embryonic survival in the cow. Article in Journal of Animal Science. https://doi.org/10.2527/2004.8213_supplE24x

Jaarsma, R.I., Sprong, H., Takumi, K., Kazimirova, M., Silaghi, C., Mysterud, A., Rudolf, I., Beck, R., Földvári, G., Tomassone, L., Groenevelt, M., Everts, R.R., Rijks, J.M., Ecke, F., Hörnfeldt, B., Modrý, D., Majerová, K., Votýpka, J., Estrada-Peña, A., 2019. Anaplasma phagocytophilum evolves in geographical and biotic niches of vertebrates and ticks. Parasit Vectors 12, 1–17. https://doi.org/10.1186/s13071-019-3583-8

Jahfari, S., Coipan, E.C., Fonville, M., Van Leeuwen, A.D., Hengeveld, P., Heylen, D., Heyman, P., Van Maanen, C., Butler, C.M., Földvári, G., Szekeres, S., Van Duijvendijk, G., Tack, W., Rijks, J.M., Van Der Giessen, J., Takken, W., Van Wieren, S.E., Takumi, K., Sprong, H., 2014. Circulation of four Anaplasma phagocytophilum ecotypes in Europe. Parasit Vectors 7, 1–11. https://doi.org/10.1186/1756-3305-7-365

Jamaluddin, A.A., Case, J.T., Hird, D.W., Blanchard, P.C., Peauroi, J.R., Anderson, M.L., 1996. Dairy cattle abortion in California: Evaluation of diagnostic laboratory data. Journal of Veterinary Diagnostic Investigation. https://doi.org/10.1177/104063879600800211

Jankowski, R.P., Aikins, H.E., Vahrson, H., Gupta, K.G., 1977. Antibacterial activity of amniotic fluid against Staphylococcus aureus, Candida albicans and Brucella abortus. Arch Gynakol 222, 275–278. https://doi.org/10.1007/BF00717604/METRICS

Jawor, P., Król, D., Mee, J.F., Sołtysiak, Z., Dzimira, S., Larska, M., Stefaniak, T., 2017. Infection exposure, detection and causes of death in perinatal mortalities in Polish dairy

herds. Theriogenology 103, 130–136. https://doi.org/10.1016/j.theriogenology.2017.07.044

Jawor, P., Mee, J.F., Stefaniak, T., 2021. Role of Infection and Immunity in Bovine Perinatal Mortality: Part 2. Fetomaternal Response to Infection and Novel Diagnostic Perspectives. Animals 2021, Vol. 11, Page 2102 11, 2102. https://doi.org/10.3390/ANI11072102

Johnston, R., Jones, K., Manley, D., 2018. Confounding and collinearity in regression analysis: a cautionary tale and an alternative procedure, illustrated by studies of British voting behaviour. Qual Quant 52, 1957. https://doi.org/10.1007/S11135-017-0584-6

Jouglin, M., Chagneau, S., Faille, F., Verheyden, H., Bastian, S., Malandrin, L., 2017. Detecting and characterizing mixed infections with genetic variants of Anaplasma phagocytophilum in roe deer (Capreolus capreolus) by developing an ankA cluster-specific nested PCR. Parasit Vectors 10, 1–9. https://doi.org/10.1186/s13071-017-2316-0

Kahl, O., Gern, L., Eisen, L., Lane, R.S., 2002. Ecological research on Borrelia burgdorferi sensu lato: terminology and some methodological pitfalls. . Lyme borreliosis: biology, epidemiology and control 29–46. https://doi.org/10.1079/9780851996325.0029

Kaltenboeck, B., Hehnen, H.R., Vaglenov, A., 2005. Bovine Chlamydophila spp. infection: Do we underestimate the impact on fertility? Vet Res Commun 29, 1–15. https://doi.org/10.1007/S11259-005-0832-4/METRICS

Kauffold, J., Henning, K., Bachmann, R., Hotzel, H., Melzer, F., 2007. The prevalence of chlamydiae of bulls from six bull studs in Germany. Anim Reprod Sci 102, 111–121. https://doi.org/10.1016/j.anireprosci.2006.10.013

Kelling, C.L., 2007. Viral diseases of the fetus, in: Youngquist, R.S., Threlfall, W. (Eds.), Current Therapy in Large Animal Theriogenology. WB Saunders Co, pp. 399–408.

Kelly, P., 2004. Q fever, in: Coetzer, J.A.W., Tustin, R.C. (Eds.), Infectious Diseases of Livestock. Oxford University Press, Cape Town, pp. 565–572.

Kemmerling, K., Müller, U., Mielenz, M., Sauerwein, H., 2009. Chlamydophila species in dairy farms: Polymerase chain reaction prevalence, disease association, and risk factors identified in a cross-sectional study in western Germany. J Dairy Sci 92, 4347–4354. https://doi.org/10.3168/jds.2009-2051

Kennedy, P.C., Richards, W.P.C., 1964. The Pathology of Abortion Caused by the Virus of Infectious Bovine Rhinotracheitis. Pathol Vet 1, 7–17. https://doi.org/10.1177/030098586400100103

Keshavamurthy, R., Singh, B.B., Kalambhe, D.G., Aulakh, R.S., Dhand, N.K., 2020. Identification of risk factors associated with Coxiella burnetii infection in cattle and buffaloes in India. Prev Vet Med 181, 105081. https://doi.org/10.1016/j.prevetmed.2020.105081

Khodakaram-Tafti, A., Ikede, B.O., 2005. A retrospective study of sporadic bovine abortions, stillbirths, and neonatal abnormalities in Atlantic Canada, from 1990 to 2001. Canadian Veterinary Journal 46, 635–637.

Khurana, S.K., Sehrawat, A., Tiwari, R., Prasad, M., Gulati, B., Shabbir, M.Z., Chhabra, R., Karthik, K., Patel, S.K., Pathak, M., Iqbal Yatoo, M., Gupta, V.K., Dhama, K., Sah, R., Chaicumpa, W., 2021. Bovine brucellosis - a comprehensive review. Vet Q 41, 61–88. https://doi.org/10.1080/01652176.2020.1868616

Kiernan, J.A., 2008. Histological and histochemical methods: theory and practice, 4th ed. Scion Publishing Ltd, UK.

Kinsel, M.L., 1999. An Epidemiologic Approach to Investigating Abortion Problems in Dairy Herds. The Bovine Proceedings 32, 152–160.

Kirkbride, C.A., 1993. Bacterial agents detected in a 10-year study of bovine abortions and stillbirths. J Vet Diagn Invest 5, 64–8. https://doi.org/10.1177/104063879300500114

Kirkbride, C.A., Gates, C.E., Libal, M.C., 1989. Ovine and bovine abortion associated with Fusobacterium nucleatum. Journal of Veterinary Diagnostic Investigation 1, 272–273. https://doi.org/10.1177/104063878900100318

Kiros, A., Asgedom, H., Abdi, R.D., 2016. A Review on Bovine Brucellosis: Epidemiology, Diagnosis and Control Options. ARC Journal of Animal and Veterinary Sciences 2, 8–21. https://doi.org/10.20431/2455-2518.0203002

Knudtson, W.U., Kirkbride, C.A., 1992. Fungi associated with bovine abortion in the northern plains states (USA). Journal of Veterinary Diagnostic Investigation 4, 181–185. https://doi.org/10.1177/104063879200400211

König, P., Wernike, K., Hechinger, S., Tauscher, K., Breithaupt, A., Beer, M., 2019. Fetal infection with Schmallenberg virus - An experimental pathogenesis study in pregnant cows. Transbound Emerg Dis 66, 454–462. https://doi.org/10.1111/tbed.13045

Kristensen, E., Jakobsen, E., 2011. Challenging the myth of the irrational dairy farmer; understanding decision-making related to herd health. N Z Vet J 59, 1–7.

Krog, C.H., Agerholm, J.S., Nielsen, S.S., 2018. Fetal age assessment for Holstein cattle. PLoS One 13. https://doi.org/10.1371/journal.pone.0207682

Lagrée, A.C., Rouxel, C., Kevin, M., Dugat, T., Girault, G., Durand, B., Pfeffer, M., Silaghi, C., Nieder, M., Boulouis, H.J., Haddad, N., 2018. Co-circulation of different A. phagocytophilum variants within cattle herds and possible reservoir role for cattle. Parasit Vectors 11, 1–10. https://doi.org/10.1186/S13071-018-2661-7/FIGURES/1

Lanyon, Sasha R., Hill, F.I., Reichel, M.P., Brownlie, J., 2014. Bovine viral diarrhoea: Pathogenesis and diagnosis. Veterinary Journal. https://doi.org/10.1016/j.tvjl.2013.07.024

Lanyon, Sasha R, Sims, S.K., Cockcroft, P.D., Reichel, M.P., 2014. Comparison of serum, ear notches, and nasal and saliva swabs for Bovine viral diarrhea virus antigen detection in colostrum-fed persistently infected (PI) calves and non-PI calves. J Vet Diagn Invest 26, 783–7. https://doi.org/10.1177/1040638714550181

Laroucau, K., Ortega, N., Vorimore, F., Aaziz, R., Mitura, A., Szymanska-Czerwinska, M., Cicerol, M., Salinas, J., Sachse, K., Caro, M.R., 2020. Detection of a novel Chlamydia species in captive spur-thighed tortoises (Testudo graeca) in southeastern Spain and proposal of Candidatus Chlamydia testudinis. Syst Appl Microbiol 43, 126071. https://doi.org/10.1016/j.syapm.2020.126071

Laroucau, K., Vorimore, F., Aaziz, R., Solmonson, L., Hsia, R.C., Bavoil, P.M., Fach, P., Hölzer, M., Wuenschmann, A., Sachse, K., 2019. Chlamydia buteonis, a new Chlamydia species isolated from a red-shouldered hawk. Syst Appl Microbiol 42, 125997. https://doi.org/10.1016/j.syapm.2019.06.002

Laureyns, J., Moyaert, H., Werbrouck, H., Catry, B., de Kruif, A., Pasmans, F., 2008. Pustular dermatitis by Listeria monocytogenes after the assisted delivery of a dead calf. Vlaams Diergeneeskd Tijdschr 77, 29–34.

Lee, J.I., Kim, I.H., 2007. Pregnancy loss in dairy cows: The contributing factors, the effects on reproductive performance and the economic impact. J Vet Sci 8, 283–288. https://doi.org/10.4142/jvs.2007.8.3.283

Lempereur, L., Lebrun, M., Cuvelier, P., Sépult, G., Caron, Y., Saegerman, C., Shiels, B., Losson, B., 2012. Longitudinal field study on bovine Babesia spp. and Anaplasma phagocytophilum infections during a grazing season in Belgium. Parasitol Res 110, 1525–1530. https://doi.org/10.1007/s00436-011-2657-0

Lernout, T., De Meyst, A., Vanrompay, D., 2022a. Epidemiologische surveillance van psittacose Chlamydia psittaci - 2019-2021. Brussels.

Lernout, T., Vanhonsebrouck, A., Claes, V., 2022b. Epidemiologische surveillance van anaplasmose Anaplasma phagocytophilum - 2019-2021. Brussels.

Lienard, J., Croxatto, A., Aeby, S., Jaton, K., Posfay-Barbe, K., Gervaix, A., Greub, G., 2011. Development of a new chlamydiales-specific real-time PCR and its application to respiratory clinical samples. J Clin Microbiol 49, 2637–2642. https://doi.org/10.1128/JCM.00114-11

Lin, Q., Rikihisa, Y., Felek, S., Wang, X., Massung, R.F., Woldehiwet, Z., 2004. Anaplasma phagocytophilum has a functional msp2 gene that is distinct from p44. Infect Immun 72, 3883–3889. https://doi.org/10.1128/IAI.72.7.3883-3889.2004

Lindsay, D.S., Dubey, J.P., 2020. Neosporosis, Toxoplasmosis, and Sarcocystosis in Ruminants: An Update. Veterinary Clinics of North America: Food Animal Practice 36, 205–222. https://doi.org/10.1016/J.CVFA.2019.11.004

Livingstone, M., Longbottom, D., 2006. What is the prevalence and economic impact of chlamydial infections in cattle? The need to validate and harmonise existing methods of detection. Veterinary Journal 172, 3–5.

Loeliger, M., Watson, C.S., Reynolds, J.D., Penning, D.H., Harding, R., Bocking, A.D., Rees, S.M., 2003. Extracellular glutamate levels and neuropathology in cerebral white matter following repeated umbilical cord occlusion in the near term fetal sheep. Neuroscience 116, 705–714. https://doi.org/10.1016/S0306-4522(02)00756-X

Lonergan, P., Fair, T., Forde, N., Rizos, D., 2016. Embryo development in dairy cattle. Theriogenology 86, 270–277. https://doi.org/10.1016/J.THERIOGENOLOGY.2016.04.040

Lonergan, P., Sánchez, J.M., 2020. Symposium review: Progesterone effects on early embryo development in cattle. J Dairy Sci 103, 8698–8707. https://doi.org/10.3168/JDS.2020-18583

Longbottom, D., Livingstone, M., Maley, S., van der Zon, A., Rocchi, M., Wilson, K., Wheelhouse, N., Dagleish, M., Aitchison, K., Wattegedera, S., Nath, M., Entrican, G., Buxton, D., 2013. Intranasal Infection with Chlamydia abortus Induces Dose-Dependent Latency and Abortion in Sheep. PLoS One 8, e57950. https://doi.org/10.1371/JOURNAL.PONE.0057950

Longbottom, D., Pathology, L.C.-J. of comparative, 2003, U., 2003. Animal chlamydioses and zoonotic implications. J Comp Pathol 128, 217–244.

López-Gatius, F., 2000. Site of semen deposition in cattle: A review. Theriogenology 53, 1407–1414. https://doi.org/10.1016/S0093-691X(00)00283-1

López-Gatius, F., Hunter, R.H.F., 2005. Spontaneous reduction of advanced twin embryos: its occurrence and clinical relevance in dairy cattle. Theriogenology 63, 118–125. https://doi.org/10.1016/J.THERIOGENOLOGY.2004.03.006

López-Gatius, F., Santolaria, P., Yániz, J., Rutllant, J., López-Béjar, M., 2002. Factors affecting pregnancy loss from gestation day 38 to 90 in lactating dairy cows from a single herd. Theriogenology 57, 1251–1261. https://doi.org/10.1016/S0093-691X(01)00715-4

López-Gatius, F., Yániz, J., Madriles-Helm, D., 2003. Effects of body condition score and score change on the reproductive performance of dairy cows: a meta-analysis. Theriogenology 59, 801–812. https://doi.org/10.1016/S0093-691X(02)01156-1

López-Gatius, F., Santolaria, P., Yániz, J.L., Garbayo, J.M., Hunter, R.H.F., 2004. Timing of early foetal loss for single and twin pregnancies in dairy cattle. Reproduction in Domestic Animals 39, 429–433. https://doi.org/10.1111/j.1439-0531.2004.00533.x

López-Gatius, F., Szenci, O., Bech-Sàbat, G., García-Ispierto, I., Serrano, B., Santolaria, P., Yániz, J., 2009. Factors of non-infectious nature affecting late embryonic and early foetal loss in high producing dairy herds in north-eastern spain | A nem fertozo környezet egyes tényezoinek hatása a késoi embrionális és a korai magzati mortalitásra északkelet-spanyolo. Magy Allatorvosok Lapja 131, 515–531.

López-Gatius, F., Garcia-Ispierto, I., Hanzen, C., 2021. Unilateral twin pregnancy: A non-infectious factor required for the etiological diagnosis of abortion in dairy herds. Journal of Reproduction and Development 67, 337–339. https://doi.org/10.1262/JRD.2021-090

López-Gatius, F., Szenci, O., 2021. Late embryonic/early foetal mortality (LEM/EFM). In Bovine Prenatal, Perinatal and Neonatal Medicine. Hungarian Association for Buiatrics Hungary, 49-53.

Loureiro, A.P., Lilenbaum, W., 2020. Genital bovine leptospirosis: A new look for an old disease. Theriogenology 141, 41–47. https://doi.org/10.1016/J.THERIOGENOLOGY.2019.09.011

Macías-Rioseco, M., Riet-Correa, F., Miller, M.M., Sondgeroth, K., Fraga, M., Silveira, C., Uzal, F.A., Giannitti, F., 2019. Bovine abortion caused by Coxiella burnetii: report of a cluster of cases in Uruguay and review of the literature. J Vet Diagn Invest 31, 634–639. https://doi.org/10.1177/1040638719856394

Macías-Rioseco, M., Silveira, C., Fraga, M., Casaux, L., Cabrera, A., Francia, M., Robello, C., Maya, L., Zarantonelli, L., Suanes, A., Colina, R., Buschiazzo, A., Giannitti, F., Riet-Correa, F., 2020. Causes of abortion in dairy cows in Uruguay. Pesquisa Veterinaria Brasileria., 40, 325–332.

Maclachlan, N.J., Conley, A.J., Kennedy, P.C., 2000. Bluetongue and equine viral arteritis viruses as models of virus-induced fetal injury and abortion, Animal Reproduction Science.

Majazki, J., Wüppenhorst, N., Hartelt, K., Birtles, R., Von Loewenich, F.D., 2013. Anaplasma phagocytophilum strains from voles and shrews exhibit specific ankA gene sequences. BMC Vet Res 9, 27–29. https://doi.org/10.1186/1746-6148-9-235

Mao, W.H., Albrecht, E., Teuscher, F., Yang, Q., Zhao, R.Q., Wegner, J., 2008. Growth- and breed-related changes of fetal development in cattle. Asian-Australas J Anim Sci 21, 640–647. https://doi.org/10.5713/ajas.2008.70293

Marugan-Hernandez, V., 2017. Neospora caninum and Bovine Neosporosis: Current Vaccine Research. J Comp Pathol 157, 193–200. https://doi.org/10.1016/j.jcpa.2017.08.001

McCausland, I.P., Slee, K.J., Hirst, F.S., 1987. Mycotic abortion in cattle. Aust Vet J 64, 129–132. https://doi.org/10.1111/j.1751-0813.1987.tb09659.x

McClurkin, A.W., Littledike, E.T., Cutlip, R.C., Frank, G.H., Coria, M.F., Bolin, S.R., 1984. Production of cattle immunotolerant to bovine viral diarrhea virus. Canadian Journal of Comparative Medicine 48, 156–161.

McPherron, S.P., Alemseged, Z., Marean, C.W., Wynn, J.G., Reed, D., Geraads, D., Bobe, R., Béarat, H.A., 2010. Evidence for stone-tool-assisted consumption of animal tissues before 3.39 million years ago at Dikika, Ethiopia. Nature 2010 466:7308 466, 857–860. https://doi.org/10.1038/nature09248

Meadows, C., Rajala-Schultz, P.J., Frazer, G.S., 2005. A spreadsheet-based model demonstrating the nonuniform economic effects of varying reproductive performance in Ohio dairy herds. J Dairy Sci 88, 1244–1254. https://doi.org/10.3168/JDS.S0022-0302(05)72791-0

Medlock, J.M., Hansford, K.M., Bormane, A., Derdakova, M., Estrada-Peña, A., George, J.C., Golovljova, I., Jaenson, T.G.T., Jensen, J.K., Jensen, P.M., Kazimirova, M., Oteo, J.A., Papa, A., Pfister, K., Plantard, O., Randolph, S.E., Rizzoli, A., Santos-Silva, M.M., Sprong, H., Vial, L., Hendrickx, G., Zeller, H., Van Bortel, W., 2013. Driving forces for

changes in geographical distribution of Ixodes ricinus ticks in Europe. Parasit Vectors 6, 1–11. https://doi.org/10.1186/1756-3305-6-1/FIGURES/1

Mee, J.F., Berry, D.P., Cromie, A.R., 2008. Prevalence of, and risk factors associated with, perinatal calf mortality in pasture-based Holstein-Friesian cows. Animal 2, 613–620. https://doi.org/10.1017/S1751731108001699

Mee, J., 2013. Why Do So Many Calves Die on Modern Dairy Farms and What Can We Do about Calf Welfare in the Future? Animals 3, 1036–1057. https://doi.org/10.3390/ani3041036

Mee, J.F., 2015. Investigating stillbirths in cattle – What should a practitioner do? Cattle Practice 23, 114–121.

Mee, J.F., Sánchez-Miguel, C., Nosov, M., Gilmore, J., 2016. Are we misdiagnosing many Neospora and Leptospira-positive bovine aborted and stillborn foetuses using foetal serology?, in: Proceedings of the 20th Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR) and the 13th Conference of the Spanish Association for Animal Reproduction (AERA), Lisbon, Portugal.

Mee, J.F., 2020. Investigation of bovine abortion and stillbirth/perinatal mortality - Similar diagnostic challenges, different approaches. Ir Vet J. https://doi.org/10.1186/s13620-020-00172-0

Mee, J.F., Jawor, P., Stefaniak, T., 2021. Role of Infection and Immunity in Bovine Perinatal Mortality: Part 1. Causes and Current Diagnostic Approaches. Animals 2021, Vol. 11, Page 1033 11, 1033. https://doi.org/10.3390/ANI11041033

Mee, J.F., Ley, R., 2021. How to set up a postmortem facility in a farm animal practice. Livestock 26, 196–201. https://doi.org/10.12968/live.2021.26.4.196

Mee, J.F., 2023. Invited review: Bovine abortion—Incidence, risk factors and causes. Reproduction in Domestic Animals. https://doi.org/10.1111/RDA.14366

Melendez, P., Donovan, A., Hernandez, J., Bartolome, J., Risco, C.A., Staples, C., Thatcher, W.W., 2003. Milk, plasma, and blood urea nitrogen concentrations, dietary protein, and fertility in dairy cattle. J Am Vet Med Assoc 223, 628–634. https://doi.org/10.2460/JAVMA.2003.223.628

Méroc, E., Poskin, A., Van Loo, H., Quinet, C., Van Driessche, E., Delooz, L., Behaeghel, I., Riocreux, F., Hooyberghs, J., De Regge, N., Caij, A.B., Van Den Berg, T., Van Der Stede, Y., 2013a. Large-Scale Cross-Sectional Serological Survey of Schmallenberg

Virus in Belgian Cattle at the End of the First Vector Season. Transbound Emerg Dis 60, 4–8. https://doi.org/10.1111/tbed.12042

Méroc, Estelle, Poskin, A., Van Loo, H., Quinet, C., Van Driessche, E., Delooz, L., Behaeghel, I., Riocreux, F., Hooyberghs, J., De Regge, N., Cay, B., van den Berghe, T., Van Der Stede, Y., 2013b. Schmallenberg virus: Results of Belgian cross-sectional serological surveys, in: Epizone Abstracts, 7th Annual Meeting "Nothing Permanent, except Change." p. 58.

Méroc, E., Poskin, A., Van Loo, H., Van Driessche, E., Czaplicki, G., Quinet, C., Riocreux, F., De Regge, N., Caij, B., van den Berg, T., Hooyberghs, J., Van der Stede, Y., 2015. Follow-up of the Schmallenberg Virus Seroprevalence in Belgian Cattle. Transbound Emerg Dis 62, e80–e84. https://doi.org/10.1111/tbed.12202

Meyer, C.L., Berger, P.J., Koehler, K.J., Thompson, J.R., Sattler, C.G., 2001. Phenotypic trends in incidence of stillbirth for Holsteins in the United States. J Dairy Sci 84, 515–523. https://doi.org/10.3168/jds.S0022-0302(01)74502-X

Miller, 1977. A summary of some of the pathogenetic mechanisms involved in bovine abortion. Canadian Veterinary Journal 1.

Miller, J.M., Van der Maaten, M.J., 1986. Experimentally induced infectious bovine rhinotracheitis virus infection during early pregnancy: effect on the bovine corpus luteum and conceptus. Am J Vet Res 47, 223–228.

Monteiro, P. L.J., Borsato, M., Silva, F.L.M., Prata, A.B., Wiltbank, M.C., Sartori, R., 2015. Increasing estradiol benzoate, pretreatment with gonadotropin-releasing hormone, and impediments for successful estradiol-based fixed-time artificial insemination protocols in dairy cattle. J Dairy Sci 98, 3826–3839. https://doi.org/10.3168/JDS.2014-9040

Monteiro, Pedro L.J., Nascimento, A.B., Pontes, G.C.S., Fernandes, G.O., Melo, L.F., Wiltbank, M.C., Sartori, R., 2015. Progesterone supplementation after ovulation: Effects on corpus luteum function and on fertility of dairy cows subjected to AI or ET. Theriogenology 84, 1215–1224. https://doi.org/10.1016/J.THERIOGENOLOGY.2015.06.023

Muñoz-Zanzi, C.A., Hietala, S.K., Thurmond, M.C., Johnson, W.O., 2003. Quantification, risk factors, and health impact of natural congenital infection with bovine viral diarrhea virus in dairy calves. Am J Vet Res 64, 358–365.

Murray, R.D., Williams, A.J., Sheldon, I.M., 2008. Field investigation of perinatal mortality in Friesian cattle associated with myocardial degeneration and necrosis. Reproduction in Domestic Animals 43, 339–345. https://doi.org/10.1111/j.1439-0531.2007.00911.x

Murray, R.D., Patch, A., 2011. Bovine abortion Part 1: Still one of the most difficult diagnoses to make. Livestock 16, 13–17. https://doi.org/10.1111/j.2044-3870.2011.00065.x

Murray, R.D., 2015. Abortion and perinatal mortality in cattle, in: Cockcroft, P.D. (Ed.), Bovine Medicine. John Wiley & Sons, Ltd, pp. 312–322.

Muylkens, B., Thiry, J., Kirten, P., Schynts, F., Thiry, E., 2007. Bovine herpesvirus 1 infection and infectious bovine rhinotracheitis. Vet Res 38, 181–209. https://doi.org/10.1051/VETRES:2006059

Myczka, A.W., Steiner-Bogdaszewska, Ż., Filip-Hutsch, K., Oloś, G., Czopowicz, M., Laskowski, Z., 2021. Detection of Anaplasma phagocytophilum in wild and farmed cervids in poland. Pathogens 10, 13–19. https://doi.org/10.3390/pathogens10091190

Nadri S., Zamani, P., Sadeghi-Sefidmazgi, A., Abdoli, R., Ghazi Khani Shad, A., 2021. Iranian Journal of Applied Animal Science 11, 79-85

Neta, A.V.C., Mol, J.P.S., Xavier, M.N., Paixão, T.A., Lage, A.P., Santos, R.L., 2010. Pathogenesis of bovine brucellosis. Veterinary Journal 184, 146–155. https://doi.org/10.1016/J.TVJL.2009.04.010

Newcomer, B.W., 2021. 75 years of bovine viral diarrhea virus: Current status and future applications of the use of directed antivirals. Antiviral Res 196.

Nieder, M., Silaghi, ; C, Hamel, ; D, Pfister, ; K, Schmäschke, ; R, Pfeffer, ; M, 2012. Tick-borne fever caused by Anaplasma phagocytophilum in Germany First laboratory confirmed case in a dairy cattle herd. Tierärztliche Praxis Großtiere 2.

Noden, D.M., DeLahunta, A., 1985. The embryology of domestic animals: developmental mechanisms and malformations. Williams & Wilkins, Baltimore MD.

Norman, H.D., Miller, R.H., Wright, J.R., Hutchison, J.L., Olson, K.M., 2012. Factors associated with frequency of abortions recorded through Dairy Herd Improvement test plans. J Dairy Sci 95, 4074–4084. https://doi.org/10.3168/jds.2011-4998

Norquay, R., Orr, J., Norquay, B., Ellis, K.A., Mee, J.F., Reeves, A., Scholes, S., Geraghty, T., 2020. Perinatal mortality in 23 beef herds in Orkney: Incidence, risk factors and aetiology. Veterinary Record. https://doi.org/10.1136/vr.105536

Ohmann, H.B., 1981. Immunoglobulin levels in non-aborted and aborted fetuses from Danish herds of cattle. Acta Vet Scand 22, 428–434.

Ooshiro, M., Zakimi, S., Matsukawa, Y., Katagiri, Y., Inokuma, H., 2008. Detection of Anaplasma bovis and Anaplasma phagocytophilum from cattle on Yonaguni Island, Okinawa, Japan. Vet Parasitol 154, 360–364. https://doi.org/10.1016/J.VETPAR.2008.03.028

Page, L.A., 1966. Revision of the family Chlamydiaceae Rake (Rickettsiales): unification of the psittacosis-lymphogranuloma venereum-trachoma group of organisms in the genus Chlamydia Jones, Rake and Stearns. Int J Syst Bacteriol 16, 223–252. https://doi.org/10.2307/2752507

Pantchev, A., Sting, R., Bauerfeind, R., Tyczka, J., Sachse, K., 2010. Detection of all Chlamydophila and Chlamydia spp. of veterinary interest using species-specific real-time PCR assays. Comp Immunol Microbiol Infect Dis 33, 473–484. https://doi.org/10.1016/J.CIMID.2009.08.002

Pascottini, O.B., Hostens, M., Dini, P., Van Eetvelde, M., Vercauteren, P., Opsomer, G., 2016. Prevalence of cytological endometritis and effect on pregnancy outcomes at the time of insemination in nulliparous dairy heifers. J Dairy Sci 99, 9051–9056. https://doi.org/10.3168/JDS.2016-11348

Paredes, E., Moroni, M., Jara, K., (2010). Main causes of bovine abortion in the south of Chile. Proceedings of the 26th World Buiatrics Congress, Santiago, Chile, 345

Pereira-Bueno, J., Quintanilla-Gozalo, A., Seijas-Carballedo, A., Costas, E., Ortega, L.M., 2000. Observational studies in Neospora caninum infected dairy cattle: pattern of transmission and age-related antibody fluctuations. International Journal of Parasitology 30, 877–924.

Piérard, D., Levtchenko, E., Dawson, J.E., Lauwers, S., 1995. Ehrlichiosis in Belgium. Lancet 346, 1233–1234.

Pinedo, P.J., Manríquez, D., Ciarletta, C., Azocar, J., De Vries, A., 2022. Association between body condition score fluctuations and pregnancy loss in Holstein cows. J Anim Sci 100. https://doi.org/10.1093/JAS/SKAC266

Plaizier, J.C.B., King, G.J., Dekkers, J.C.M., Lissemore, K., 1997. Estimation of economic values of indices for reproductive performance in dairy herds using computer simulation. J Dairy Sci 80, 2775–2783. https://doi.org/10.3168/JDS.S0022-0302(97)76240-4

Pospischil, A., Thoma, R., Von Bomhard, W., Reitt, K., Cantieni, J., Zimmermann, D., Polkinghorne, A., 2002. Abort beim Rind durch Chlamydia psittaci. Schweiz Arch Tierheilkd 144, 467–472.

Prévot, C., Licoppe, A., 2013. Comparing red deer (Cervus elaphus L.) and wild boar (Sus scrofa L.) dispersal patterns in southern Belgium. Eur J Wildl Res 59, 795–803.

Pusterla, N., Braun, U., 1997. Clinical Findings in Cows after Experimental Infection with Ehrlichia phagocytophila. Zentralblatt fur Veterinarmedizin 44, 385–390. https://doi.org/10.1111/j.1439-0442.1997.tb01123.x

Pusterla, N., Braun, U., Wolfensberger, C., Lutz, H., 1997. Intrauterine infection with Ehrlichia phagocytophila in a cow. Vet Rec 141, 101–102. https://doi.org/10.1136/VR.141.4.101

Pusterla, N., Pusterla, J.B., Braun, U., Lutz, H., 1998. Serological, Hematologic, and PCR Studies of Cattle in an Area of Switzerland in Which Tick-Borne Fever (Caused by Ehrlichia phagocytophila) Is Endemic. Clin Diagn Lab Immunol 5, 325. https://doi.org/10.1128/CDLI.5.3.325-327.1998

Rafati, N., Mehrabani-Yeganeh, H., Hanson, T.E., 2010. Risk factors for abortion in dairy cows from commercial Holstein dairy herds in the Tehran region. Prev Vet Med 96, 3-4

Rar, V., Tkachev, S., Tikunova, N., 2021. Genetic diversity of Anaplasma bacteria: Twenty years later. Infection, Genetics and Evolution 91. https://doi.org/10.1016/J.MEEGID.2021.104833

Reese, S.T., Franco, G.A., Poole, R.K., Hood, R., Fernadez Montero, L., Oliveira Filho, R. V., Cooke, R.F., Pohler, K.G., 2020. Pregnancy loss in beef cattle: A meta-analysis. Anim Reprod Sci 212. https://doi.org/10.1016/J.ANIREPROSCI.2019.106251

Regan, E.J., Harrison, G.A.J., Butler, S., McLauchlin, J., Thomas, M., Mitchell, S., 2005. Primary cutaneous listeriosis in a veterinarian. Vet Rec 157, 207.

Reggiardo, C., Kaeberle, M.L., 1981. Detection of bacteremia in cattle inoculated with bovine viral diarrhea virus. Am J Vet Res 42, 218–21.

Reinhold, P., Sachse, K., Kaltenboeck, B., 2011. Chlamydiaceae in cattle: Commensals, trigger organisms, or pathogens? The Veterinary Journal 189, 257–267. https://doi.org/10.1016/J.TVJL.2010.09.003

Remesar, S., Díaz, P., Prieto, A., García-Dios, D., Fernández, G., López, C.M., Panadero, R., Díez-Baños, P., Morrondo, P., 2020. Prevalence and molecular characterization of

Anaplasma phagocytophilum in roe deer (Capreolus capreolus) from Spain. Ticks Tick Borne Dis 11, 101351. https://doi.org/10.1016/j.ttbdis.2019.101351

Reppert, E., Galindo, R.C., Breshears, M.A., Kocan, K.M., Blouin, E.F., de la Fuente, J., 2013. Demonstration of transplacental transmission of a human isolate of Anaplasma phagocytophilum in an experimentally infected sheep. Transbound Emerg Dis 60 Suppl 2, 93–96. https://doi.org/10.1111/TBED.12120

Revah, I., Butler, W.R., 1996. Prolonged dominance of follicles and reduced viability of bovine oocytes. J Reprod Fertil 106, 39–47. https://doi.org/10.1530/JRF.0.1060039

Ribbens, S., De Bleecker, K., Van Crombrugge, J.M., De Meulemeester, L., Mijten, E., Stoop, S., De Kruif, A., Dewulf, J., 2009. Serosurvey of four 'emerging' cattle diseases (Q-fever, Neosporosis, Leptospirosis and Salmonellosis) in Northern-Belgian dairy herds using bulk-milk samples.

Ribeiro, E., Gomes, G., Greco, L., Cerri, R., Vieira-Neto, A., Monteiro Jr, P., Lima, F., Bisinotto, R., Thatcher, W., Santos, J., 2016. Carryover effect of postpartum inflammatory diseases on developmental biology and fertility in lactating dairy cows. J Dairy Sci 99, 2201–2220. https://doi.org/10.3168/jds.2015-10337

Ribeiro, E.S., Lima, F.S., Greco, L.F., Bisinotto, R.S., Monteiro, A.P.A., Favoreto, M., Ayres, H., Marsola, R.S., Martinez, N., Thatcher, W.W., Santos, J.E.P., 2013. Prevalence of periparturient diseases and effects on fertility of seasonally calving grazing dairy cows supplemented with concentrates. J Dairy Sci 96, 5682–5697. https://doi.org/10.3168/jds.2012-6335

Roberson, M.S., Wolfe, M.W., Stumpf, T.T., K11tok, R.J., Kinder2, J.E., 1989. Luteinizing Hormone Secretion and Corpus Luteum Function in Cows Receiving Two Levels of Progesterone1. Biol Reprod 41, 997–1003.

Roelandt, S., Van Der Stede, Y., Czaplicki, G., Van Loo, H., Van Driessche, E., Dewulf, J., Hooyberghs, J., Faes, C., 2015. Serological diagnosis of bovine neosporosis: A Bayesian evaluation of two antibody ELISA tests for in vivo diagnosis in purchased and abortion cattle. Veterinary Record 176, 598. https://doi.org/10.1136/VR.102872

Rosef, O., Paulauskas, A., Radzijevskaja, J., 2009. Prevalence of Borrelia burgdorferi sensu lato and Anaplasma phagocytophilum in questing Ixodes ricinus ticks in relation to the density of wild cervids. Acta Vet Scand 51, 47. https://doi.org/10.1186/1751-0147-51-47/TABLES/4

Roth, J.A., Kaeberle, M.L., Griffith, R.W., 1981. Effects of bovine viral diarrhea virus infection on bovine polymorphonuclear leukocyte function. Am J Vet Res 42, 244–50.

Rousseau, R., Delooz, L., Dion, E., Quinet, C., Vanwambeke, S.O., 2021. Environmental determinants of Anaplasma phagocytophilum infection in cattle using a kernel density function. Ticks Tick Borne Dis 12. https://doi.org/10.1016/j.ttbdis.2021.101814

Ruhl, S., Casson, N., Kaiser, C., Thoma, R., Pospischil, A., Greub, G., Borel, N., 2009. Evidence for Parachlamydia in bovine abortion. Vet Microbiol 135, 169–174. https://doi.org/10.1016/J.VETMIC.2008.09.049

Rzewuska, M., Kwiecień, E., Chrobak-Chmiel, D., Kizerwetter-Świda, M., Stefańska, I., Gieryńska, M., 2019. Pathogenicity and virulence of trueperella pyogenes: A review. Int J Mol Sci 20. https://doi.org/10.3390/ijms20112737

Sachse, K., Bavoil, P.M., Kaltenboeck, B., Stephens, R.S., Kuo, C.C., Rosselló-Móra, R., Horn, M., 2015. Emendation of the family Chlamydiaceae: Proposal of a single genus, Chlamydia, to include all currently recognized species. Syst Appl Microbiol 38, 99–103. https://doi.org/10.1016/j.syapm.2014.12.004

Sager, H., Fischer, I., Furrer, K., Strasser, M., Waldvogel, A., Boerlin, P., Audigé, L., Gottstein, B., 2001. A Swiss case-control study to assess Neospora caninum-associated bovine abortions by PCR, histopathology and serology. Vet Parasitol 102, 1–15. https://doi.org/10.1016/S0304-4017(01)00524-6

Salopek, S., Nelson, D., McConnel, C., 2016. Factors influencing dairy veterinarian necropsy practices and their use of diagnostic laboratories. Bov Pract (Stillwater) 50, 140–152.

Samartino, L.E., Enright, F.M., 1996. Brucella abortus differs in the multiplication within bovine chorioallantoic membrane explants from early and late gestation. Comp Immunol Microbiol Infect Dis 19, 55–63. https://doi.org/10.1016/0147-9571(95)00018-6

Sammin, D., Markey, B., Bassett, H., Buxton, D., 2009. The ovine placenta and placentitis-A review. Vet Microbiol 135, 90–97. https://doi.org/10.1016/j.vetmic.2008.09.054

Sánchez-Miguel, C., Crilly, J., Grant, J., Mee, J.F., 2018. Sensitivity, specificity and predictive probability values of serum agglutination test titres for the diagnosis of Salmonella Dublin culture-positive bovine abortion and stillbirth. Transbound Emerg Dis 65, 676–686. https://doi.org/10.1111/tbed.12784

Sangsritavong, S., Combs, D.K., Sartori, R., Armentano, L.E., Wiltbank, M.C., 2002. High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17β

in dairy cattle. J Dairy Sci 85, 2831–2842. https://doi.org/10.3168/jds.S0022-0302(02)74370-1

Santos, J.E.P., Thatcher, W.W., Chebel, R.C., Cerri, R.L.A., Galvão, K.N., 2004. The effect of embryonic death rates in cattle on the efficacy of estrus synchronization programs. Anim Reprod Sci 82–83, 513–535. https://doi.org/10.1016/J.ANIREPROSCI.2004.04.015

Santos, J.E.P., Rutigliano, H.M., Filho, M.F.S., 2009. Risk factors for resumption of postpartum estrous cycles and embryonic survival in lactating dairy cows. Anim Reprod Sci 110, 207–221. https://doi.org/10.1016/J.ANIREPROSCI.2008.01.014

Sarrazin, S., Veldhuis, A., Méroc, E., Vangeel, I., Laureyns, J., Dewulf, J., Caij, A.B., Piepers, S., Hooyberghs, J., Ribbens, S., Van Der Stede, Y., 2013. Serological and virological BVDV prevalence and risk factor analysis for herds to be BVDV seropositive in Belgian cattle herds. Prev Vet Med 108, 28–37. https://doi.org/10.1016/j.prevetmed.2012.07.005

Sarrazin, S., Cay, A.B., Laureyns, J., Dewulf, J., 2014. A survey on biosecurity and management practices in selected Belgian cattle farms. Prev Vet Med 117, 129–139. https://doi.org/10.1016/J.PREVETMED.2014.07.014

Sarre, C., De Bleecker, K., Deprez, P., Levecke, B., Charlier, J., Vercruysse, J., Claerebout, E., 2012. Risk factors for Psoroptes ovis mange on Belgian Blue farms in Northern Belgium. Vet Parasitol 190, 216–221. https://doi.org/10.1016/J.VETPAR.2012.05.026

Sartori, R., Sartor-Bergfelt, R., Mertens, S.A., Guenther, J.N., Parrish, J.J., Wiltbank, M.C., 2002. Fertilization and early embryonic development in heifers and lactating cows in summer and lactating and dry cows in winter. J Dairy Sci 85, 2803-2812.

Scharf, W., Schauer, S., Freyburger, F., Petrovec, M., Schaarschmidt-Kiener, D., Liebisch, G., Runge, M., Ganter, M., Kehl, A., Dumler, J.S., Garcia-Perez, A.L., Jensen, J., Fingerle, V., Meli, M.L., Ensser, A., Stuen, S., Von Loewenich, F.D., 2011. Distinct host species correlate with Anaplasma phagocytophilum ankA gene clusters. J Clin Microbiol 49, 790–796. https://doi.org/10.1128/JCM.02051-10

Schimmer, B., Schotten, N., van Engelen, E., Hautvast, J.L.A., Schneeberger, P.M., van Duijnhoven, Y.T.H.P., 2014. Coxiella burnetii seroprevalence and risk for humans on dairy cattle farms, the Netherlands, 2010-2011. Emerg Infect Dis 20, 417–425. https://doi.org/10.3201/eid2003.131111

Schlafer, D., Foster, R., 2016. Female genital system, in: Maxie, M.G. (Ed.), Jubb, Kennedy, and Palmer's Pathology of Domestic Animals. Saunders, St. Louis, pp. 358–464.

Schlafer, D.H., Fisher, P.J., Davies, C.J., 2000. The bovine placenta before and after birth: placental development and function in health and disease. Anim Reprod Sci 60–61.

Schneider, D., 2004. Listeriosis, in: Coetzer, J.A.W., Tustin, R.C. (Eds.), Infectious Diseases of Livestock. Oxford University Press, Cape Town, pp. 1904–1907.

Seleem, M.N., Boyle, S.M., Sriranganathan, N., 2010. Brucellosis: A re-emerging zoonosis. Vet Microbiol 140, 392–398. https://doi.org/10.1016/J.VETMIC.2009.06.021

Shanks, R.D., Robinson, J.L., 1989. Embryonic Mortality Attributed to Inherited Deficiency of Uridine Monophosphate Synthase. J Dairy Sci 72, 3035–3039. https://doi.org/10.3168/jds.S0022-0302(89)79456-X

Sheridan, P., 2021. Bovine abortion. All-Island Disease Surveillance Report 2020, DAFM/AFBI, 134-137.

Shewen, P.E., Mary, S.M., 1980. Chlamydial Infection in Animals: A Review. The Canadian Veterinary Journal 21, 2.

Sigdel, A., Bisinotto, R.S., Peñagaricano, F., 2022. Genetic analysis of fetal loss in Holstein cattle. J Dairy Sci 105, 9012-9020.

Silaghi, C., Nieder, M., Sauter-Louis, C., Knubben-Schweizer, G., Pfister, K., Pfeffer, M., 2018. Epidemiology, genetic variants and clinical course of natural infections with Anaplasma phagocytophilum in a dairy cattle herd. Parasit Vectors 11. https://doi.org/10.1186/s13071-017-2570-1

Silke, V., Diskin, M.G., Kenny, D.A., Boland, M.P., Dillon, P., Mee, J.F., Sreenan, J.M., 2002. Extent, pattern and factors associated with late embryonic loss in dairy cows. Anim Reprod Sci 71, 1-2

Sinclair, K.D., Kuran, M., Gebbie, F.E., Webb, R., McEvoy, T.G., 2000. Nitrogen metabolism and fertility in cattle: II. Development of oocytes recovered from heifers offered diets differing in their rate of nitrogen release in the rumen. J Anim Sci 78, 2670–2680. https://doi.org/10.2527/2000.78102670X

Smith, K.C., 1997. Herpesviral abortion in domestic animals. The Veterinary Journal 153, 253–268. https://doi.org/10.1016/S1090-0233(97)80061-5

Smyth, J.A., McNamee, P.T., Kennedy, D.G., McCullough, S.J., Logan, E.F., Ellis, W.A., 1992. Stillbirth/perinatal weak calf syndrome: preliminary pathological, microbiological and biochemical findings. Vet Rec 130, 237–240. https://doi.org/10.1136/vr.130.12.237

Smyth, J.A., Fitzpatrick, D.A., Ellis, W.A., 1999. Stillbirth/perinatal weak calf syndrome: A study of calves infected with Leptospira. Veterinary Record 145, 539–542. https://doi.org/10.1136/vr.145.19.539

Sohier, C., Deblauwe, I., Van Loo, T., Hanon, J.B., Cay, A.B., De Regge, N., 2017. Evidence of extensive renewed Schmallenberg virus circulation in Belgium during summer of 2016 – increase in arthrogryposis-hydranencephaly cases expected. Transbound Emerg Dis 64, 1015–1019. https://doi.org/10.1111/TBED.12655

Spiteri, C.D., Gillis, R.E., Roffet-Salque, M., Navarro, L.C., Guilaine, J., Manen, C., Muntoni, I.M., Segui, M.S., Urem-Kotsou, D., Whelton, H.L., Craig, O.E., Vigne, J.D., Evershed, R.P., 2016. Regional asynchronicity in dairy production and processing in early farming communities of the northern Mediterranean. Proc Natl Acad Sci U S A 113, 13594–13599. https://doi.org/10.1073/PNAS.1607810113/SUPPL_FILE/PNAS.1607810113.ST06.DOCX

SRUC, 2022. Review of bovine stillbirth and abortion investigations in Scotland. The Vet Rec, 190, 233.

Staub, E., Marti, H., Biondi, R., Levi, A., Donati, M., Leonard, C.A., Ley, S.D., Pillonel, T., Greub, G., Seth-Smith, H.M.B., Borel, N., 2018. Novel Chlamydia species isolated from snakes are temperature-sensitive and exhibit decreased susceptibility to azithromycin. Sci Rep 8, 1–14. https://doi.org/10.1038/s41598-018-23897-z

Stigum, V.M., Jaarsma, R.I., Sprong, H., Rolandsen, C.M., Mysterud, A., 2019. Infection prevalence and ecotypes of Anaplasma phagocytophilum in moose Alces alces, red deer Cervus elaphus, roe deer Capreolus capreolus and Ixodes ricinus ticks from Norway. Parasit Vectors 12, 1–8. https://doi.org/10.1186/s13071-018-3256-z

Storz, J., Carroll, E.J., Ball, L., Faulkner, L.C., 1968. Isolation of a psittacosis agent (Chlamydia) from semen and epididymitis of bulls with seminal vesiculitis syndrome. Am J Vet Res 29, 54–555.

Stuen, S., Bergström, K., 2001. The Effect of Two Different Oxytetracycline Treatments in Experimental Ehrlichia phagocytophila Infected Lambs. Acta Vet Scand 42, 339–346.

Stuen, S., Granquist, E.G., Silaghi, C., 2013. Anaplasma phagocytophilum—a widespread multi-host pathogen with highly adaptive strategies. Front Cell Infect Microbiol 3. https://doi.org/10.3389/FCIMB.2013.00031

Szulowski, K., Iwaniak, W., Weiner, M., Złotnicka, J., 2013. Brucella suis biovar 2 isolations from cattle in Poland. Annals of Agricultural and Environmental Medicine 20, 672–675.

Tack, W., Madder, M., Baeten, L., De Frenne, P., Verheyen, K., 2012. The abundance of Ixodes ricinus ticks depends on tree species composition and shrub cover. Parasitology 139, 1273–1281.

Tavernier, P., Sys, S.U., De Clercq, K., De Leeuw, I., Caij, A.B., De Baere, M., De Regge, N., Fretin, D., Roupie, V., Govaerts, M., Heyman, P., Vanrompay, D., Yin, L., Kalmar, I., Suin, V., Brochier, B., Dobly, A., De Craeye, S., Roelandt, S., Goossens, E., Roels, S., 2015. Serologic screening for 13 infectious agents in roe deer (Capreolus capreolus) in Flanders. Infect Ecol Epidemiol 5. https://doi.org/10.3402/IEE.V5.29862

Taylor, R.F., Njaa, Bradley L., 2012. General Approach to fetal and neonatal loss, in: Njaa, Bradley L; (Ed.), Kirkbride's Diagnosis of Abortion and Neonatal Loss in Animals. John Wiley & Sons, Oxford: UK, pp. 1–12.

Taylor-Brown, A., Bachmann, N.L., Borel, N., Polkinghorne, A., 2016. Culture-independent genomic characterisation of Candidatus Chlamydia sanzinia, a novel uncultivated bacterium infecting snakes. BMC Genomics 17. https://doi.org/10.1186/s12864-016-3055-x

Taylor-Brown, A., Spang, L., Borel, N., Polkinghorne, A., 2017. Culture-independent metagenomics supports discovery of uncultivable bacteria within the genus Chlamydia. Sci Rep 7, 1–9. https://doi.org/10.1038/s41598-017-10757-5

Thobokwe, G., Heuer, C., 2011. Incidence of abortion and association with putative causes in dairy herds in New Zealand. http://dx.doi.org/10.1080/00480169.2004.36411 52, 90–94. https://doi.org/10.1080/00480169.2004.36411

Thomas, K., Kibona, T., Claxton, J., de Glanville, W., Lankester, F., Amani, N., Buza, J., Carter, R., Chapman, G., Crump, J., Dagleish, P., Halliday, J., Hamilton, C., Innes, E., Katzer, F., Livingstone, M., Longbottom, D., Millins, C., Mmbaga, B.T., Mosha, V., Nyarobi, J., Nyasebwa O.M., Russell, G.C., Sanka, P.N., Semango, P., Wheelhouse, N., Willett, B.J., Cleaveland, S., Allan, K.J., 2022. Prospective cohort study reveals unexpected aetiologies of livestock abortion in northern Tanzania. Sci Rep, 12, 11669.

Thomsen, P.T., Capion, N., Foldager, L., 2020. Higher odds of abortion in dairy cows hoof trimmed late in gestation. Res Vet Sci 133, 1-3

Thorning, T.K., Raben, A., Tholstrup, T., Soedamah-Muthu, S.S., Givens, I., Satrup, A., 2016. Milk and dairy products: Good or bad for human health? An assessment of the totality of scientific evidence. Food Nutr Res 60.

Thurmond, M.C., Blanchard, P.C., Anderson, M.L., 1994. An example of selection bias in submissions of aborted bovine fetuses to a diagnostic laboratory. J Vet Diagn Invest 6, 269–271. https://doi.org/10.1177/104063879400600224

Thurmond, M.C., Anderson, M.L., Blanchard, P.C., 1995. Secular and seasonal trends of Neospora abortion in California dairy cows. J Parasitol 81, 364–7.

Tipples, R., Trafford, S., Callister, P., 2010. The factors which have resulted in migrant workers being "essential" workers on New Zealand dairy farms, in: Labour, Employment and Work Conference.

Tipples, R., Hill, R., Wilson, K., 2012. How did dairy fatigue research come about and what are we doing?

Tomaiuolo, S., Boarbi, S., Fancello, T., Michel, P., Desqueper, D., Grégoire, F., Callens, J., Fretin, D., Devriendt, B., Cox, E., Mori, M., 2021. Phylogeography of Human and Animal Coxiella burnetii Strains: Genetic Fingerprinting of Q Fever in Belgium. Front Cell Infect Microbiol 10, 625576. https://doi.org/10.3389/FCIMB.2020.625576/BIBTEX

Toussaint, J.F., Sailleau, C., Mast, J., Houdart, P., Czaplicki, G., Demeestere, L., VandenBussche, F., Van Dessel, W., Goris, N., Bréard, E., Bounaadja, L., Thiry, E., Zientara, S., De Clercq, K., 2007. Bluetongue in Belgium, 2006. Emerg Infect Dis 13, 614–616. https://doi.org/10.3201/eid1304.061136

Toyokawa, T., Ohnishi, M., Koizumi, N., 2011. Diagnosis of acute leptospirosis. Expert Rev Anti Infect Ther. https://doi.org/10.1586/eri.10.151

Trees, A.J., Davison, H.C., Innes, E.A., Wastling, J.M., 1999. Towards evaluating the economic impact of bovine neosporosis. International Journal of Parasitology 29, 1195–1200.

Tsoumani, M.E., Papailia, S.I., Papageorgiou, E.G., Voyiatzaki, C., 2023. Climate Change Impacts on the Prevalence of Tick-Borne Diseases in Europe, in: COMECAP 2023. MDPI, Basel Switzerland, p. 18. https://doi.org/10.3390/environsciproc2023026018

Tuomi, J., 1967. Experimental studies on bovine tick-borne fever. 1. Clinical and haematological data, some properties of the causative agent, and homologous immunity. Acta Pathol Microbiol Scand 70, 429–445.

Turin, L., Surini, S., Wheelhouse, N., Rocchi, M.S., 2022. Recent advances and public health implications for environmental exposure to Chlamydia abortus: from enzootic to zoonotic disease. Veterinary Research 2022 53:1 53, 1–17. https://doi.org/10.1186/S13567-022-01052-X

van Andel, M., Jones, G., Buckle, K., Phiri, B., McFadden, A., Dacre, I., Bingham, P., Heuer, C., Abila, R., Win, H.H., Lwin, K.O., Binney, B., Zaari, S., Gates, M.C., 2020. Estimating foot-and-mouth disease (FMD) prevalence in central Myanmar: Comparison of village headman and farmer disease reports with serological findings. Transbound Emerg Dis 67, 778–791. https://doi.org/10.1111/TBED.13397

Van Loo, H., Méroc, E., Cay, B., Van de Stede, Y., De Bleecker, K., 2013. Use of abortion surveillance in cattle to verify the introduction of Schmallenberg Virus in Flanders., in: Epizone Abstracts, 7th Annual Meeting "Nothing Permanent, except Change." p. 101.

Van Loo, H., Pardon, B., De Schutter, P., De Bleecker, K., Vanrompay, D., Deprez, P., Maris, J., 2014. Detection of Chlamydia psittaci in Belgian cattle with signs of respiratory disease and milk drop syndrome. Veterinary Record 175, 562. https://doi.org/10.1136/VR.102527

Van Loo, H., Pascottini, O.B., Ribbens, S., Hooyberghs, J., Pardon, B., Opsomer, G., 2021. Retrospective study of factors associated with bovine infectious abortion and perinatal mortality. Prev Vet Med 105366. https://doi.org/10.1016/j.prevetmed.2021.105366

Van Loock, M., Verminnen, K., Messmer, T.O., Volckaert, G., Goddeeris, B.M., Vanrompay, D., 2005. Use of a nested PCR-enzyme immunoassay with an internal control to detect Chlamydophila psittaci in turkeys. BMC Infect Dis 5, 1–9. https://doi.org/10.1186/1471-2334-5-76/TABLES/3

van Roon, A.M., Mercat, M., van Schaik, G., Nielen, M., Graham, D.A., More, S.J., Guelbenzu-Gonzalo, M., Fourichon, C., Madouasse, A., Santman-Berends, I.M.G.A., 2020. Quantification of risk factors for bovine viral diarrhea virus in cattle herds: A systematic search and meta-analysis of observational studies. J Dairy Sci 103, 9446–9463. https://doi.org/10.3168/jds.2020-18193

Vandekerkhove, K., 2013. Integration of Nature Protection in Forest Policy in Flanders (Belgium). Freiburg.

Vangeel, I., Méroc, E., Roelandt, S., Welby, S., Driessche, E. V., Czaplicki, G., Schoubroeck, L. V., Quinet, C., Riocreux, F., Hooyberghs, J., Houdart, P., Stede, Y. V.D., 2012.

Seroprevalence of Neospora caninum, paratuberculosis and Q fever in cattle in Belgium in 2009-2010. Veterinary Record 171, 477. https://doi.org/10.1136/vr.101016

Vasconcelos, J.L.M., Sangsritavong, S., Tsai, S.J., Wiltbank, M.C., 2003. Acute reduction in serum progesterone concentrations after feed intake in dairy cows. Theriogenology 60, 795–807. https://doi.org/10.1016/S0093-691X(03)00102-X

Vor, T., Kiffner, C., Hagedorn, P., Niedrig, M., Rühe, F., 2010. Tick burden on European roe deer (Capreolus capreolus). Exp Appl Acarol 51, 405–417. https://doi.org/10.1007/S10493-010-9337-0/TABLES/5

Vorimore, F., Hsia, R. ching, Huot-Creasy, H., Bastian, S., Deruyter, L., Passet, A., Sachse, K., Bavoil, P., Myers, G., Laroucau, K., 2013. Isolation of a New Chlamydia species from the Feral Sacred Ibis (Threskiornis aethiopicus): Chlamydia ibidis. PLoS One 8, 1–11. https://doi.org/10.1371/journal.pone.0074823

Waldner, C.L., Kennedy, R.I., Rosengren, L.B., Pollock, C.M., Clark, E.G., 2010. Article Gross postmortem and histologic examination findings from abortion losses and calf mortalities in western Canadian beef herds, Can Vet J 51, 11.

Waldner, C.L., 2014. Cow attributes, herd management and environmental factors associated with the risk of calf death at or within 1 h of birth and the risk of dystocia in cow–calf herds in Western Canada. Livestock Science 163, 126-139.

Waldner, C.L., Parker, S., Campbell, J.R., 2019. Identifying performance benchmarks and determinants for reproductive performance and calf survival using a longitudinal field study of cow-calf herds in western Canada. PLoS One 14, 7

Walker, E., Lee, E.J., Timms, P., Polkinghorne, A., 2015. Chlamydia pecorum infections in sheep and cattle: A common and under-recognised infectious disease with significant impact on animal health. https://doi.org/10.1016/j.tvjl.2015.09.022

Wang, C., Gao, D., Kaltenboeck, B., 2009. Acute Chlamydia pneumoniae Reinfection Accelerates the Development of Insulin Resistance and Diabetes in Obese C57BL/6 Mice. J Infect Dis 200, 279–287. https://doi.org/10.1086/599796

Wang, F.I., Shieh, H., Liao, Y.K., 2001. Prevalence of Chlamydophila abortus infection in domesticated ruminants in Taiwan. J Vet Med Sci 63, 1215–1220. https://doi.org/10.1292/JVMS.63.1215

Welby, S., Letellier, C., Fretin, D., Hooyberghs, J., Vanholme, L., Godfroid, J., Vanderstede, Y., 2009. Evaluation du programme de surveillance pour la brucellose et la leucose bovine enzootique en Belgique. Epidémiologie et Santé Animale 55, 1–6.

Wernike, K., Holsteg, M., Schirrmeier, H., Hoffmann, B., Beer, M., 2014. Natural Infection of Pregnant Cows with Schmallenberg Virus – A Follow-Up Study. PLoS One 9, e98223. https://doi.org/10.1371/JOURNAL.PONE.0098223

Wheelhouse, N., Katzer, F., Wright, F., Longbottom, D., 2010. Novel chlamydia-like organisms as cause of bovine abortions, UK. Emerg Infect Dis 16, 1323–1324.

Wheelhouse, N., Howie, F., Gidlow, J., Greub, G., Dagleish, M., Longbottom, D., 2012. Involvement of Parachlamydia in bovine abortions in Scotland. The Veterinary Journal 193, 586–588. https://doi.org/10.1016/J.TVJL.2012.01.008

Wheelhouse, N., Mearns, R., Willoughby, K., Wright, E., Turnbull, D., Longbottom, D., 2015. Evidence of members of the Chlamydiales in bovine abortions in England and Wales. Vet Rec 176, 465. https://doi.org/10.1136/vr.103075

Wheelhouse, N., Hearn, J., Livingstone, M., Flockhart, A., Dagleish, M., Longbottom, D., 2022. Identification of Parachlamydiaceae DNA in nasal and rectal passages of healthy dairy cattle. J Appl Microbiol 132, 2642–2648. https://doi.org/10.1111/JAM.15422

Wickham, H., 2009. ggplot2 Elegant Graphics for Data Analysis, 1st ed. Springer-Verlag New York, New York.

Willock, J., Deary, I.J., Edwards-Jones, G., Gibson, G.J., McGregor, M.J., Sutherland, A., Dent, J.B., Morgan, O., Grieve, R., 1999a. The Role of Attitudes and Objectives in Farmer Decision Making: Business and Environmentally-Oriented Behaviour in Scotland. J Agric Econ 50, 286–303.

Willock, J., Deary, I.J., McGregor, M.J., Sutherland, A., Edwards-Jones, G., Morgan, O., Dent, J.B., Grieve, R., Gibson, G.J., Austin, E., 1999b. Farmers' Attitudes, Objectives, Behaviors, and Personality Traits: The Edinburgh Study of Decision Making on Farms. J Vocat Behav 54, 5–36.

Wilson, A., Darpel, K., Mellor, P.S., 2008. Where does bluetongue virus sleep in the winter? PLoS Biol. https://doi.org/10.1371/journal.pbio.0060210

Wilson, D.J., Orsel, K., Waddington, J., Rajeev, M., Sweeny, A.R., Joseph, T., Grigg, M.E., Raverty, S.A., 2016. Neospora caninum is the leading cause of bovine fetal loss in British Columbia, Canada. Vet Parasitol 15, 46-51.

Wilson, J.C., Foggie, A., Carmichael, M.A., 1964. Tick-borne fever as a cause of abortion and stillbirhts in cattle. Vet Rec 76, 1081–1081.

Wiltbank, M.C., Garcia-Guerra, A., Carvalho, P.D., Hackbart, K.S., Bender, R.W., Souza, A.H., Toledo, M.Z., Baez, G.M., Surjus, R.S., Sartori, R., 2014. Effects of energy and protein nutrition in the dam on embryonic development. Anim. Reprod., v 11, 168–182.

Wiltbank, M.C., Baez, G.M., Garcia-Guerra, A., Toledo, M.Z., Monteiro, P.L.J., Melo, L.F., Ochoa, J.C., Santos, J.E.P., Sartori, R., 2016. Pivotal periods for pregnancy loss during the first trimester of gestation in lactating dairy cows. Theriogenology 86, 239–253. https://doi.org/10.1016/J.THERIOGENOLOGY.2016.04.037

WOAH (Ed.), 2022. Brucellosis, in: Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. pp. 1–48.

Woldehiwet, Z., 1987. The effects of tick-borne fever on some functions of polymorphonuclear cells of sheep. J Comp Pathol 97, 481–485. https://doi.org/10.1016/0021-9975(87)90026-0

Woldehiwet, Z., 2006. Anaplasma phagocytophilum in ruminants in Europe. Ann N Y Acad Sci 1078, 446–460. https://doi.org/10.1196/ANNALS.1374.084

Woldehiwet, Z., 2010. The natural history of Anaplasma phagocytophilum. Vet Parasitol 167, 108–122. https://doi.org/10.1016/J.VETPAR.2009.09.013

Wolf-Jäckel, G.A., Hansen, M.S., Larsen, G., Holm, E., Agerholm, J.S., Jensen, T.K., 2020. Diagnostic studies of abortion in Danish cattle 2015-2017. Acta Vet Scand 62, 1. https://doi.org/10.1186/s13028-019-0499-4

Wouda, W., Moen, A.R., Visser, I.J., van Knapen, F., 1997. Bovine fetal neosporosis: a comparison of epizootic and sporadic abortion cases and different age classes with regard to lesion severity and immunohistochemical identification of organisms in brain, heart, and liver. J Vet Diagn Invest 9, 180–5. https://doi.org/10.1177/104063879700900212

Wouda, W., Bartels, C.J.M., Moen, A.R., 1999. Characteristics of Neospora caninum-associated abortion storms in dairy herds in the Netherlands (1995 to 1997). Theriogenology 52, 233–245. https://doi.org/10.1016/S0093-691X(99)00125-9

Yaeger, M.J., Holler, L.D., 2007. Bacterial causes of bovine infertility and abortion, in: Youngquist, R.S., Threlfall, W.R. (Eds.), Current Therapy in Large Animal Theriogenology. WB Saunders Co, pp. 389–398.

Yagupsky, P., 1999. Detection of brucellae in blood cultures. J Clin Microbiol 37, 3437–3442. https://doi.org/10.1128/jcm.37.11.3437-3442.1999

Yin, L., Schautteet, K., Kalmar, I.D., Bertels, G., Van Driessche, E., Czaplicki, G., Borel, N., Longbottom, D., Frétin, D., Dispas, M., Vanrompay, D., 2014. Prevalentie van Chlamydia abortus bij herkauwers in België. Vlaams Diergeneeskd Tijdschr 83, 164–170. https://doi.org/10.21825/VDT.V83I4.16642

Chapter 2

Scientific Aims

The general objective of this PhD thesis was to broaden our knowledge on the epidemiology of bovine abortion and perinatal mortality (APM) in Belgium, particularly in Flanders. More specifically, we aimed to study the main infectious agents involved in APM in this region, based on the analyses of cases that were submitted as part of the national mandatory abortion monitoring program. Moreover, we aimed to evaluate trends in case submissions in the context of this abortion monitoring program.

In the first section (**Chapter 3**), we evaluated the compliance of farmers with the current mandatory abortion monitoring program. To do so, APM submissions between 2006 and 2021 were analysed, and factors which might influence the number of submitted APM cases were identified.

In the second section (**Chapter 4**), we studied the prevalence of the most important infectious causes of bovine APM in Flanders, based on the analytical results of the abortion monitoring program. We also determined differences in production type (dairy versus beef), gestation length, parity, and seasonality of the different pathogens.

In the third section, the presence of the zoonotic abortifacient pathogens *Anaplasma phagocytophilum*, *Chlamydia* spp., and *Chlamydia*-like organisms was studied. In a first study (**Chapter 5**), we investigated the presence of *Anaplasma phagocytophilum* in placental and fetal spleen samples from bovine cases of APM. Subsequently (**Chapter 6**), we analysed the presence of *Chlamydia* spp. (*Chlamydia abortus* and *Chlamydia psittaci*) and *Parachlamydia acanthamoebae* in bovine placental tissue, and the potential involvement of these pathogens in APM.

The scientific aims of this thesis are summarized in **Figure 2.1.**

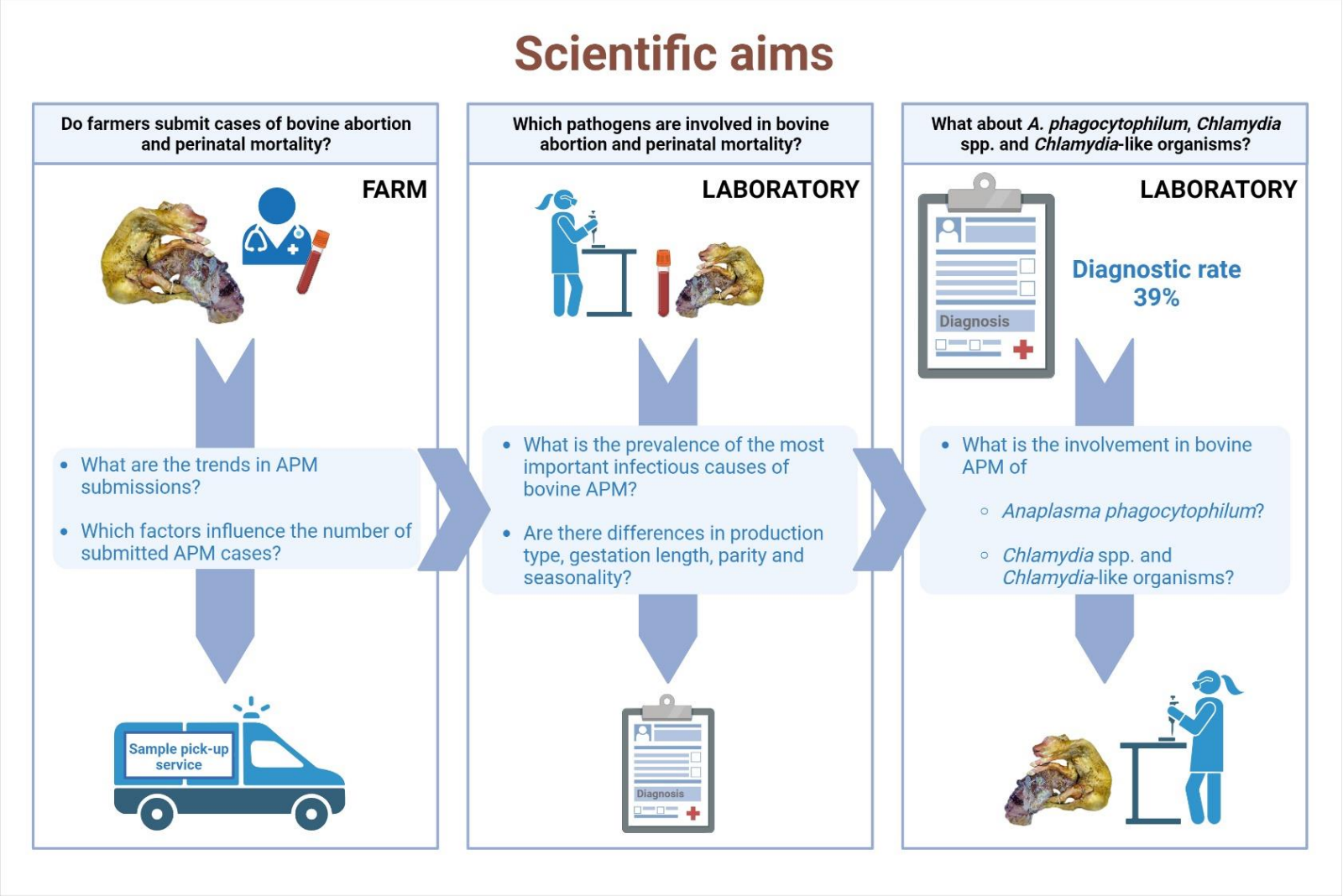

Figure 2.1. Graphical summary of the scientific aims.

Chapter 3

Enhancing bovine abortion surveillance: a learning experience

Hans Van Loo[1], Osvaldo Bogado Pascottini[1], Stefaan Ribbens[2], Jozef Hooyberghs[3], Geert Opsomer[1] and Bart Pardon[1]

[1] Department of Internal Medicine, Reproduction and Population Medicine, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium
[2] Animal Health Services Flanders (Dierengezondheidszorg Vlaanderen), Torhout, Belgium
[3] Federal Agency for the Safety of the Food Chain, Brussels, Belgium

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Abstract

Abortions and perinatal mortalities (APM) significantly impact cattle industry efficiency. Various infectious and non-infectious factors have been associated with bovine APM worldwide. Infections are often considered pivotal due to their abortifacient potential, leading laboratories to primarily investigate relevant infectious agents for APM cases. Some infectious causes, like *Brucella abortus*, have also a zoonotic impact, necessitating monitoring for both animal and human health. However, under-reporting of bovine APM is a global issue, affecting early detection of infectious and zoonotic causes. Previous studies identified factors influencing case submission, but regional characteristics may impact results. In Belgium, farmers are obliged to report cases of APM within the context of a national brucellosis monitoring program. The inclusion criteria for this monitoring program cover abortions (gestation length of 42–260 days) and perinatal mortalities of (pre)mature calves following a gestation length of more than 260 days, which were stillborn or died within 48 h after birth. The objective of the present study was to describe the evolution in submission of APM cases within a mandatory abortion monitoring program in relation to subsidised initiatives in the northern part of Belgium. Based on the proportion of APM submissions versus the proportion of bovine reproductive females, an APM proportion (APM_{PR}) was calculated, and factors at both animal and herd level which may influence this APM_{PR} were explored by using linear models. This evaluation revealed that the APM_{PR} increased with the introduction of an extensive analytical panel of abortifacient agents and a free on-farm sample collection from 0.44 to 0.94%. Additionally, an increase of the APM_{PR} was associated with an outbreak of an emerging abortifacient pathogen (Schmallenberg virus) (1.23%), and the introduction of a mandatory eradication program for bovine viral diarrhea virus (BVDv) (1.20%). The APM_{PR} was higher in beef compared to dairy cattle, and it was higher in winter compared to fall, spring, and summer. Smaller herds categorized in the first quartile had a higher APM_{PR} compared to larger herds. Herds that submitted an APM in the previous year had a higher APM_{PR} in the next year compared to herds without an APM submission. Finally, herds for which there was evidence of the presence of BVDv had a higher APM_{PR} compared to herds without evidence of the presence of BVDv. In conclusion, the number of APM submissions increased after the introduction of a free on-farm sample collection and an extensive pathogen screening panel. Production type (beef), season (winter), smaller herd size, previous APM, and presence of BVDv seemed to have a positive effect on APM_{PR}. However, even under mandatory circumstances, APM still seems to be under-

reported, since the APM_{PR} was lower than the expected minimal rate of 2%. Therefore, further research to identify the drivers that convince farmers to submit APM cases in order to improve submission rates and ensure an efficient monitoring program for APM and eventually associated zoonotic pathogens is necessary.

Key words: cattle; abortion; perinatal mortality; disease monitoring.

Introduction

Abortions and perinatal mortalities (APM) have a major economic impact on reproduction and production efficiency in the cattle industry. The term “abortion” typically refers to pregnancy loss in the fetal stage, which spans from 42 to 260 d of gestation, including early fetal loss (EFL) occurring between 45 and 60 d of gestation, and late fetal loss (LFL) occurring between 60 and 260 d of gestation. In dairy cattle, the expected incidence of EFL is around 7%, while LFL ranges from 1 to 3% (Wiltbank et al., 2016; Albaaj et al., 2023). However, for beef cattle, specific abortion thresholds have not been determined. Perinatal mortality has been defined as the loss of a non-viable fetus beyond 260 d of gestation, and deaths of full-term calves up to 48 h of age (Mee, 2013). In dairy cattle, the perinatal mortality rate ranges from 2.4 to 9.7%, with a median of 6.6% (Cuttance and Laven, 2019), while this rate also remains unknown for beef cattle.

Many infectious and non-infectious factors have been described to be involved in bovine APM in different regions and countries worldwide (Mee, 2013; Clothier & Anderson, 2016; Wolf-Jäckel et al., 2020; Van Loo et al., 2021). Although non-infectious causes are important, especially in cases of perinatal mortality (Jawor et al., 2017), infections are generally considered to be more relevant due to their significant abortifacient potential. As a result, in most laboratories, the primary emphasis is placed on investigating the infectious agents that are most relevant and prevalent in a particular region when studying cases of APM. Depending on the region, numerous infectious agents may be involved in bovine APM, including bacteria (e.g., *Brucella abortus*, *Pajaroellobacter abortibovis*, and *Trueperella pyogenes*), yeasts/molds, viruses (e.g., bovine herpes virus type 1, bovine viral diarrhea virus, and Schmallenbergvirus), and parasites (e.g., *Neospora caninum*). Several infectious causes of APM have also a zoonotic impact (e.g., *Brucella abortus*, *Salmonella* spp., *Coxiella burnetii*, *Chlamydia* spp.), which makes monitoring of infectious causes of APM crucial for both animal and human health. As for all diseases, the monitoring of APM relies on the notification and reporting of suspected cases (Bronner et al., 2014, 2013). However, under-reporting of detected bovine APM is a major issue worldwide, although it is mandatory to report in many countries. For instance, in Canada and France it was reported that less than 40% of farmers were motivated to submit cases of APM for analysis (Bronner et al., 2014; Denis-Robichaud et al., 2019), while in New Zealand, it was only 5.5% (Thobokwe and Heuer, 2011). This under-reporting may hamper the

early detection of zoonotic and other infectious causes of APM. Previous studies have identified several factors that influence the decision of farmers and veterinarians to submit APM cases, such as the perceived risk of causes of APM, the adopted definition of APM, the cost-benefit of case analysis, and practical considerations related to submitting a case (Bronner et al., 2014; Clothier et al., 2020). However, it is important to note that the results of these studies may be affected by regional characteristics. Therefore, the objectives of the present study were, 1) to evaluate trends in APM submissions and 2) to identify factors which may influence the number of submitted APM cases in the northern region of Belgium (Flanders).

Materials and methods

Background

In Belgium, the prevalence of bovine brucellosis has significantly decreased over the years, leading to the country being declared officially free of the disease by the European Union in 2003 (Commission Decision 2003/467/EC). However, surveillance remains essential, as demonstrated by the re-emergence of *Brucella* outbreaks in Belgium between 2010 and 2013. Since 1978 onwards, one of the main pillars of bovine brucellosis surveillance in Belgium has been the obligatory reporting of cases of APM by farmers to their corresponding regional sanitary veterinarian, responsible for the epidemiological surveillance of the herd. The inclusion criteria for this APM monitoring program cover abortions (gestation length of 42–260 d) and perinatal mortalities of (pre)mature calves following a gestation length of more than 260 d, which were stillborn or died within 48 h after birth. When a farmer reports an APM case, the sanitary veterinarian must collect a blood sample from the corresponding dam. This blood sample is sent to one of the accredited laboratories along with the associated fetus/calf and/or placenta for *Brucella*-specific analyses. The Budgetary Fund for the Health and Quality of Animals and Animal Products funds the sampling by the veterinarian, while the Federal Agency for the Safety of the Food Chain (FASFC) funds the laboratory *Brucella*-specific analyses.

At the end of 2009, the APM monitoring program was expanded to encompass a broader spectrum of analyses (**Table 3.1.**). This extension allowed for the monitoring of the most relevant infectious causes of APM in the country (Van Loo et al., 2021). Simultaneously, to streamline the process and increase efficiency, daily (weekends/holidays not included) sample collection (including maternal blood, placenta, and fetus/calf samples) at the farm level was organized by the diagnostic laboratories. As an incentive, the FASFC fully funds both the on-farm sample pick up, and the newly introduced laboratory analyses, in addition to the *Brucella*-specific analyses that were already funded. To stimulate the reporting of bovine APM, a communication campaign to emphasize the importance of APM monitoring was initiated at the end of 2009. This campaign involved distributing information leaflets that highlighted the importance of reporting and submitting cases of APM. Moreover, the renewed APM monitoring program and its related regulations were communicated to cattle farmers, farmer associations, and bovine veterinary practitioners through leaflets, newsletters, and articles in agricultural and veterinary media. Additionally, two veterinarians were hired and stationed at accredited

diagnostic regional laboratories. These veterinarians visited cattle herds facing APM issues, particularly those with an annual abortion rate exceeding 5%, or those experiencing a cluster of APM cases within a short time period. During dedicated meetings organized by the involved laboratories, they also educated farmers and their veterinarians on the topic. Between 2010 and 2021, the Belgian APM monitoring program underwent several revisions, resulting in the removal of some analyses (**Table 3.1.**), mainly because of budget reallocations. However, other analyses, such as a PCR test for Schmallenberg virus (SBv) for cases with arthrogryposis-hydranencephaly syndrome, were included.

Table 3.1. Background of the Belgian abortion monitoring program between the years 1978 and 2021, showing the different pathogens. Blue bars indicate in which time period each analysis was performed. The orange line indicates when the extensive abortion monitoring program was introduced.

			Time period					
Pathogen	**Test**	**Sample**	**1978 - 10/2009**	**10/2009 - 10/2011**	**10/2011 - 01/2012**	**01/2012 - 03/2012**	**03/2012 - 04/2014**	**04/2014 - 2021**
Brucella abortus	ELISA Ab	Maternal serum						
	SAT Ab	Maternal serum						
	ZN staining	Fetal abomasal content or placenta						
	Culture	Fetal abomasal content or placenta						
BVDv	ELISA Ab	Maternal serum						
	ELISA Ag	Fetal spleen						
BHV-1	ELISA Ab	Maternal serum						
BTv	PCR	Fetal spleen						
SBv	PCR	Fetal spleen						
Aerobic bacteria	Culture	Fetal abomasal content and lungs						
	Histology	Fetal lungs						
Coxiella burnetiid	ELISA Ab	Maternal serum						
	PCR	Fetal abomasal content						
L. interrogans serovar Hardjo	ELISA Ab	Maternal serum						
Listeria spp.	Culture	Fetal abomasal content and lungs						
Yeast/mold	Culture	Fetal abomasal content						
Neospora caninum	ELISA Ab	Maternal serum						
	Histology	Fetal heart and brain						

BVDv = bovine viral diarrhea virus, BHV-1 = bovine herpes virus type 1, BTv = bluetongue virus, SBv = Schmallenberg virus, *L. interrogans* serovar Hardjo = *Leptospira interrogans* serovar Hardjo, ELISA = enzyme-linked immunosorbent assay, SAT = serum agglutination test, Ab = antibody, Ag = antigen, ZN = Ziehl Neelsen, PCR = polymerase chain reaction

It is worth investigating whether the new APM monitoring program, its revisions, or other events (such as the outbreak of an emerging abortifacient agent, or the introduction of an eradication program for a specific abortifacient agent) may have affected the number of submitted APM cases. Therefore, a longitudinal observational retrospective cohort study was conducted, utilizing two datasets. The first dataset (Dataset 1) covered the time period between January 2006 and December 2021, while the second dataset (Dataset 2) covered the time period between January 2009 and December 2021. Both datasets included the total number of bovine APM submissions, and the total number of calf births in Flanders. The number of APM submissions was extracted from the regional laboratory information management system, and the number of births was available from the national identification and registration system (Sanitel). Notably, cases of APM were not registered as births in Sanitel.

Dataset 1 (Type-Year-Season)

For this dataset, the total number of bovine APM submissions and the total number of calf births per month in Flanders were available per production type (dairy, beef, and double purpose) for the time period between January 2006 and December 2021. The production type for each birth and APM case was extracted from the national identification and registration system. The month of submission of APM cases was used to group the APM cases per meteorological season. Winter cases were submitted from December to February, spring cases from March to May, summer cases from June to August, and fall cases from September to November.

Data from double purpose cattle (809,710 births and 2,142 APM cases) were excluded from statistical analyses, because it was not clear under which type of management these animals were kept.

Dataset 2 (Herd)

For this dataset, the number of APM submissions and the number of births per year for each active cattle herd in Flanders were collected for the time period between January 2009 and December 2021. A cattle herd was defined as active when at least 1 birth or APM event was registered each consecutive year from 2009 until 2021. A total of 13,664 active herds were available for this time period. Herds with less than 25 reproductive females (cows that gave birth or experienced an APM event) each consecutive year were considered as non-professional, and were excluded from the data set, resulting in a final study population of 4,164 herds. For each year, herds were grouped in quartiles based on herd size, i.e., the number of reproductive

females. Herds smaller than the first quartile were classified as small. Medium-sized herds had a total number of bovine females in reproductive age that fell in the interquartile range, while large herds were larger than the third quartile. Depending on the year, the first quartile was found between 44 and 53, and the third quartile ranged between 80 and 120 bovine females in reproductive age.

Starting from 2015, the Belgian government implemented the national bovine viral diarrhea virus (BVDv) eradication program. As part of this program, every newborn calf and aborted fetus underwent mandatory sampling using ear notches to detect BVDv antigen through ELISA or PCR tests. In dataset 2, information on the number of submitted APM cases that tested positive for BVDv and the annual count of immunotolerant persistently infected calves (IPIs) born at the herd level were available for each herd since 2015.

APM proportion

To evaluate the number of APM submissions, an APM proportion (APM_{PR}) was calculated by dividing the number of APM submissions by the total number of reproductive bovine females (Formula [1]). The total number of reproductive bovine females is the sum of the number of APM submissions and the number of registered births.

$$\frac{number\ of\ APM\ submissions}{(number\ of\ births + number\ of\ APM\ submissions)} \times 100 = APM\ proportion\ (\%) \quad [1]$$

Statistical analysis

All statistical analyses were performed using R Studio (version 3.6.1; R Core Team, Vienna, Austria). Binomial generalized linear mixed models were constructed to identify variables that are associated with APM_{PR}. This was performed within each dataset separately (Datasets 1 and 2). In both cases, the dependent variable was APM_{PR}, and the independent variables were different depending on the dataset. Model fit was evaluated using R-squared.

For Dataset 1, the variables were composed of *production type*, *year*, and *season* (model 1).

For Dataset 2, three separate models were constructed each within a specific time period. This allowed the inclusion of an increasing number of variables. Herd ID was defined as a random effect for each of these models. The used time periods in each of the three models were the following:

1) 2009-2021: With the following variables *herd size group*, *year*, and their interaction (model 2).

2) 2010-2021: Next to herd size and year variables, *APM submissions in the previous year (yes/no)* was added in this model (model 3). The year 2009 was excluded from this model because no data was available for the previous year (2008).

3) 2015-2021: For this model (model 4), the variable *presence of BVDv* was added. Presence was established in a yearly window (-1, 0, +1) effect. BVDv presence was defined as positive when one or more APM events tested positive for BVDv, or when one or more neonatal calves were identified as an IPI. For this period, data of BVDv presence at herd level was only available from 2015.

In all models only significant ($P < 0.05$) variables were retained. For those variables, least-squares means (LSM) were calculated and pairwise comparisons were computed using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). To avoid collinearity, the variation inflation factor (VIF) was evaluated for the different variables within each dataset, threshold was put at 2.5 (Johnston et al., 2018).

Results

Dataset 1

Between January 2006 and December 2021, a total of 63,221 APM cases were submitted. In 55.7% (35,188/63,221) of the cases, the production type was beef (> 90% Belgian Blue), while this was dairy (> 90% Holstein) in 41% (25,891/63,221), and double purpose in 3.4% (2,142/63,221) of the cases. In this time period, 7,699,289 calf births were registered, of which 40.4% were beef, 49.1% dairy, and 10.5% double purpose. The mean APM_{PR} from 2006 till 2021 was 0.81% (± 0.26%). This was 0.68% (± 0.19%) for dairy, 1.12% (± 0.40%) for beef, and 0.26% (± 0.11%) for double purpose cattle. Before the introduction of the extended analytical panel and the free on-farm sample collection (2006 till 2009), the mean number of submitted APM cases was 2,076 per year (SD = ± 77). The total APM_{PR} for this period was 0.44%. This was 0.43% (3,176 submissions versus 727,588 births) in dairy, 0.47% (3,928 submissions versus 829,704 births) in beef, and 0.23% (542 submissions versus 230,460 births) in double purpose cattle. After the introduction of the extended analytical panel and the free on-farm sample collection (2010 till 2021), the mean number of submitted APM cases was 4,805 per year (SD = ± 610). The total APM_{PR} for this period was 0.94%, while this was 0.79% (17,930 submissions versus 2,239,318 births) in dairy, 1.37% (24,608 submissions versus 1,776,009 births) in beef, and 0.33% (1,365 submissions versus 416,385 births) in double purpose cattle. Descriptive results of APM_{PR} per month and per year are depicted in **Figures 3.1.** and **3.2.** for both dairy and beef cattle. In general, APM_{PR} declined post 2015 from 1.2% (2015) to 0.86% (2021). This decline may be attributed mostly to dairy cattle, as depicted in **Figure 3.2**.

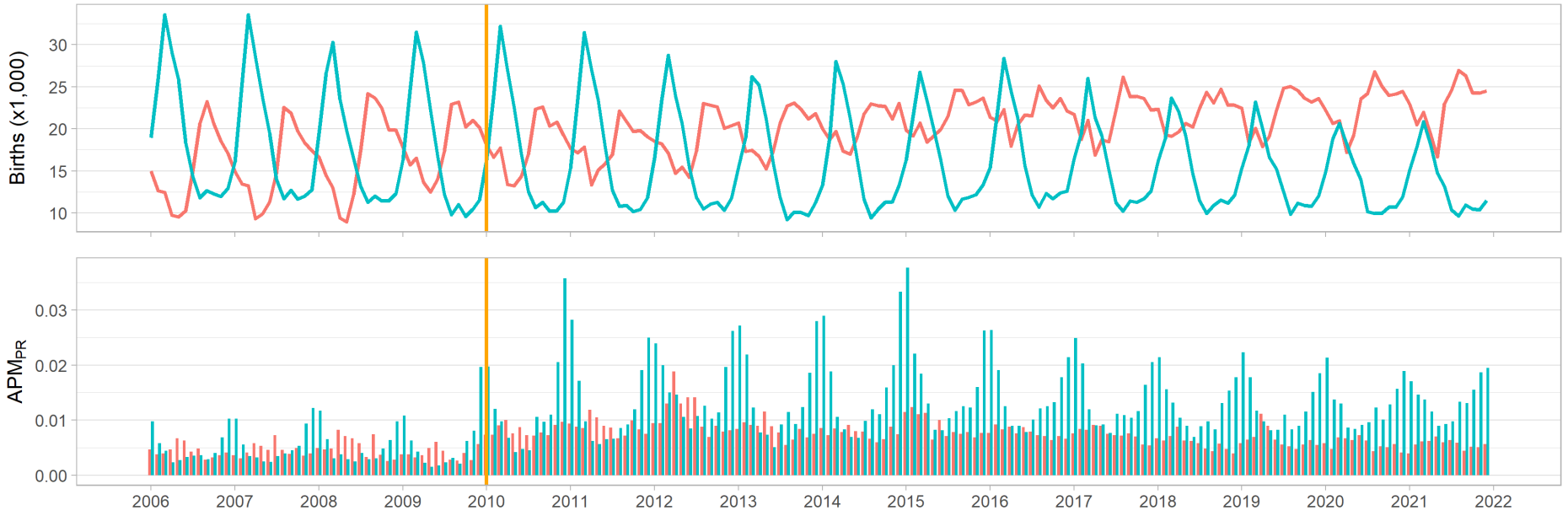

Figure 3.1. Monthly number of births, and proportion of abortion, stillbirth and perinatal mortality (APM_{PR}) in Flanders between 2006 and 2021 in beef (blue) and dairy (red) cattle. The extensive APM monitoring program was introduced since 2010 (orange line).

Figure 3.2. Descriptive statistics showing bovine abortion, stillbirth and perinatal mortality proportion (APM_{PR}) per year (2006-2021) in beef (blue) and dairy (red) cattle in Flanders. The extensive APM monitoring program was introduced since 2010 (orange line).

No collinearity was detected within this dataset using the VIF. Results from the statistical analyses through model 1 are shown in **Table 3.2.** R-squared for model 1 was 0.65. Our analysis revealed that between 2006 and 2021, the LSM of APM_{PR} was higher ($P < 0.001$) in beef (1.05 ± 0.06%) compared to dairy (0.61 ± 0.04%) cattle. The LSM of APM_{PR} was the highest ($P < 0.001$) in winter (1.11 ± 0.08%), followed by fall (0.78 ± 0.07%), spring (0.71 ± 0.06%), and summer (0.67 ± 0.06%). The LSM of APM_{PR} of the years between 2006 and 2009 ranged from 0.41 ± 0.096% (2006) to 0.46 ± 0.102% (2008), while this ranged from 0.84 ± 0.14% (2020) to 1.23 ± 0.17% (2012) between 2010 and 2021. Results of pairwise testing of the LSM of APM_{PR} per year are depicted in **Figure 3.3.**, which shows that the LSM of APM_{PR} was higher in 2012 (1.23 ± 0.17%) and 2015 (1.20 ± 0.16%) ($P < 0.05$) compared to all the other years.

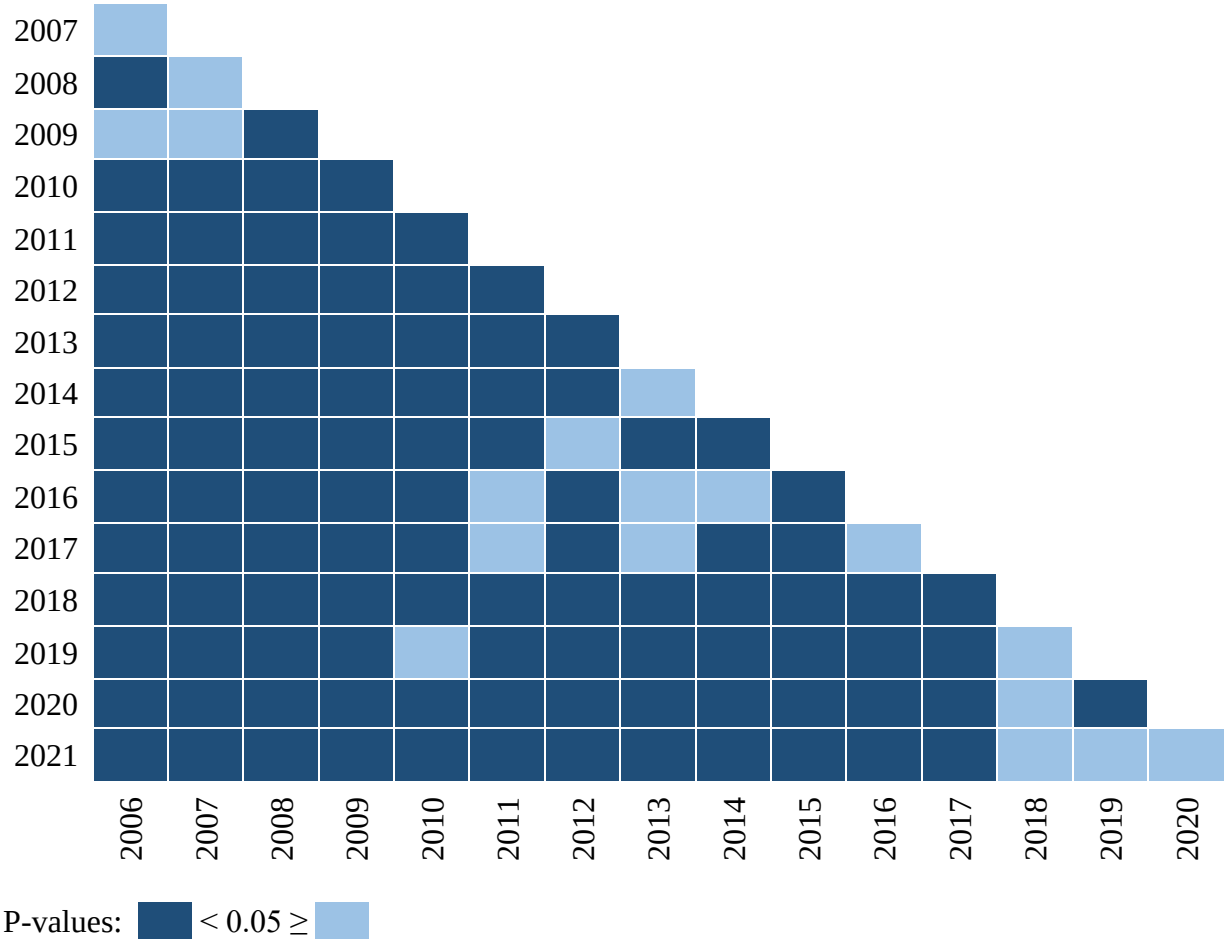

Figure 3.3. Heatmap showing the results of pairwise testing of abortion and perinatal mortality proportion per year (2006-2021).

Table 3.2. Least-squares means ± standard error of the mean of abortion/perinatal mortality proportion (APM_{PR}) for each year (2006-2021), season (winter, spring, summer, and fall), and production type (dairy and beef).

Variable		**APM_{PR} (LSM ± SEM)**	**Variable**		**APM_{PR} (LSM ± SEM)**
Year	2006	0.41 ± 0.096	**Season**	Winter	1.11 ± 0.079
	2007	0.44 ± 0.100		Spring	0.71 ± 0.058
	2008	0.46 ± 0.102		Summer	0.67 ± 0.064
	2009	0.43 ± 0.098		Fall	0.78 ± 0.070
	2010	0.92 ± 0.143	**Production type**	Dairy	0.61 ± 0.040
	2011	0.98 ± 0.149		Beef	1.05 ± 0.059
	2012	1.23 ± 0.167			
	2013	1.03 ± 0.154			
	2014	1.03 ± 0.151			
	2015	1.20 ± 0.160			
	2016	1.02 ± 0.146			
	2017	0.99 ± 0.147			
	2018	0.85 ± 0.137			
	2019	0.89 ± 0.141			
	2020	0.84 ± 0.138			
	2021	0.86 ± 0.140			

LSM = least-squares means; SEM = standard error of the mean

Dataset 2

Fourteen percent (589/4,164) of the selected active cattle herds did not submit any APM case for analysis during the analyzed time period (2009 till 2021). Overall, the mean number of APM submissions was 7.7 cases per herd, ranging from 0 to 94, while the median number was 5. Of the selected active herds, 33.9% submitted at least 1 APM case per year between 2010 and 2021, ranging from 25.1% (1,047/4,164) in 2010 to 41.2% (1,715/4,164) in 2015, while this was only 9.7% in 2009 (405/4,164).

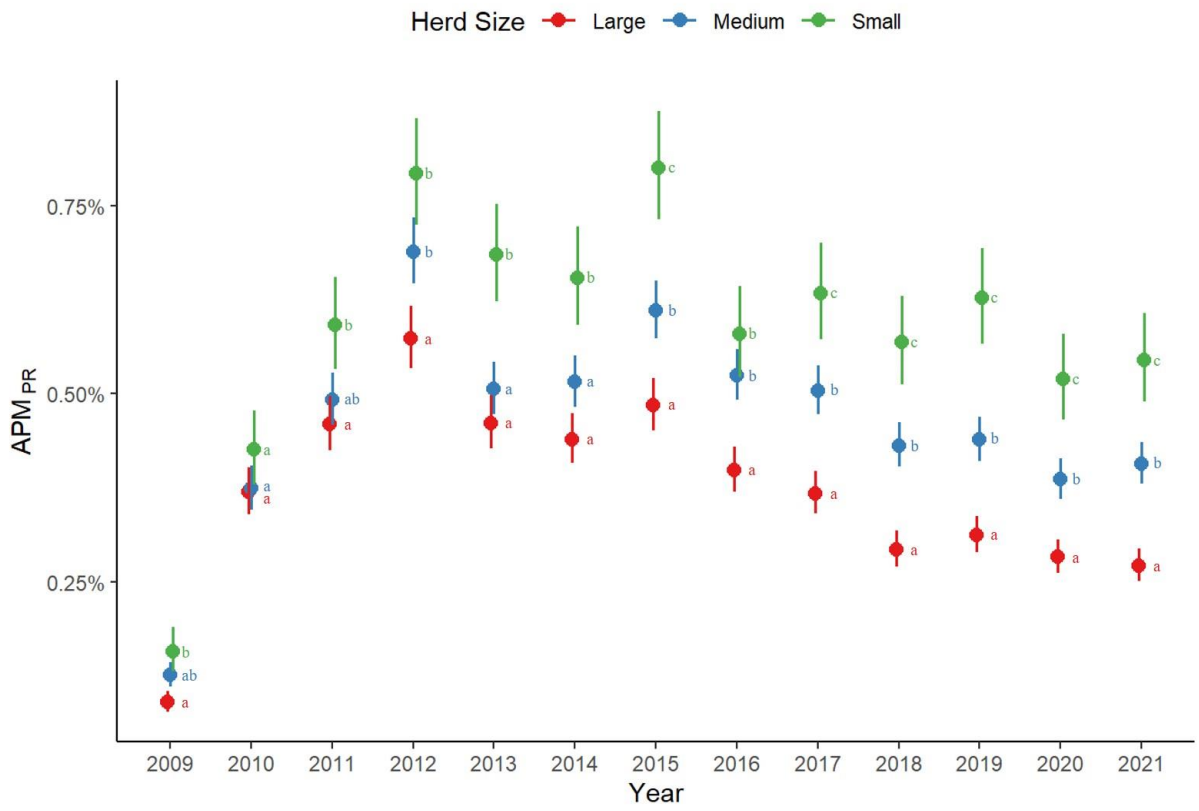

Figure 3.4. Abortion/perinatal mortality proportion (APM_{PR}) for each year between 2009-2021, stratified for herd size (small, medium, large). Herd size was based on the number of reproductive females and categorized based on quartiles (small = 1st quartile – medium sized = 2 interquartiles – large = 4th quartile) Different letters (a, b, c) within the same year indicate significant difference ($P < 0.05$).

No collinearity was detected within this dataset using the VIF. Results from the statistical analyses through model 2 are shown in **Figure 3.4.** R-squared for model 2 was 0.29. In general, APM_{PR} was higher ($P < 0.001$) in small-sized compared to medium and large herds. Differences in APM_{PR} between small, medium, and large herds were more significant after the introduction of the extensive analytical panel and the free on-farm sample pick up in 2010. Results of pairwise testing of APM_{PR} per year, stratified for herd size, are shown in **Figure 3.5.** R-squared for model 3 was 0.25. This model revealed that herds with one or more APM submissions in the previous year had a higher ($P < 0.001$) APM_{PR} in the subsequent year. Model 4 displayed that herds in which at least one APM or a (live) neonatal calf tested positive for BVDv during the defined yearly window, had a higher ($P < 0.01$) APM_{PR} compared to herds without a BVDv positive fetus or calf. R-squared for model 4 was 0.28.

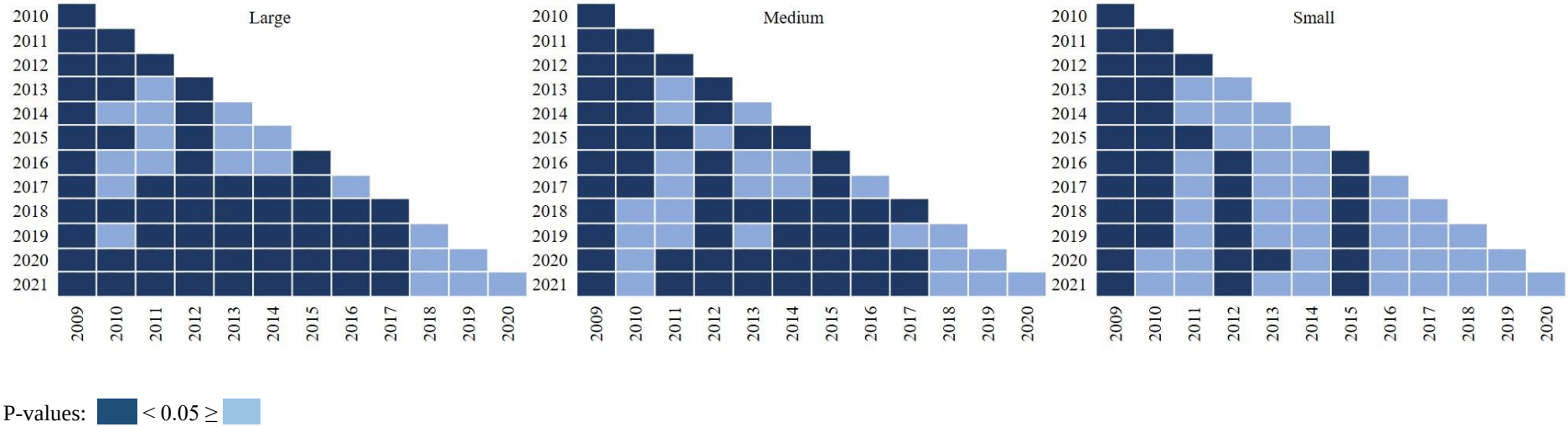

Figure 3.5. Heatmaps showing the results of pairwise testing of abortion and perinatal mortality proportion (APM_{PR}) per year (2009-2021) on large, medium, and small cattle herds. Per year, herd size was based on the number of reproductive females and categorized based on quartiles (small = 1st quartile – medium = 2 interquartiles – large = 4th quartile).

Discussion

To the best of our knowledge, the present study documents the largest dataset on bovine APM dynamics so far, covering an extensive period of 16 years of surveillance (2006 till 2021).

Our main finding revealed a significant increase in the APM_{PR} across all production types following the introduction of a more extensive APM monitoring program and an on-farm sample pick up service, both fully funded and promoted by the government. Notably, the submission of APM cases nearly doubled in dairy and almost tripled in beef cattle. Multiple factors could account for this increase in submissions. In a previous study, identifying the cause of an abortion was recognized as the main motivator for farmers to submit samples for APM investigation, rather than just the legal requirement (Clothier et al., 2020). However, in our study, it might be possible that also the availability of on-farm sample collection and the access to accredited and free diagnostic services since the end of 2009 may have been one of the primary motivators to submit a case of bovine APM for analysis. It is worth mentioning that this convenient on-farm sample collection feature was not present in the study conducted by Clothier et al., (2020), where farmers must take the time to bring the fetus to the laboratory, which might be located at a considerable distance. Farmers mentioned laboratory accessibility and the time cost involved in submitting APM samples as a great barrier (Clothier et al., 2020). In the present study, the introduction of daily on-farm sample collection by the diagnostic laboratory (Animal Health Services Flanders) was entirely government-funded and concurrent with the implementation of the extensive analytical panel. Prior to this initiative, the free on-farm collection of APM cases was not organized by the government. Moreover, broadening the analytical panel of the presented abortion monitoring program might have significantly contributed to the increased APM_{PR} since 2010. This extension resulted in an increased diagnostic rate of up to almost 40% (Van Loo et al., 2021), which likely encouraged farmers to submit more cases of APM for analysis. Before the implementation of the extensive analytical panel, APM cases were only analyzed for brucellosis, despite the country already being officially declared free of the disease since 2003. This limited focus might have resulted in low farmer interest in participating in the previous abortion monitoring program. Farmers are more likely to prioritize biosecurity measures during an outbreak situation for diseases they are aware of, while diseases absent in the country are often considered to pose minimal risk (Ekboir, 1999; Bronner et al., 2014). Furthermore, the intensive communication campaign launched during the

early stages of the extensive APM monitoring program may have also contributed to the increased APM_{PR}. Previous evidence suggests that educating farmers, as well as their veterinarians, may increase the likelihood to report signs of disease (Garner et al., 2016). Based on this, it may be concluded that an accessible, comprehensive, government-funded and -promoted APM surveillance program effectively encourages farmers and their veterinarians to report and submit cases of bovine APM.

In the present study, a higher APM_{PR} could be observed for the years 2012 and 2015. The higher APM_{PR} in 2012 may be explained by the SBv outbreak in Belgium during fall 2011 (Méroc et al., 2013b; Van Loo et al., 2013). Following the implementation of an SBv PCR test in the APM monitoring program and the detection of the first SBv positive bovine neonate in January 2012, more (malformed) cases of APM were submitted for analysis between January and August 2012 (Van Loo et al., 2013). Beyond August 2012, the number of APM cases with SBv-associated lesions decreased, which might be explained by the findings of Méroc et al., (2013a), who concluded that after the first SBv outbreak in 2011 and 2012, almost every cow in Belgium has been in contact with the virus. The between-herd seroprevalence in 2012 in Belgian cattle was estimated at 99.76%, and the within-herd seroprevalence at 86.3%. Additionally, a long persistence of immunity against the virus after seroconversion of at least 1 year was demonstrated (Méroc et al., 2015). Consequently, the vast majority of the Belgian cattle population should have developed post infection protective immunity against the virus. As a result, the number of APM cases associated with SBv decreased after the first outbreak of the virus, which might explain the decrease of APM submissions beyond the peak of 2012. This observation reveals that APM monitoring may be a valuable tool to detect outbreaks of emerging causes of APM, although vigilance of farmers and their veterinarians is essential in initial reporting and submitting of cases to the laboratories. Additionally, it should be noted that agricultural media coverage regarding the clinical consequences of SBv infection may have motivated farmers to report suspected APM cases with potential SBv infection. The second peak in APM_{PR} in 2015 may be related to the introduction of the mandatory BVDv eradication program in Belgium in the same year. This BVDv eradication program was based on compulsory BVDv analysis of ear notch samples from neonatal calves, but also from cases of APM. As APM is a potential outcome of an intrauterine BVDv infection (Kelling, 2007; Mee, 2013), reporting and submission for analysis of APM cases were stimulated during the communication campaign of the BVDv eradication program, leading to a higher APM_{PR} at the beginning of this program. For the years beyond the beginning of the mandatory BVDv

eradication, it could be observed that herds where BVDv was detected in an APM case or in a (live) neonatal calf, had a higher APM_{PR}. This may be explained by the fact that BVDv is a well known abortifacient agent in cattle. As a result, the presence of the virus in a cattle herd may lead to a higher APM_{PR}. Another explanation for this finding may be that having previous personal experience with the disease, may increase the likelihood that involved farmers correctly identify clinical signs (e.g., APM), leading to a higher submission of APM cases (Guinat et al., 2016; van Andel et al., 2020; Gates et al., 2021).

Interestingly, we observed a decrease in APM_{PR} after the year 2015, especially in large-sized farms. This decrease may be explained by the fact that it might be difficult to sustain long-term engagement with disease reporting in enhanced passive surveillance programs like the presented abortion monitoring program, even when incentives are provided (Gates et al., 2021). Also the removal of some analyses from the analytical panel since October 2011, may have had a negative effect on the motivation of farmers to submit an APM case, resulting in a decreased number of submissions, although this could not be fully substantiated from the results of the present study.

In the present study, we observed that the APM_{PR} was higher in beef compared to dairy cattle, which corresponds with the findings of Sarrazin et al. (2014), who reported that Belgian beef farmers were more inclined to submit each case of abortion (88%), compared to dairy farmers (42%). Clothier et al. (2020) also identified a higher motivation among beef farmers to submit APM cases for analysis compared to dairy farmers. In contrast, other studies observed that dairy farmers were more likely than beef farmers to contact a veterinarian to report (re)-emerging diseases (Gilbert et al., 2014), and cases of APM (Bronner et al., 2013). This discrepancy could not be clearly explained, but the higher submission rate of APM cases in beef cattle in the present study may be attributed to the high proportion of Belgian Blue cattle in the Belgian beef cattle population. Belgian Blue calves hold a higher economic value compared to most other beef cattle breeds, and especially when compared to dairy breeds, with the price of Belgian Blue beef calves being about a nine-fold higher than that of dairy calves. This may make Belgian Blue beef cattle breeders more inclined to submit APM cases for further analysis in order to find out the underlying cause of foetal/calf mortality. Moreover, in beef cattle, the calf is the primary source of income, leading to much more focus on the birth of a healthy calf in this production type. Both male and female calves are important in beef cattle, while in the dairy industry, male calves are often considered by-products destined for the veal calf or dairy beef industry. The latter may lead dairy farmers to be less interested in the cause

of an APM in a male calf. Unfortunately, we did not have information on the fetal sex of the included cases to further investigate this aspect. Furthermore, the management practices in beef cattle vary from those in the dairy industry in several ways, including housing, vaccination rate, and nutrition. These variations may lead to a difference in prevalence of certain abortifacient pathogens in both production types (Van Loo et al., 2021), which may also explain the differences in APM_{PR} observed between beef and dairy cattle in our study.

A seasonal distribution in APM_{PR} was observed, with a higher number of cases submitted during fall and winter compared to spring and summer. This may be attributed to the breeding season on pasture typically applied in many Belgian Blue beef herds between April and October. During this period on pasture, the detection and reporting of abortions may decrease, as mentioned by Bronner et al. (2014). Additionally, due to the typical breeding season, most of the Belgian Blue cows are non-pregnant or in the first trimester of gestation in spring and summer. Previous studies (Forar et al., 1996; Mee, 2020; Norman et al., 2012) reported that detecting fetal loss is less likely in early pregnancy, which could be another reason for the observed seasonal submission pattern of beef cases.

To calculate the APM_{PR}, we assumed that all detectable APM cases were submitted for analysis. However, one of the limitations of the present study is that we believe that the submitted numbers do not fully reflect the actual APM numbers. Previous research has suggested that the real number of abortions may be approximately 2.2 to 5 times higher than the submitted number (Forar et al., 1995; Kinsel, 1999). This estimation is based on the consideration that when only observed abortions are taken into account, normal abortion rates beyond 120 d of gestation appear to be around 2 to 5% (Kinsel, 1999; Hovingh, 2009; Mee, 2020). Additionally, perinatal mortality rates after 265 d of gestation have been reported to vary between 2.4 and 9.7% (Cuttance and Laven, 2019). In the present study, APM_{PR} was analyzed in commercial herds with at least 25 reproductive females per year, which means that at least 1 APM event every 2 years on each commercial herd should have been happened, assuming a minimal APM rate of 2%. However, 14% of the included herds never submitted any APM case over a period of 13 years, suggesting that even under mandatory conditions, where collection and extensive analysis of the samples were completely funded by the government, many APM cases remain unreported. Even with mandatory reporting, farmers and their veterinarians often fail to report APM cases, guided by self-interest and other reasons such as health aspects, financial loss, practical obstructions, peer influence, and fear of consequences such as farm isolation in case of reporting of a suspected case of brucellosis or another notifiable pathogen

(Elbers et al., 2010; Bronner et al., 2014). Moreover, depending on risk aversion, some farmers need multiple cases of APM before deciding to report. Furthermore, especially in large herds, sporadic abortion is considered to be a normal event, and farmers are not prompted to report each case (Bronner et al., 2014). The latter is confirmed by the present study, where a lower APM_{PR} was observed in larger compared to smaller herds. However, this is in contrast with a previous study from the UK, where it was found that farmers from larger dairy herds were willing to pay more for APM analysis, potentially due to the larger impact on the herd, or the overall more intense herd health management taking place in larger dairy cattle farms (Clothier and Anderson, 2016; Clothier et al., 2020). Based on this, Clothier et al. (2020) assumed that the motivation to investigate cases of APM will remain or grow, as there is a trend towards larger herds. Although there is no clear explanation for our contradictory finding, there may be a correlation with the growing issue of staff retention and turnover in large-scale, labour intensive, dairy farms (Tipples et al., 2012, 2010). Because of this, it could be possible that the employed staff on larger commercial farms is less experienced (or motivated) with recognising abnormal behaviours and clinical signs of disease (e.g., abortion) compared to the owner of a smaller family owned and operated farm (Gates et al., 2021). All these factors may cause underreporting of APM cases, which makes it challenging to estimate the real prevalence of APM.

To ensure freedom of brucellosis with a 99% confidence level, Welby et al. (2009) calculated that a minimum of 8,000 submitted APM cases per year would be required in Belgium, with 4,000 each in Flanders and Wallonia, in combination with the other components within the national brucellosis surveillance system (e.g., serological analyses). However, in the present study, before the introduction of an extensive analytical panel and the on-farm sample pick up, the mean number of submitted APM cases per year in Flanders was only 2,076. Broadening the analytical panel and free on-farm sample collection resulted in an increase of the mean number of submitted cases per year up to 4,576, which seems to be sufficient to guarantee the brucellosis-free status in Flanders.

Conclusion

This study provides a general overview and valuable insights from the APM screening approach in cattle in Flanders (northern Belgium), which may have relevance for other surveillance programs worldwide. However, it is essential to recognize that certain factors, such as the presence of Belgian Blue cattle, a free on-farm sample pick up service, the mandatory BVDv eradication program, and the fully funded extended analytical panel, are specific for the region, which may limit the direct applicability of all our results to other countries. Nevertheless, it can be concluded that an accessible, comprehensive, and low cost APM surveillance program with an acceptable diagnostic rate effectively encourages farmers and their veterinarians to report and submit cases of bovine APM. However, even with mandatory reporting, these factors may not completely eliminate the issue of under-reporting, which could hamper surveillance of infectious and zoonotic causes of APM. Therefore, it remains crucial to identify the drivers that convince farmers to submit APM cases in order to further improve submission rates and ensure the effectiveness of an APM monitoring program.

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References

Albaaj, A., Durocher, J., Leblanc, S.J., Dufour, S., 2023. Meta-analysis of the incidence of pregnancy losses in dairy cows at different stages to 90 days of gestation. JDSC 4, 144–148. https://doi.org/10.3168/jdsc.2022-0278

Benjamini, Y., Hochberg, Y., 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society. Series B (Methodological) 57, 289–300.

Bronner, A., Hénaux, V., Fortané, N., Hendrikx, P., Calavas, D., 2014. Why do farmers and veterinarians not report all bovine abortions, as requested by the clinical brucellosis surveillance system in France? BMC Vet Res 10, 93. https://doi.org/10.1186/1746-6148-10-93

Bronner, A., Hénaux, V., Vergne, T., Vinard, J.-L., Morignat, E., Hendrikx, P., Calavas, D., Gay, E., 2013. Assessing the mandatory bovine abortion notification system in France using unilist capture-recapture approach. PLoS One 8, e63246. https://doi.org/10.1371/journal.pone.0063246

Clothier, G., Wapenaar, W., Kenny, E., Windham, E., 2020. Farmers' and veterinary surgeons' knowledge, perceptions and attitudes towards cattle abortion investigations in the UK. Veterinary Record 187. https://doi.org/10.1136/vr.105921

Clothier, K., Anderson, M., 2016. Evaluation of bovine abortion cases and tissue suitability for identification of infectious agents in California diagnostic laboratory cases from 2007 to 2012. Theriogenology 85, 933–938. https://doi.org/10.1016/j.theriogenology.2015.11.001

Cuttance, E., Laven, R., 2019. Estimation of perinatal mortality in dairy calves: a review. Vet J. 252. http://dx.doi.org/10.1016/j.tvjl.2019.105356

Denis-Robichaud, J., Kelton, D.F., Bauman, C.A., Barkema, H.W., Keefe, G.P., Dubuc, J., 2019. Biosecurity and herd health management practices on Canadian dairy farms. J Dairy Sci 102, 9536–9547. https://doi.org/10.3168/jds.2018-15921

Ekboir, J.M., 1999. The role of the public sector in the development and implementation of animal health policies. Prev Vet Med 40, 101–115. https://doi.org/10.1016/S0167-5877(99)00015-X

Elbers, A.R.W., Gorgievski-Duijvesteijn, M.J., Zarafshani, K., Koch, G., 2010. To report or not to report: A psychosocial investigation aimed at improving early detection of avian influenza outbreaks. OIE Revue Scientifique et Technique 29, 435–449. https://doi.org/10.20506/rst.29.3.1988

Forar, A.L., Gay, J.M., Hancock, D.D., 1995. The frequency of endemic fetal loss in dairy cattle: A review. Theriogenology. https://doi.org/10.1016/0093-691X(95)00063-E

Forar, A.L., Gay, J.M., Hancock, D.D., Gay, C.C., 1996. Fetal loss frequency in ten Holstein dairy herds. Theriogenology 45, 1505–1513.

Garner, M.G., East, I.J., Kompas, T., Ha, P. V., Roche, S.E., Nguyen, H.T.M., 2016. Comparison of alternatives to passive surveillance to detect foot and mouth disease incursions in Victoria, Australia. Prev Vet Med 128, 78–86. https://doi.org/10.1016/J.PREVETMED.2016.04.009

Gates, M.C., Earl, L., Enticott, G., 2021. Factors influencing the performance of voluntary farmer disease reporting in passive surveillance systems: A scoping review. Prev Vet Med 196, 105487. https://doi.org/10.1016/J.PREVETMED.2021.105487

Gilbert, W.H., Häsler, B.N., Rushton, J., 2014. Influences of farmer and veterinarian behaviour on emerging disease surveillance in England and Wales. Epidemiol Infect 142, 172–186. https://doi.org/10.1017/S0950268813000484

Guinat, C., Wall, B., Dixon, L., Pfeiffer, D.U., 2016. English Pig Farmers' Knowledge and Behaviour towards African Swine Fever Suspicion and Reporting. PLoS One 11. https://doi.org/10.1371/JOURNAL.PONE.0161431

Hovingh, E., 2009. Abortions in Dairy Cattle - II: Diagnosing and Preventing Abortion Problems | VCE Publications | Virginia Tech, Virginia Cooperative Extension.

Jawor, P., Król, D., Mee, J.F., Sołtysiak, Z., Dzimira, S., Larska, M., Stefaniak, T., 2017. Infection exposure, detection and causes of death in perinatal mortalities in Polish dairy herds. Theriogenology 103, 130–136. https://doi.org/10.1016/j.theriogenology.2017.07.044

Johnston, R., Jones, K., Manley, D., 2018. Confounding and collinearity in regression analysis: a cautionary tale and an alternative procedure, illustrated by studies of British voting behaviour. Qual Quant 52, 1957. https://doi.org/10.1007/S11135-017-0584-6

Kelling, C.L., 2007. Viral diseases of the fetus, in: Youngquist, R.S., Threlfall, W. (Eds.), Current Therapy in Large Animal Theriogenology. WB Saunders Co, pp. 399–408.

Kinsel, M.L., 1999. An Epidemiologic Approach to Investigating Abortion Problems in Dairy Herds. The Bovine Proceedings 32, 152–160.

Mee, J.F., Berry, D.P., Cromie, A.R., 2008. Prevalence of, and risk factors associated with, perinatal calf mortality in pasture-based Holstein-Friesian cows. Animal 2, 613–620. https://doi.org/10.1017/S1751731108001699

Mee, J., 2013. Why Do So Many Calves Die on Modern Dairy Farms and What Can We Do about Calf Welfare in the Future? Animals 3, 1036–1057. https://doi.org/10.3390/ani3041036

Mee, J.F., 2020. Investigation of bovine abortion and stillbirth/perinatal mortality - Similar diagnostic challenges, different approaches. Ir Vet J. https://doi.org/10.1186/s13620-020-00172-0

Méroc, E., Poskin, A., Van Loo, H., Quinet, C., Van Driessche, E., Delooz, L., Behaeghel, I., Riocreux, F., Hooyberghs, J., De Regge, N., Caij, A.B., Van Den Berg, T., Van Der Stede, Y., 2013a. Large-Scale Cross-Sectional Serological Survey of Schmallenberg Virus in Belgian Cattle at the End of the First Vector Season. Transbound Emerg Dis 60, 4–8. https://doi.org/10.1111/tbed.12042

Méroc, Estelle, Poskin, A., Van Loo, H., Quinet, C., Van Driessche, E., Delooz, L., Behaeghel, I., Riocreux, F., Hooyberghs, J., De Regge, N., Cay, B., van den Berghe, T., Van Der Stede, Y., 2013b. Schmallenberg virus: Results of Belgian cross-sectional serological surveys, in: Epizone Abstracts, 7th Annual Meeting “Nothing Permanent, except Change.” p. 58.

Méroc, E., Poskin, A., Van Loo, H., Van Driessche, E., Czaplicki, G., Quinet, C., Riocreux, F., De Regge, N., Caij, B., van den Berg, T., Hooyberghs, J., Van der Stede, Y., 2015. Follow-up of the Schmallenberg Virus Seroprevalence in Belgian Cattle. Transbound Emerg Dis 62, e80–e84. https://doi.org/10.1111/tbed.12202

Norman, H.D., Miller, R.H., Wright, J.R., Hutchison, J.L., Olson, K.M., 2012. Factors associated with frequency of abortions recorded through Dairy Herd Improvement test plans. J Dairy Sci 95, 4074–4084. https://doi.org/10.3168/jds.2011-4998

Sarrazin, S., Cay, A.B., Laureyns, J., Dewulf, J., 2014. A survey on biosecurity and management practices in selected Belgian cattle farms. Prev Vet Med 117, 129–139. https://doi.org/10.1016/J.PREVETMED.2014.07.014

Thobokwe, G., Heuer, C., 2011. Incidence of abortion and association with putative causes in dairy herds in New Zealand. http://dx.doi.org/10.1080/00480169.2004.36411 52, 90–94. https://doi.org/10.1080/00480169.2004.36411

Tipples, R., Hill, R., Wilson, K., 2012. How did dairy fatigue research come about and what are we doing?

Tipples, R., Trafford, S., Callister, P., 2010. The factors which have resulted in migrant workers being "essential" workers on New Zealand dairy farms, in: Labour, Employment and Work Conference.

van Andel, M., Jones, G., Buckle, K., Phiri, B., McFadden, A., Dacre, I., Bingham, P., Heuer, C., Abila, R., Win, H.H., Lwin, K.O., Binney, B., Zaari, S., Gates, M.C., 2020. Estimating foot-and-mouth disease (FMD) prevalence in central Myanmar: Comparison of village headman and farmer disease reports with serological findings. Transbound Emerg Dis 67, 778–791. https://doi.org/10.1111/TBED.13397

Van Loo, H., Méroc, E., Cay, B., Van de Stede, Y., De Bleecker, K., 2013. Use of abortion surveillance in cattle to verify the introduction of Schmallenberg Virus in Flanders., in: Epizone Abstracts, 7th Annual Meeting "Nothing Permanent, except Change." p. 101.

Van Loo, H., Pascottini, O.B., Ribbens, S., Hooyberghs, J., Pardon, B., Opsomer, G., 2021. Retrospective study of factors associated with bovine infectious abortion and perinatal mortality. Prev Vet Med 105366. https://doi.org/10.1016/j.prevetmed.2021.105366

Welby, S., Letellier, C., Fretin, D., Hooyberghs, J., Vanholme, L., Godfroid, J., Vanderstede, Y., 2009. Evaluation du programme de surveillance pour la brucellose et la leucose bovine enzootique en Belgique. Epidémiologie et Santé Animale 55, 1–6.

Wiltbank, M.C., Baez, G.M., Garcia-Guerra, A., Toledo, M.Z., Monteiro, P.L.J., Melo, L.F., Ochoa, J.C., Santos, J.E.P., Sartori, R., 2016. Pivotal periods for pregnancy loss during

the first trimester of gestation in lactating dairy cows. Theriogenology 86, 239–253. https://doi.org/10.1016/J.THERIOGENOLOGY.2016.04.037

Wolf-Jäckel, G.A., Hansen, M.S., Larsen, G., Holm, E., Agerholm, J.S., Jensen, T.K., 2020. Diagnostic studies of abortion in Danish cattle 2015-2017. Acta Vet Scand 62, 1. https://doi.org/10.1186/s13028-019-0499-4

Chapter 4

Retrospective study of factors associated with bovine infectious abortion and perinatal mortality

Hans Van Loo[1], Osvaldo Bogado Pascottini[1], Stefaan Ribbens[2], Jozef Hooyberghs[3], Bart Pardon[1†], and Geert Opsomer[1†]

[1] Department of Internal Medicine, Reproduction and Population Medicine, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium
[2] Animal Health Services Flanders (Dierengezondheidszorg Vlaanderen), Torhout, Belgium
[3] Federal Agency for the Safety of the Food Chain, Brussels, Belgium
[†]These authors have contributed equally to this work

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Abstract

Abortion and perinatal mortality, leading causes of economic loss in cattle industry, are the consequence of both non-infectious and a wide range of infectious causes. However, the relative contribution of pathogens to bovine abortion and perinatal mortality is poorly documented, since available studies involved only a limited number of pathogens. Therefore, the objectives of the present monitoring study were to determine the prevalence of infectious agents associated with bovine abortion and perinatal mortality, and to identify differences in production type, gestation length, parity and seasonality by using mixed effect models (logistic regression). A pre-established sampling protocol based on the collection of the aborted fetus/calf and a corresponding maternal blood sample, involving diagnostic testing for 10 pathogens, was performed. At least one potential causal agent of the abortion or perinatal mortality was detected in 39% of cases. In these diagnosed cases, *Neospora caninum* was the most detected pathogen, followed by *Trueperella pyogenes*, BVDv, *Escherichia coli*, and *Aspergillus fumigatus*. *Neospora caninum* [odds ratio (OR): 0.4; 95% confidence interval (CI): 0.3–0.7] and *Aspergillus fumigatus* (OR: 0.1; 95% CI: 0.1–0.3) were detected less in late versus early gestation. *Aspergillus fumigatus* was less common in dairy in comparison to beef abortion cases (OR: 0.2; 95% CI: 0.1–0.6). Winter was associated with a lower positivity for *Neospora caninum* and BVDv in comparison to warmer seasons. Despite extensive diagnostic testing, an etiological diagnosis was not reached in 61% of cases, highlighting the need for even more extensive (non-)infectious disease testing or more accurate tests.

Keywords: dairy cow; beef cow; abortion diagnosis; infectious abortion; infectious perinatal mortality.

Introduction

Abortion and perinatal mortality (APM) have a detrimental effect on the performance of cattle operations. Losses of pregnancies (and calves) largely jeopardize the production efficiency of the farm (Meyer et al., 2001; De Vries, 2006; Lee and Kim, 2007; Hovingh, 2009) and indicate poor animal welfare (Mee, 2013). In this regard, preventing and controlling APM require a deep understanding of the (multi)causality of potential factors involved (Jamaluddin et al., 1996). Numerous infectious and non-infectious causes of bovine APM have been reported in literature (Smyth et al., 1992; Anderson, 2007; Givens and Marley, 2008; Clothier and Anderson, 2016; Norquay et al., 2020), but, because of financial limitations, it is not possible to analyse all of them. Therefore, and because infections are generally considered to be more important due to their significant abortifacient potential or zoonotic impact (e.g. brucellosis) (Wolf-Jäckel et al., 2020), laboratory investigation of bovine APM cases is mostly focused on infectious causes. However, evidencing a definite cause of APM is challenging because pathognomonic gross lesions are rare and may be shaded by tissue autolysis (Anderson, 2007), or may even be absent. Additionally, the lack of available diagnostic tests, or the lack of suitable samples may interfere with the detection of causative agents (Taylor and Njaa, 2012; Clothier and Anderson, 2016;). This can partially explain the high percentage of non-diagnosed cases (Mee, 2020). Even when applying multiple analyses, only in 20 to 50% of the analyzed abortions (Wheelhouse et al., 2015), and in 30 to 75% of the perinatal mortalities (Mee, 2015) an infectious or non-infectious cause of fetal/calf death could be identified. In literature, several reports on the prevalence of infectious causes of APM in cattle are available (Carpenter et al., 2006; Clothier and Anderson, 2016; Wolf-Jäckel et al., 2020), but regional differences highlight the importance of local, systematic monitoring.

The objectives of the present study were (1) to determine the prevalence of the most important infectious causes of bovine APM, and (2) to identify differences in production type, gestation length, parity and seasonality of the different pathogens in the northern part of Belgium (Flanders) between January 2010 and December 2011.

Materials and methods

Background and sampling protocol

In Belgium, cattle farmers are obliged to report each bovine APM to the Federal Agency for the Safety of the Food Chain (FASFC) in the context of the abortion monitoring program since 1978, as part of the national brucellosis monitoring program. Inclusion criteria for this abortion monitoring program are abortions (gestation length of 42 to 260 days) and perinatal mortalities of (pre)mature calves following a gestation length of more than 260 days, that died within 48 hours after birth. To stimulate the reporting of bovine APM, and based on previous studies performed in the region (Toussaint et al., 2007; Wilson et al., 2008; Callens et al., 2009; Ribbens et al., 2009; Garigliany et al., 2011; Vangeel et al., 2012), an extensive set of analyses for the most relevant abortifacient pathogens [*Neospora caninum* (*N. caninum*), *Coxiella burnetii* (*C. burnetii*), bovine herpesvirus type-1 (BHV-1), bovine viral diarrhea virus (BVDv), bluetongue virus (BTv), *Leptospira interrogans* serovar Hardjo (*L. interrogans* serovar Hardjo), *Listeria* spp., *Salmonella* spp. and opportunistic aerobic bacteria] was included to the abortion monitoring program at the end of 2009. Collection of the samples and analyses were completely funded by the FAFSC. From 2012 onwards fetal histology, analyses for *C. burnetii*, and serological tests for BHV-1, BVDv, and *L. interrogans* serovar Hardjo were excluded from the funded program because of financial limitations. After the outbreak of the previously unknown schmallenbergvirus (SBv) in 2012 (Méroc et al., 2013; Van Loo et al., 2013), analysis for SBv was included in cases with arthrogryposis-hydranencephaly syndrome.

Within 24 hours of case declaration by the farmer or the sanitary veterinarian, who is responsible for the epidemiological surveillance of the herd, sample sets were collected at the farm by the transport service of the accredited regional laboratory. Each sample set consisted of a maternal blood sample (tubes without anticoagulant) and the corresponding fetus/calf or placenta. Samples were transported in individual plastic boxes that were cleaned and disinfected with Virocid 0.5% (CID Lines, Ieper, Belgium) after usage. Shipment and storage conditions were at 4°C, and fetuses/calves/placentas were processed within 24 hours after arrival at the laboratory facilities.

Gestational age of the fetus/calf was estimated using crown-rump length (CRL) measurement at time of necropsy [gestational age in days = 68 + 2.25 x CRL (in cm); (Noden and DeLahunta, 1985)]. Then, the abdomen and thorax were cut-opened to allow microbiologic

sampling. Lung tissue was cut-opened with a sterile scalpel, and a sample was collected using a sterile cotton swab. Afterwards, the abomasum was opened with a sterile scalpel, and sampling by aspiration of abomasal content was performed with a sterile 10 ml syringe. Spleen tissue was collected by cutting with a sterile scalpel. The skull was disarticulated at the atlantooccipital joint, and the brain was removed by sagittal dissection. The brain was sliced, fixed in formalin, and stored in screw cap containers. Similarly, lung and heart samples were sliced and formalin-fixed for further histopathologic analyses. Blood samples were centrifuged at 1,500 × g for 15 min at 18°C, serum aliquoted, and stored at −20°C until further analysis.

Abortion screening approach from January 2010 to December 2011

The most extensive panel of analyses was performed from January 2010 to December 2011. The analytical approach and methods used to evaluate the association of APM with eventual infectious agents are shown in Table 2. Briefly, the serum sample of the dam was analyzed for antibodies against *Brucella* spp., BVDv, BHV-1, *L. interrogans*, *N. caninum,* and *C. burnetii*. In the case of a seropositive analysis for *N. caninum* in the dam, histological brain and heart samples from the fetus/calf were processed (hematoxylin and eosin) as described by Kiernan (2008) and checked for microscopic lesions. The abomasal content of each fetus/calf was analyzed by RT-PCR for *C. burnetii* and cultured for yeasts and molds. The lung tissue and abomasal content were cultured for *Brucella* spp., aerobic bacteria, and *Listeria* spp. In the case of a pure aerobic bacterial culture in both the lung tissue and the abomasal content, histological examination of the lung tissue was also performed (hematoxylin and eosin) as described by Kiernan (2008). On the spleen tissue samples, a BVDv Ag ELISA and a RT-PCR for BTv were performed. In cases where no fetus was available, only culture for *Brucella* spp. was performed on placental tissue.

Case definitions

The schematic illustration for each case definition, based on our sampling approach, is depicted in **Figure 4.1.** Briefly, cases in which the dam was seropositive for *N. caninum* and *N. caninum*-associated histopathological lesions (focal to multifocal non-suppurative necrotising encephalitis and non-suppurative interstitial myocarditis) were identified in brain and/or heart from the fetus/calf, were considered as positive for *N. caninum*. Cases with a positive *Brucella* spp. culture were classified as positive for *Brucella* spp. A case positive to aerobic bacteria was defined as a case in which a bacterium was found in pure aerobic culture of the abomasal content as well as of the lung tissue and presenting concurrent histopathological lesions (suppurative

bronchopneumonia). An exception was made for *Listeria* spp. positive cases, in which no histological examination was performed when the culture was positive. Cases with a positive yeast/mold culture in the abomasal content were considered as positive for yeast/mold. When BVDv antigens were found via ELISA on the spleen of the fetus/calf, the case was considered as positive for BVDv. Cases with a positive RT-PCR for BTv were classified as positive for BTv, and cases with a positive RT-PCR for *C. burnetii* as positive for *C. burnetii*. Cases in which two or more infectious agents (co-infections) were detected, were considered as positive for each agent.

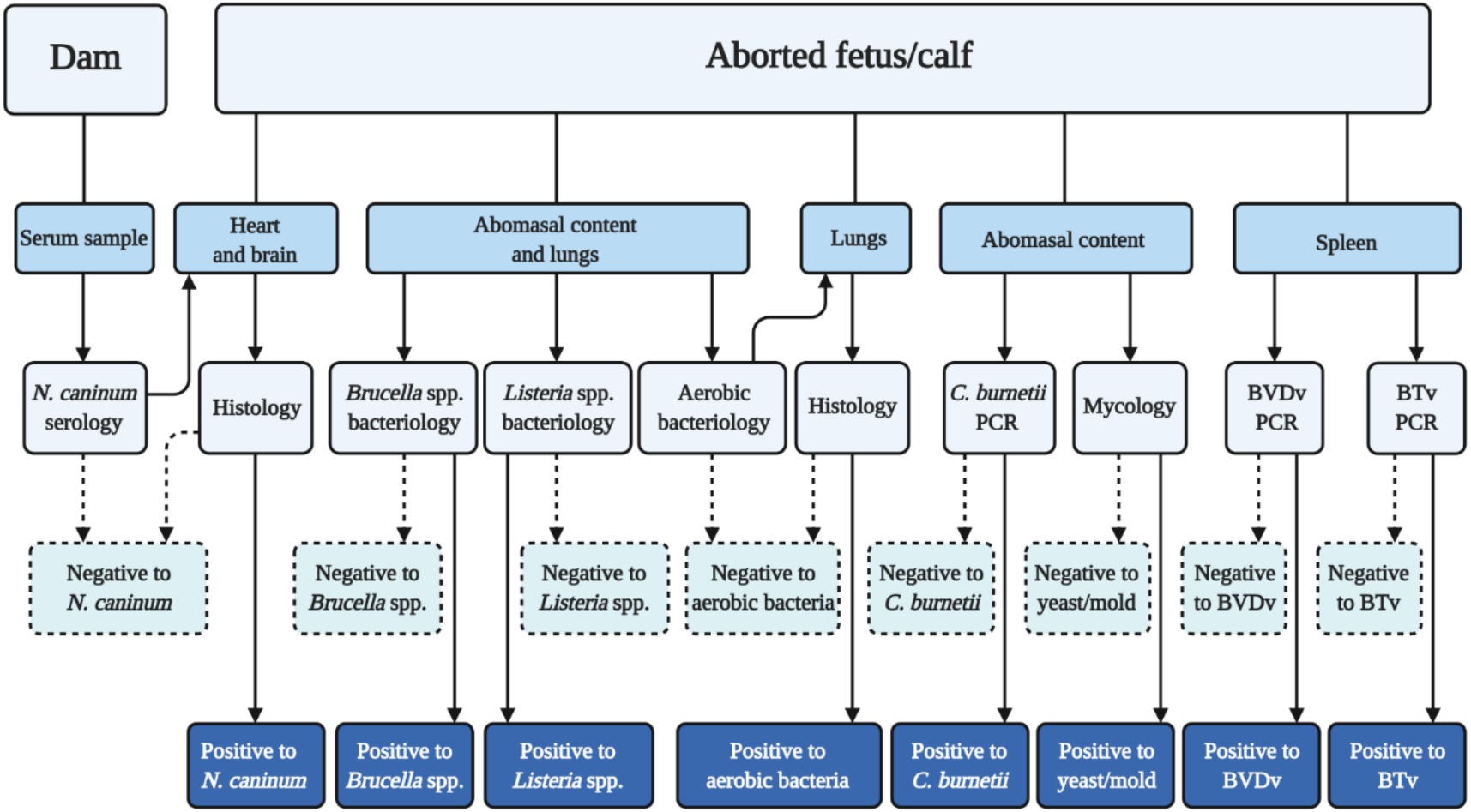

Figure 4.1. Flow chart illustrating the diagnostic approach of infectious abortions and perinatal mortalities in 4,006 cows in the northern part of Belgium in 2010 and 2011. A pathogen was identified as a possible cause of abortion/perinatal mortality in 1,574 cases. Dashed arrows indicate a negative diagnosis and solid arrows a positive diagnosis.

Study design

Between January 2010 and December 2011, 9,185 cases of bovine APM (52% beef, 40% dairy, and 8% dual-purpose) from the northern part of Belgium were submitted by farmers to the accredited regional laboratory, the Animal Health Service Flanders (DGZ Vlaanderen). The study population was limited to this time interval because, only in this period, the most extensive dataset was available, and the most extensive panel of analyses was performed. During this time period, a total of 960,885 newborn calves (43% beef, 45% dairy, and 12% dual-purpose) were registered in the national identification and registration system (Sanitel). To compare the number of APM submissions with the overall number of births per production type per season, an APM proportion was calculated by dividing the number of APM submissions for production type *x* in season *y* by the total number of pregnancies for production type *x* in season *y*. The total number of pregnancies for production type *x* in season *y* was calculated by adding the number of APM submissions to the number of births for production type *x* in season *y*. The number of births was extracted from Sanitel. For readability, the APM proportion was presented as a percentage by multiplying the numerator by 100 (Formula [1]).

$$\frac{number\ of\ submissions_{xy} \times 100}{(number\ of\ births + number\ of\ submissions)_{xy}} = APM\ proportion_{xy}\ (\%)$$

[1]

Case selection

From the initial 9,185 cases, 2,288 cases were excluded for the APM monitoring study because no fetus/calf was submitted (e.g., damage, severe autolysis, lost, etc.). Cases with a gestational length of more than 290 days (based on measurement of crown-rump length (CRL); > 99 cm) and full-term calves that received colostrum (n = 1,889) were excluded because there was no certainty about the reason of submission, and because there may be interference with bacteriological diagnostics when colostrum is present. Cases without a complete diagnostic protocol, as described in **Figure 4.1.** (n = 201), cases with no data available from the dam or fetus/calf (n = 200), and twins (n = 197) were also excluded. Because of the low number of cases in dual-purpose breeds (mainly Belgian Blue-Holstein crossbred; n = 197), these cases were also excluded. Based on these inclusion and exclusion criteria, the final population for the monitoring study to determine the infectious causes of APM in our region consisted of a total of 4,006 cases (**Figure 4.2.**), originating from 2,506 cattle farms. 1,702 cases originated from dairy (> 90% Holstein), while 2,304 from beef cattle (> 90% Belgian Blue).

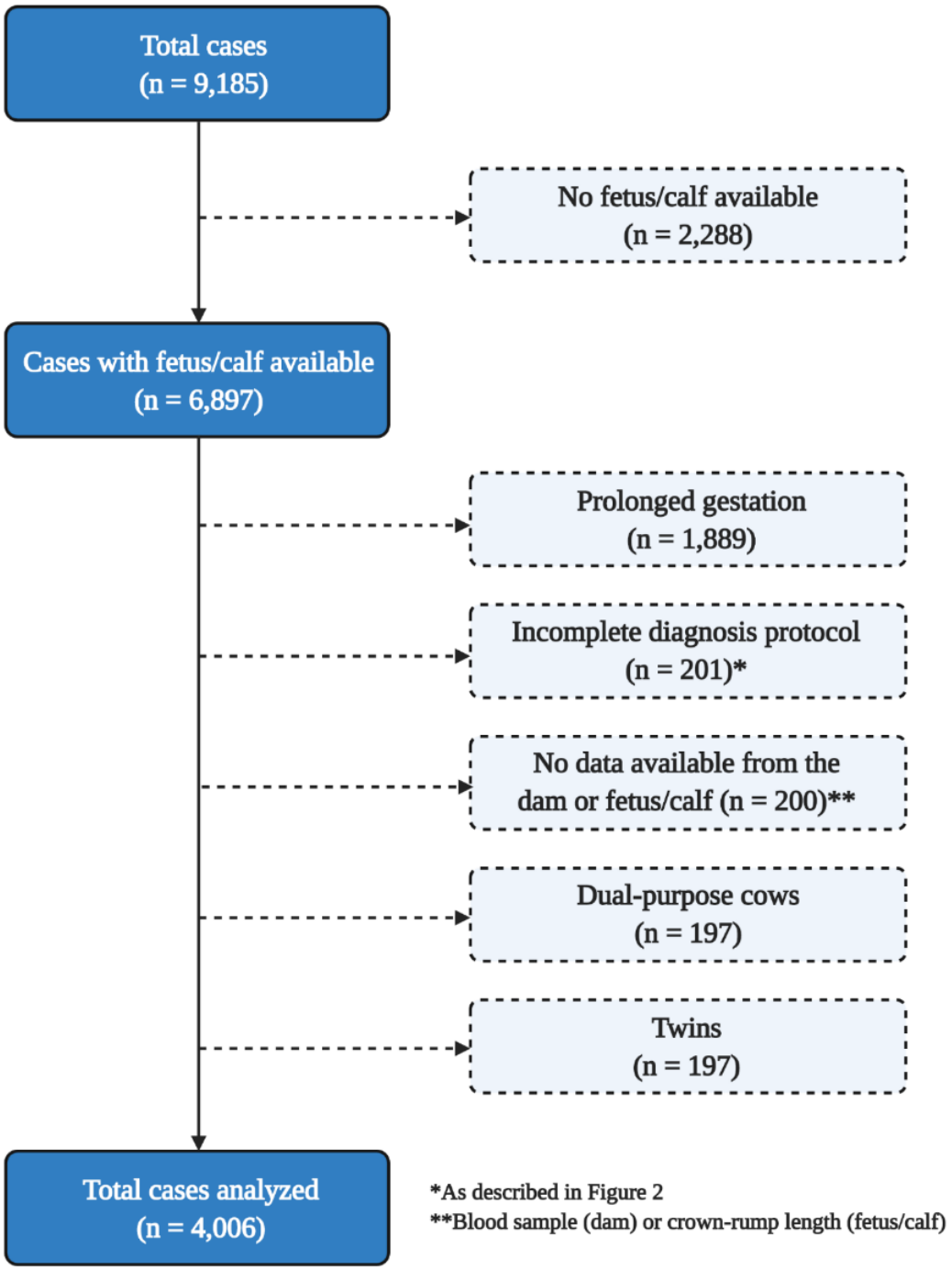

Figure 4.2. Flow chart depicting the initial population included in the study and the different reasons of exclusion for further analyses.

Statistical analyses

All data collected at the farm and from the laboratory data record system were exported and merged into a Microsoft Excel (Microsoft Corp., Redmond, WA) spreadsheet. Prime data exploration and organization were done using the PivotTables function. All statistical analyses were done using R-core version 3.6.3 (R Core Team, Vienna, Austria), considering the APM case as the unit of interest. Descriptive statistical analyses were conducted using the summary function of the R coding system (package base). The ggplot package (Wickham, 2009) was used to build a heatmap to visualize the most prevalent causes of abortion/perinatal mortality.

Descriptive statistics focused on the results of the diagnostic sampling approach as performed in the 4,006 complete cases. Descriptive statistics were used to analyse the distribution of APM cases per production type (dairy, and beef), parity of the dam [primiparous (< 30 months of age), and multiparous (≥ 30 months of age)], season [winter (January-March), spring (April-June), summer (July-September), and fall (October-December)], and month of gestation (3rd-4th, 4th-5th, 5th-6th, 6th-7th, 7th-8th, 8th-9th, and 9th) in the 4,006 cases.

Multilevel generalized mixed-effect models (package lme4, function glmer) (Bates et al., 2015) were built to determine differences of the most diagnosed agents (> 5% prevalence) associated with infectious APM. The responsive variables were binary (dichotomous), with APM cases classified as being positive or negative to *N. caninum*, yeast, *Trueperella pyogenes (T. pyogenes)*, BVDv, *Escherichia coli (E. coli)*, and *Aspergillus fumigatus (A. fumigatus)*. The fixed effects tested were gestation length (month of pregnancy) at APM (3rd to 6th, 6th to 7th, 7th to 8th, 8th to 9th, and 9th), season (winter, spring, summer, and fall), the production type of the dam (beef, dairy), and parity (primiparous, multiparous). The crown-rump length was ruled out of the model due to the high collinearity with the month of gestation (Pearson correlation < 0.9). The APM case nested within cow and nested within farm (3-level model) was chosen as random effect to correct for the fact that some farms presented APM more than once during the two-years time of the study. Multivariable models were fitted with factors found to be $P < 0.2$ in the univariable models, and only factors (and first-degree interactions) with $P < 0.05$ were retained in the final model via manual stepwise backward elimination. The distribution of the multivariable models' residuals were assessed using a scatterplot of the studentized residuals for homoscedasticity, linear predictor for linearity, and a Shapiro-Wilk test for normality. All results are expressed as odds ratios with their respective 95% confidence intervals.

Results

Descriptive statistics of the number of births, the total number of submitted APM cases, and the APM proportion per production type during 2010 and 2011 are shown in **Table 4.1.** The percentual distribution of 4,006 complete APM cases, submitted from January 2010 to December 2011, is displayed in **Figure 4.3.**, according to month of gestation, stratified by production type. Month of gestation was calculated based on the crown-rump length, which ranged from 5 cm to 99 cm, with a median of 70 cm. The results of the diagnostic analyses are summarized in **Table 4.2. Figure 4.4.** illustrates the diagnostic rate by month of gestation, which was 44%, 45%, 38%, 38%, and 33% for 3-6 months, 6-7 months, 7-8 months, 8-9 months, and 9 months of gestation, respectively. The identified pathogens and their prevalence, stratified by production type, and parity, are displayed in **Figure 4.5.** The percentual distribution of the five most detected pathogens (*N. caninum*, *T. pyogenes*, BVDv, *E. coli*, and *A. fumigatus*) for season, production type, gestation length, and parity is shown in **Table 4.3.** According to our diagnostic protocol, the general prevalence of the identified pathogens was 15,1% for *N. caninum*, 5.8% for *T. pyogenes*, 4.4% for BVDv, 3.5% for *E. coli*, 2.9% for *A. fumigatus*, 2.3% for *C. burnetii*, 1.3% for *L. monocytogenes*, 1% for *Serratia* spp., 0.6% for *Bacillus licheniformis*, 0.4% for *Staphylcoccus aureus*, 0.3% for *Serratia marcescens*, *Enterococcus faecalis*, *Salmonella* spp., and *Pseudomonas aeruginosa*, 0.2% for *Streptococcus uberis*, and *Staphylococcus hyicus*, 0.1% for BTv, *Yersinia pseudotuberculosis*, *Staphylococcus* spp., *Streptococcus dysgalactiae*, *Enterococcus* spp., and *Bacillus* spp., 0.05% for *Pseudomonas* spp., and 0.02% for *Streptococcus bovis*, *Pasteurella multocida*, *Enterobacter* spp., and *Corynebacterium* spp. In 8% of the diagnosed cases (127/1,574), co-infections (more than one infectious agent in the same case) were present. The different combinations of abortifacients detected in cases with coinfections are shown in **Figure 4.6**.

Table 4.1. Descriptive statistics showing the number of births, the number of submitted abortion and perinatal mortality (APM) cases, and the APM proportion per production type (dairy and beef) in the northern part of Belgium during 2010 and 2011, stratified by season.

	Production type	**Winter**	**Spring**	**Summer**	**Fall**
Number of births	Dairy	105,094	85,270	121,834	118,973
	Beef	142,679	135,019	68,862	64,128
Number of submitted APM cases	Dairy	892	826	910	1,036
	Beef	2,102	780	559	1,375
APM proportion (%)	Dairy	0.84	0.96	0.74	0.86
	Beef	1.45	0.57	0.81	2.10

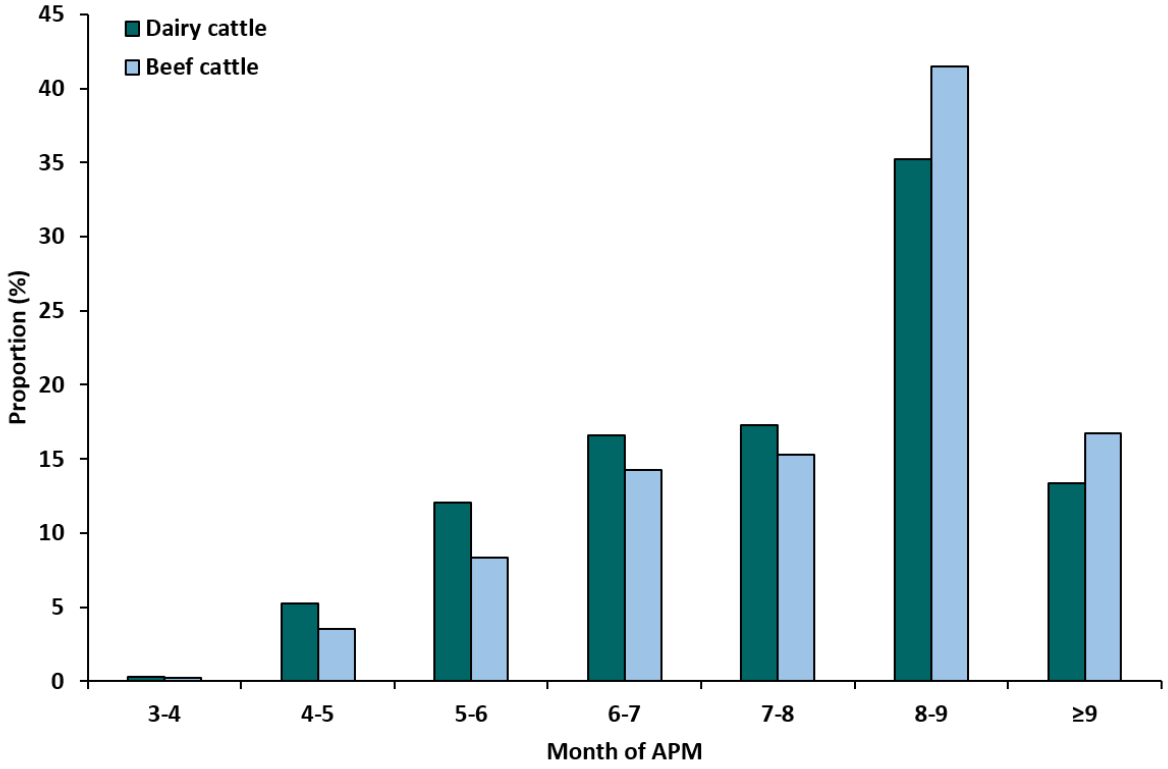

Figure 4.3. Descriptive statistics showing the percentual distribution of bovine abortion/perinatal mortality (APM) cases (n = 4,006) submitted in the northern part of Belgium in 2010 and 2011, according to month of gestation, stratified by production type (1,702 dairy, and 2,304 beef cases).

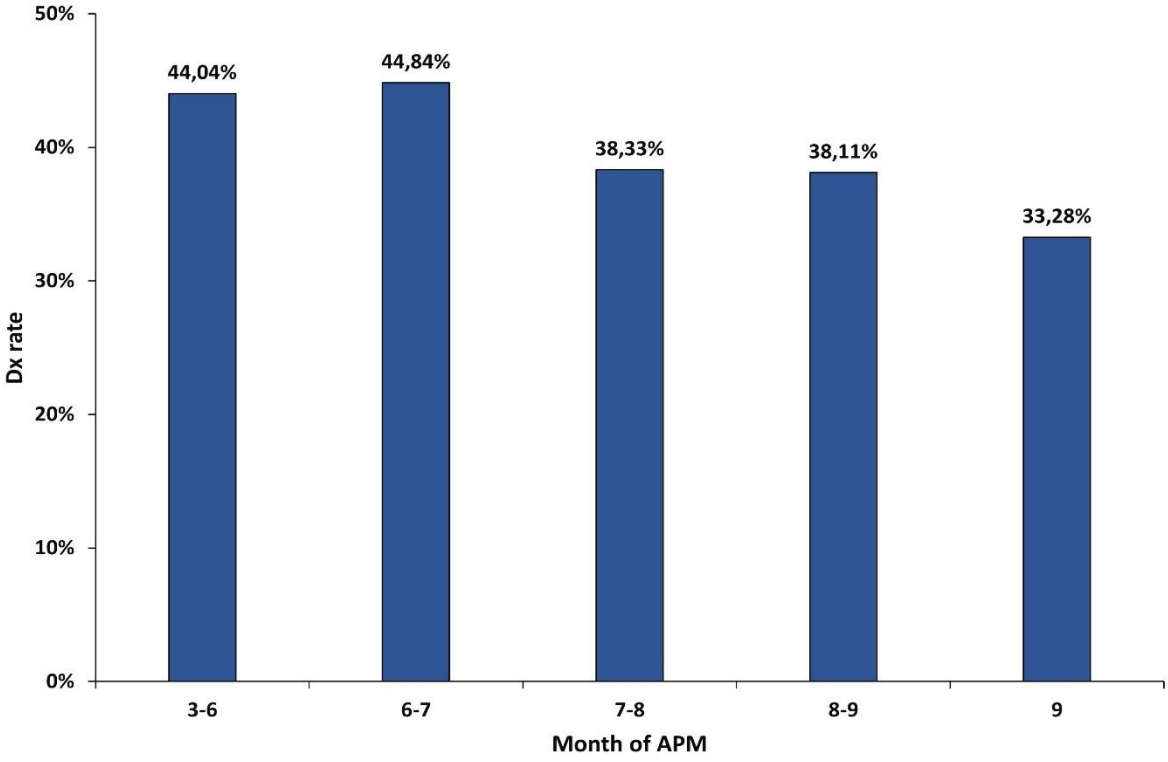

Figure 4.4. Descriptive statistics showing the percentual diagnostic rate (Dx rate) by month of gestation of abortion and perinatal mortality (APM).

Table 4.2. Results of the analytical panel performed in 4,006 aborted dams and the corresponding fetus/calf between January 2010 and December 2011 in the northern part of Belgium.

Disease	N	Dairy[1]	Beef[1]	Sample type	Test
Aerobic bacteria*	4,006	17.4	30.2	Fetal/calf abomasal content and lungs	Columbia blood agar base, Oxoid – Thermo Fisher Scientific, Waltham MA, USA
BHV-1	3,301	18.6	23.1	Maternal serum	HerdChek Anti-IBR gE Ab, IDEXX, Liebefeld-Bern, Switzerland
***Brucella* spp.**	4,005	0.1	0.4	Maternal serum	SERELISA Brucella OCB Ab Mono Indirect, SYNBIOTICS, Lyon, France
	3,993	0.8	2.5	Maternal serum	MAT, CODA-CERVA, Ukkel, Belgium
	4,006	0	0	Fetal/calf abomasal content or placenta	Brucella agar, Becton, Dickinson and company, Heidelberg, Germany
BTv	4,006	0.1	0.2	Fetal/calf spleen	Real-time q-PCR, in house kit, CODA/CERVA, Brussels, Belgium
BVDv	3,260	49.4	50.2	Maternal serum	SERELISA BVD p80 Ab Mono Blocking, SYNBIOTICS, Lyon, France
	4,006	4.7	3.3	Fetal/calf spleen	BVDv-Ag/Serum Plus, IDEXX, Liebefeld-Bern, Switzerland
Coxiella burnetii**	3,348	15.8	11.4	Maternal serum	LSIVET Ruminant MILK/SERUM Q FEVER, LSI, Lissieu, France
	2,238	1.7	1.4	Fetal/calf abomasal content	Real-Time PCR, in house kit, CODA/CERVA (as described by Klee et al., 2006)
***Leptospira interrogans* serovar Hardjo**	3,942	0.9	0.9	Maternal serum	PrioCHECK L.hardjo Ab, ThermoFisher, Belgium
***Listeria* spp.**	4,006	0.7	1.6	Fetal/calf abomasal content and lungs	PALCAM Listeria Selective Agar, Merck, Overijse, Belgium
Neospora caninum	4,006	22.7	18.2	Maternal serum	HerdChek: Anti-Neospora caninum Ab, IDEXX, Liebefeld-Bern, Switzerland
	761	90.2	76.6	Fetal/calf heart and brain	Hematoxylin and eosin staining + histopathology as described by Kiernan (2008)
Yeast/mold	4,006	6.6	4.3	Fetal/calf abomasal content	Sabouraud agar + Bacto agar, Becton, Dickinson and company, Heidelberg, Germany

[1]Percentage positives from the total of analyzed cases. *Only cases where the bacterium was detected in abomasal content or in lung tissue of the fetus/calf were considered as positive. ** In 2010, a commercial PCR kit (TAQVET C. burnetii LSI PCR TaqMan, Lissieu, France) was used to detect DNA of *C. burnetii*. Since February 2011, a RT-PCR as described by Vandenbussche et al. (2008) was used because this test had a higher specificity. Only the results of samples analyzed by the RT-PCR are presented here.

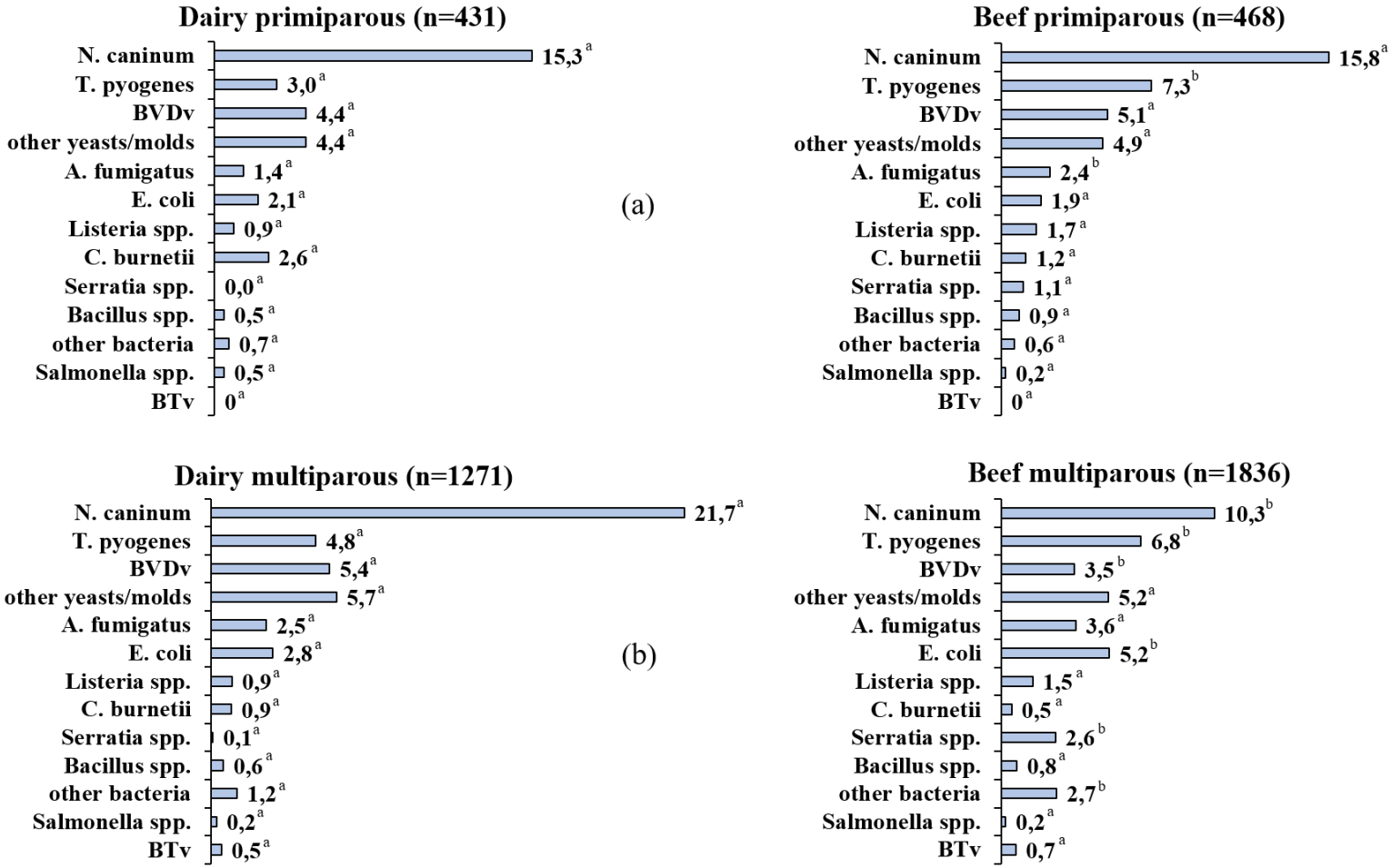

Figure 4.5. Percentual distribution of the different identified pathogens according to our diagnostic approach in (a) 899 primiparous cases of bovine abortion and perinatal mortality, stratified by production type (431 dairy, 468 beef), and (b) 3,107 multiparous cases of bovine abortion and perinatal mortality, stratified by production type (1,271 dairy and 1,836 beef) in the northern part of Belgium in 2010 and 2011. Different superscript letters (^{a,b}) within the same row indicate significant difference ($P < 0.05$).

Table 4.3. Descriptive statistics showing the percentual distribution of the five most detected pathogens (*Neospora caninum*, *Trueperella pyogenes*, BVDv, *Escherichia coli*, and *Aspergillus fumigatus*) for season, production type, gestation length, and parity in 4,006 cases of abortion and perinatal mortality in the northern part of Belgium in 2010 and 2011.

		N	***N. caninum***	***T. pyogenes***	**BVDv**	***E. coli***	***A. fumigatus***
Total		4,006	15.1	5.8	4.4	3.5	2.9
Season	Winter	1,441	12.0	9.1	4.7	4.0	3.1
	Spring	787	16.7	4.1	4.5	3.7	3.4
	Summer	633	19.3	2.1	4.9	3.5	1.6
	Fall	1,145	15.6	4.9	3.8	2.7	3.0
Production type	Dairy	1,702	20.1	4.4	5.2	2.5	2.2
	Beef	2,304	11.4	6.9	3.9	4.2	3.3
Gestation length	3rd-6th month	579	24.4	0.9	4.3	3.6	5.0
	6th-7th month	611	21.6	2.6	6.4	2.8	5.4
	7th-8th month	647	15.2	3.7	5.7	3.3	2.9
	8th-9th month	1,556	10.9	8.4	4.1	3.6	1.7
	9th month	613	10.6	9.3	2.1	4.1	1.1
Parity	Primiparous	899	15.6	5.2	4.8	2.0	1.9
	Multiparous	3,107	15.0	6.0	4.3	3.9	3.2

	N. caninum	*T. pyogenes*	BVDv	*E. coli*	*A. fumigatus*	*L. mono-cytogenes*	*Serratia* spp.	yeasts	*B. licheni-formis*	*P. aeruginosa*	*Staph*. spp.	*S. aureus*	*S. hyicus*
N. caninum		5 (6)	15 (19)	10 (13)	16 (20)	4 (5)	--	20 (26)	2 (2)	1 (1)	--	1 (1)	--
T. pyogenes	5 (6)		1 (1)	--	--	1 (1)	--	1 (1)	--	--	--	--	--
BVDv	15 (19)	1 (1)		1 (1)	3 (4)	2 (2)	1 (1)	4 (5)	1 (1)	--	1 (1)	--	1 (1)
E. coli	10 (13)	--	1 (1)		2 (2)	--	--	9 (12)	--	--	--	--	--
A. fumigatus	16 (20)	--	3 (4)	2 (2)		1 (1)	--	1 (1)	1 (1)	--	--	--	--
L. monocytogenes	4 (5)	1 (1)	2 (2)	--	1 (1)		--	2 (3)	--	--	--	--	--
***Serratia* spp.**	--	--	1 (1)	--	--	--		2 (2)	--	--	--	--	--
yeasts	20 (26)	1 (1)	4 (5)	9 (12)	1 (1)	2 (3)	2 (2)		--	--	--	--	--
B. licheniformis	2 (2)	--	1 (1)	--	1 (1)	--	--	--		--	--	--	--
P. aeruginosa	1 (1)	--	--	--	--	--	--	--	--		--	--	--
***Staph*. spp.**	--	--	1 (1)	--	--	--	--	--	--	--		--	--
S. aureus	1 (1)	--	--	--	--	--	--	--	--	--	--		--
S. hyicus	--	--	1 (1)	--	--	--	--	--	--	--	--	--	

Figure 4.6. Percentual distribution (n) of different combinations of abortifacients detected in 127 abortion and perinatal mortality cases with co-infections.

The 4,006 analyzed APM cases originated from 2,506 different cattle herds. The mean number of APM submissions per herd was 1.6, with a range from 1 to 20 (median is 1). Herds submitting multiple APM cases for analysis exhibited a higher diagnostic rate compared to those with fewer submissions, ranging from 70 to 36%, as shown in **Table 4.4**.

Table 4.4. Mean diagnostic rate (Dx rate) (%) associated with the number of submitted APM cases per herd

N° of APM cases (N° of herds)	20 (1)	9 (3)	8 (8)	7 (8)	6 (14)	5 (32)	4 (87)	3 (198)	2 (496)	1 (1,659)
Dx rate (%)	70	59	48	41	39	45	41	43	37	36

In 1,574 (39.3%) of the analyzed cases a pathogen was identified as a possible cause of APM. From these 1,574 cases, 16.2, 17.3, 15.8, 37.7, and 13% were aborted at the 3rd to 6th, 6th to 7th, 7th to 8th, 8th to 9th, and 9th month of pregnancy, respectively. Diagnosed cases by season were 39.9% in winter, 24.9% in spring, 16.2% in summer, and 18.9% in fall. Twenty-two percent of the diagnosed cases were in primiparous and 78% in multiparous cows, while 57.6% in beef, and 42.4% in dairy cattle. **Figure 4.7.** shows the proportions of identified pathogens associated with APM cases, stratified by production type, and month of gestation.

Differences in month of gestation, season, parity, and production type, associated with *N. caninum* and *A. fumigatus* positive cases, are presented in **Table 4.5.** and **Table 4.6.**, respectively. Parity and production type in *N. caninum* positive cases were not significant in the multivariable analysis, but they were retained in the final model because of some significant interactions. The month of gestation, parity, production type, and the interaction between the month of gestation and production type were associated with *A. fumigatus* positive cases. Season played a role in BVDv positive cases being lower in winter in comparison to the warmer seasons (spring, summer, and fall; $P < 0.001$). No significant differences were identified when yeasts, *T. pyogenes*, and *E. coli* were identified as a possible cause of abortion or perinatal mortality ($P > 0.1$).

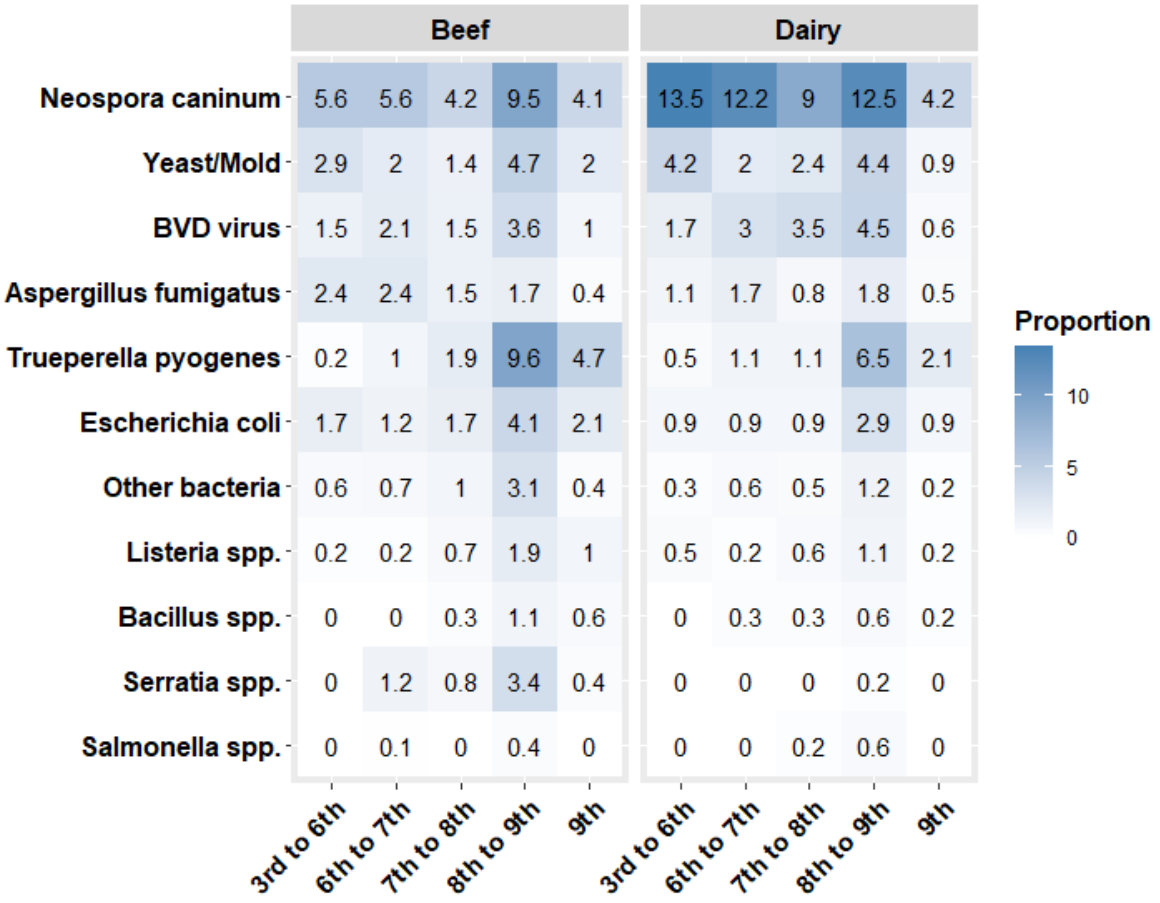

Figure 4.7. Heatmap showing the proportion of abortion/perinatal mortality cases with a diagnosis (based on our diagnostic approach; n = 1,574) in the northern part of Belgium in 2010 and 2011. The proportion of cases is stratified by month of gestation in beef (n = 908) and dairy (n = 666) cattle.

Table 4.5. Results of the multivariable mixed effects analysis on parameters associated with *Neospora caninum* abortion and perinatal mortality diagnosis in cows. From 1,574 cases with a diagnosis (based on our diagnostic approach), 38.4% (n = 605) cases were diagnosed *Neospora caninum* positive.

Variable		n	%positives	Odds ratio	95% CI	*P*-value
Month of gestation	3rd to 6th month	255	55.3	Referent		
	6th to 7th month	274	48.2	0.7	0.5 – 1.1	0.1
	7th to 8th month	248	39.5	0.5	0.3 – 0.7	< 0.001
	8th to 9th month	593	28.5	0.3	0.2 – 0.4	< 0.001
	9th month	204	31.9	0.4	0.3 – 0.7	< 0.001
Season	Winter	594	29.1	Referent		
	Spring	309	42.4	2.3	1.2–4.1	0.006
	Summer	236	51.7	3.5	1.8–6.9	0.0001
	Fall	435	41.2	4.2	2.1–8.3	< 0.001
Parity	Primiparous	318	44.0	Referent		
	Multiparous	1,256	37.0	0.8	0.5–1.2	0.3
Production type	Beef	908	28.7	Referent		
	Dairy	666	51.4	1.0	0.6–1.6	0.9
Season × parity	Spring × multiparous			0.5	0.2–1.0	0.06
	Summer × multiparous			0.5	0.2–1.0	0.07
	Fall × multiparous			0.3	0.1–0.7	0.007
Parity × production type	Multiparous × Dairy			2.4	1.4–4.2	0.001

Table 4.6. Results of the multivariable mixed effects analysis on parameters associated with *Aspergillus fumigatus* abortion and perinatal mortality diagnosis in cows. From 1,574 cases with a diagnosis (based on our diagnostic approach), 7.3% (n = 115) cases were diagnosed *Aspergillus fumigatus* positive.

Variable		n	% positives	Odds ratio	95% CI	*P*-value
Month of gestation	3rd to 6th month	255	11.4	Referent		
	6th to 7th month	274	12.0	0.8	0.4–1.5	0.5
	7th to 8th month	248	7.7	0.5	0.2–1.1	0.9
	8th to 9th month	593	4.6	0.1	0.1–0.3	< 0.001
	9th month	204	3.4	0.1	0.1–0.3	< 0.001
Parity	Primiparous	318	5.4	Referent		
	Multiparous	1,256	7.8	1.6	1.0–2.8	0.05
Production type	Beef	908	8.5	Referent		
	Dairy	666	5.7	0.2	0.1–0.6	0.002
Month of gestation × production type	6th to 7th month × dairy			1.8	0.5–6.0	0.3
	7th to 8th month × dairy			1.4	0.3–5.7	0.5
	8th to 9th month × dairy			5.2	1.6–17.1	0.06
	9th month × dairy			7.7	1.3–45.7	0.02

Discussion

In this study, a unique, extensive data-set of 9,185 cases of abortion and perinatal mortality was analyzed. In literature, bovine abortion generally refers to pregnancy loss in the fetal stage, between days 42 and 260 of gestation (Baumgartner, 2015) while the loss of a non viable fetus beyond day 260 of gestation, and deaths of full-term calves up to 48 hours of age have been defined as perinatal mortality (Mee, 2013). Although infections are less diagnosed in perinatal mortalities compared to abortions, many infectious causes of bovine abortion have also been associated with an increased risk of perinatal mortality (Kirkbride, 1993; Graham et al., 2009; Brickell et al., 2010; Mee, 2013; Smyth et al., 1999). Therefore, we included not only abortions in our study, but also perinatal mortality cases until 290 days of gestation.

From the initial 9,185 cases, 5,179 cases were excluded. Of these, 2,288 lacked a (complete) calf or fetus. In 2010, 68% of the submitted cases included a complete fetus, while 32% comprised only a maternal blood sample and placenta. In 2011, 83% of the cases included a fetus, with only 17% having a blood sample and placenta. This shift can be attributed to an intensive communication campaign that encouraged farmers and veterinarians to submit fetuses. The provision of free on-farm sample pickup further facilitated fetus submission for analysis.

In beef cattle, a seasonal distribution in submitted cases could be observed. Previous studies (Forar et al., 1996; Norman et al., 2012; Mee, 2020) reported that detecting fetal loss is more likely in late pregnancy. In many Belgian beef herds, a breeding season on pasture between April and October is typically applied. As a consequence, most of the Belgian beef cows are non-pregnant or in the first trimester of gestation during spring and summer, which may explain the observed seasonal submission pattern. Besides, Bronner et al. (2014) mentioned that when animals are on pasture, detection and reporting of abortions decreases.

Based on our analytical approach, an underlying infectious abortifacient agent could be detected in 39% of APM cases, which is comparable to previously published studies that ranged from 23 to 47% (Kirkbride, 1993; Jamaluddin et al., 1996; Khodakaram-Tafti and Ikede, 2005; Wolf-Jäckel et al., 2020). The diagnostic success would further increase if more analyses for other infectious, and also non-infectious, causes of APM could have been performed on the samples. Many infectious, and non-infectious agents are described as potential causes of bovine APM, but, due to financial constraints, we focused only on the leading infectious causes of

bovine APM in our region. One important limitation while studying the potentially underlying infectious cause of bovine APM, is the time-lag between the moment of infection, and the moment the abortion/calf death took place, which may occur days or even weeks later (Kennedy and Richards, 1964; Grooms and Bolin, 2005; Dubey et al., 2006). Therefore, at the moment of the laboratory analysis, the agent responsible for the death of the fetus or calf may no longer be present (McClurkin et al., 1984). Another plausible reason for non-diagnosis is an eventually poor quality of the sample(s) due to autolysis, mummification, or maceration. These situations are commonly faced in the diagnosis of bovine APM in the field (Khodakaram-Tafti and Ikede, 2005; Waldner et al., 2010).

In our study, serological analyses for 6 pathogens was performed on the maternal serum sample. However, the results of single blood samples from aborted cows are of very limited diagnostic value and must be interpreted in context with management data of the corresponding herd (Taylor and Njaa, 2012; Clothier and Anderson, 2016), which was not available in our study. For some diseases, e.g. *N. caninum*, a maternal seronegative result may be used to rule out the pathogen as the cause of APM (Pereira-Bueno et al., 2000; Mee, 2020). A seropositive result for an infectious abortifacient agent indicates recent exposure that may or may not be associated with the abortion, an endemic condition in the population, or previous vaccination (Taylor and Njaa, 2012; Clothier and Anderson, 2016;). Paired acute (at the time of abortion) and convalescent (2 weeks after abortion) serum samples may demonstrate an increasing titer to a particular pathogen and may provide stronger evidence of association (Anderson, 2007; Clothier and Anderson, 2016) for some infectious causes of bovine abortion (e.g., *Salmonella* Dublin, (Sánchez-Miguel et al., 2018)). However, for most of the abortifacient pathogens, maternal seroconversion may have occurred already before the abortion due to the lag phase between infection and the expulsion of the dead fetus (Taylor and Njaa, 2012). In the present study, maternal serology was used as a monitoring tool, and information on paired samples was not identified. Therefore, the results of the single serological analyses were not used in our approach to establishing the diagnosis of APM, except for *N. caninum* (Figure 1).

None of the submitted cases were positive for *Brucella* spp. culture. However, serological analyses for *Brucella* spp. indicated a small proportion of seropositive dams. As vaccination for bovine brucellosis is not allowed in Belgium, seropositivity can be explained by false-positive reactions, probably caused by infections with other gram-negative bacteria like *Yersinia enterocolitica* O:9, *E. coli* O157:H7 or *Salmonella* group N serotypes (Bonfini et al., 2018).

N. caninum was the most detected pathogen associated with APM in this study, followed by mycotic infections (mainly *A. fumigatus*), *T. pyogenes*, BVDv, and *E. coli*. Diagnosis of *N. caninum* positive APM was based on the combination of a seropositive serum sample of the dam and histopathological lesions of the corresponding fetal/calf brain and heart. Although abortions of *N. caninum* PCR positive fetuses have been rarely described in *N. caninum* seronegative cows (Sager et al., 2001), infected dams are expected to have high titers of specific antibodies at the time of abortion (Pereira-Bueno et al., 2000). Focal to multifocal non-suppurative necrotizing encephalitis and non-suppurative interstitial myocarditis are typical lesions for fetal neosporosis (Barr et al., 1991; Wouda et al., 1997). Still, these lesions can also be found in other protozoal infections like sarcocystosis or toxoplasmosis (Anderson, 2012). However, compared to neosporosis, sarcocystosis and toxoplasmosis are much more uncommon causes of bovine APM, so we assumed that most of the detected lesions were caused by a *N. caninum* infection. Based on our diagnostic protocol, *N. caninum* was the most commonly diagnosed cause of APM. Fifteen percent of the submitted cases were positive for *N. caninum*, which is in agreement with other publications (Dubey et al., 2007; Wolf-Jäckel et al., 2020). *N. caninum* positive cases were more common during the 3rd to 6th month of pregnancy compared to the 6th to 9th month. It is known that a fetal infection with *N. caninum* prior to fetal immunocompetence is more likely to result in abortion than an infection on a later stage of pregnancy. As pregnancy progresses, the bovine fetus may be able to mount an immune response to *N. caninum* infection, which may protect against fetal death (Dubey et al., 2006). Our findings show also an interaction between parity and production type (beef and dairy). Specifically, fewer *N. caninum* positive cases occurred in multiparous beef in comparison to primi- or multiparous dairy cows. In this context, previous Belgian studies noted a higher seroprevalence of *N. caninum* in beef compared to dairy herds (Vangeel et al., 2012), but a lower risk of abortion in *N. caninum* infected beef compared to *N. caninum* infected dairy cows. No clear explanation for this difference between dairy and beef cattle could be found (Hemphill and Gottstein, 2000; De Meerschman et al., 2002). Interestingly, we found fewer cases positive for *N. caninum* in winter than in other seasons, which contradicts the findings of Thurmond et al. (1995). However, Wouda et al. (1999) described an apparent seasonal pattern in the occurrence of *N. caninum*-associated abortion storms with more abortions during summer and early fall. Bartels et al. (1999) mentioned favorable conditions of temperature and humidity during summer and early fall for fungal growth (according to the authors, a risk factor for *N. caninum* abortion) and for oocyst sporulation as risk factors for *N. caninum* abortion storms in

the Netherlands. As climate conditions in the Netherlands are similar to those in Belgium, this may explain the seasonal pattern of *N. caninum* positive cases in our study.

Bacteriological examination should be combined with histopathology from placental or fetal/calf tissue to detect lesions associated with bacterial APM cases (Yaeger and Holler, 2007). As APM cases caused by opportunistic agents more commonly start as a primary placentitis, and reach the fetus via ingestion and/or inhalation causing bronchopneumonia, gastroenteritis, and dermatitis (Yaeger and Holler, 2007; Anderson, 2012; Taylor and Njaa, 2012), we limited histopathology to fetal/calf lung tissue. Bacteria that reach the fetus via the umbilical vasculature, e.g. *Listeria* spp., *Salmonella enterica*, and *Campylobacter* spp., lead to systemic, but especially hepatic, lesions (Anderson, 2012; Taylor and Njaa, 2012; Baumgartner, 2015). However, liver tissue collection and histological analysis were not included in our study. Anaerobes have also been reported as potential infectious causes of bovine abortion (Kirkbride et al., 1989), but anaerobic culture was not performed in the present study.

Another limitation of our study is the fact that no analysis on placental tissue was performed. Placental tissue is often contaminated with environmental or vaginal bacteria (Yaeger and Holler, 2007), which may bias the diagnostic accuracy. However, it can be valuable to submit placental tissue for histological examination, because some infections [e.g., *Chlamydia* spp., *C. burnetii*, and *Salmonella* Dublin (Hall and Jones, 1977; Clothier and Anderson, 2016)] may be limited to the placenta. In those cases, the etiological agent may not be detected in fetal/calf samples (Yaeger and Holler, 2007), which could contribute to missed diagnoses. However, Botta et al. (2019) reported that histological lesions in the bovine placenta must be interpreted with caution, because lesions might not be necessarily linked to an infectious etiological cause and can even be part of physiological processes during pregnancy or placental expulsion.

To confirm the diagnosis of mycotic APM cases, and to exclude environmental fungal contamination, histological examination of the placenta may be necessary (Baumgartner, 2015), but this was not performed in our study. The majority of mycotic bovine abortions are linked to an *Aspergillus* spp. infection (Glover et al., 2011; Anderson, 2012), which is in agreement with our findings. We found more mycotic cases in the 3rd to 6th month of gestation, but Anderson (2012) mentioned that mycotic abortions usually occur in the third trimester of gestation. Similar to us, McClausland et al. (1987) reported that abortions due to *A. fumigatus* tend to occur in the second and third trimester of gestation. Based on our data, beef cattle seem

to be significantly more sensitive to experience an *A. fumigatus* positive case of APM compared to dairy.

A high seroprevalence for BVDv (50%) could be detected in aborted dams whereas in only 4% of the analyzed fetuses/calves, BVDv antigen could be detected. As BVDv vaccines are no DIVA (Differentiating Infected from Vaccinated Animals) vaccines, the high maternal seroprevalence for BVDv may be partially explained by vaccination. Unfortunately, in the present study, no data on vaccination status of the included dams was available. Besides, as antibodies induced by a BVDv infection are detectable for several years (Fredriksen et al., 1999), exposure to the virus before pregnancy could also be a potential cause of seropositivity. Another explanation for this discrepancy is the fact that the virus may already be eliminated at the time of abortion/birth (Hansen et al., 2010). In addition, specific fetal or maternal antibodies can interfere with BVDv antigen detection in the fetus (Lanyon et al., 2014), leading to (false) negative results. Thus, calculating the prevalence of APM caused by BVDv, based only on fetal BVDv antigen detection, may lead to an underestimation of the true prevalence. However, Borel et al. (2014) mentioned that the use of ELISA Ag tests on fetal tissue might result in false-positive results, perhaps because of the effect of autolysis. As the fetus is able to produce BVDv specific antibodies when an infection is acquired after 150-180 days of gestation (Hansen et al., 2010), demonstrating antibodies in fetal/calf fluids may confirm intrauterine infection (Lanyon et al., 2014). However, in cases where the pregnant dam becomes infected with non-cytopathic BVDv prior to fetal immunocompetence (between 30 and 125 days of gestation), the fetus develops immunotolerance to the infecting BVDv strain, and will typically test negative for BVDv specific antibodies (Evans et al., 2019).

Fetal and perinatal mortality may be caused by more than one infectious or non-infectious agent in the same case (co-mortality or polypathia) (Macías-Rioseco et al., 2019; Henker et al., 2020; Mee, 2020). In 8% of the diagnosed cases, co-infections (more than one infectious agent in the same case) were present. In 26% of these cases, BVDv was detected in the fetus/calf. It is known that BVDv can induce immunosuppression (Roth et al., 1981), which may make cows and fetuses/perinates more sensitive to opportunistic bacteria or saprophytic fungi (Reggiardo and Kaeberle, 1981; Kirkbride, 1993), and which may also lead to a reactivation of quiescent *N. caninum* bradyzoites to differentiate into tachyzoites, allowing the parasite to infect the placenta and the fetus (Marugan-Hernandez, 2017).

Using the CRL measurements, it became clear that most submitted cases involved fetuses/calves in the second half of gestation. This reporting bias is caused by the fact that early abortions are more difficult to detect (Forar et al., 1996). Besides, lower diagnostic success in early abortions may also discourage submission of cases (Thurmond et al., 1994). Therefore, it needs to be mentioned that conclusions on prevalence of infectious causes of APM based on laboratory diagnostic results must be drawn with caution, because the submitted fetuses/calves are not representative of the real population of bovine fetal loss.

It is important to mention that the statistical methods for the differences associated with specific causes of APM was performed only on cases with at least one positive result following our analytical approach as depicted in **Figure 4.1.**, and only for pathogens with a prevalence ≥ 5%. We aimed to balance the dataset since we used conventional statistical methods for our data analysis. Future studies should aim to use more advanced analytical methods such as machine learning for more in depth evaluation and interpretation of the results.

Despite analyzing a significant number of APM cases, it's important to note that our study was conducted within a relatively short time frame (2010-2011). The relative contribution of different pathogens and the number of submitted APM cases may vary significantly between years, emphasizing the need for ongoing APM monitoring. Additionally, a two-year study period may be too brief to draw conclusive insights into seasonal effects on the prevalence of specific pathogens.

Conclusion

Although, even under mandatory circumstances, analysis of APM cases is susceptible to submission bias, trends in infectious abortifacient agents can be determined by collecting retrospective data. Despite the inclusion of an extensive diagnostic workout, in only 39% of the abortion or perinatal mortality cases, an underlying infectious cause could be identified. Either the diagnostic panel should be enlarged towards more infectious and non-infectious causes, or more laboratory diagnostics should be included to confirm associated causes. However, in cases

in which no cause of APM can be detected, ruling out typical abortive agents may be useful, especially at herd level.

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References

Anderson, M.L., 2012. Disorders of cattle, in: Njaa, B.L. (Ed.), Kirkbride's Diagnosis of Abortion and Neonatal Loss in Animals. Oxford: UK, pp. 13–48.

Anderson, M.L., 2007. Infectious causes of bovine abortion during mid- to late-gestation. Theriogenology 68, 474–486. https://doi.org/10.1016/j.theriogenology.2007.04.001

Barr, B.C., Conrad, P.A., Dubey, J.P., Anderson, M.L., 1991. Neospora-like encephalomyelitis in a calf: pathology, ultrastructure, and immunoreactivity. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 3, 39–46. https://doi.org/10.1177/104063879100300109

Bartels, C.J.M., Wouda, W., Schukken, Y.H., 1999. Risk factors for Neospora caninum-associated abortion storms in dairy herds in the Netherlands (1995 to 1997). Theriogenology 52, 247–257. https://doi.org/10.1016/S0093-691X(99)00126-0

Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. lme4: Linear mixed-effects models using Eigen and S4. R package version 1:1-7 [WWW Document]. URL http://cran.r-project.org/package=lme4

Baumgartner, W., 2015. Fetal disease and abortion: diagnosis and causes, in: Hopper, R.M. (Ed.), Bovine Reproduction. John Wiley & Sons, Oxford: UK, pp. 481–517.

Bonfini, B., Chiarenza, G., Paci, V., Sacchini, F., Salini, R., Vesco, G., Villari, S., Zilli, K., Tittarelli, M., 2018. Cross-reactivity in serological tests for brucellosis: a comparison of immune response of Escherichia coli O157:H7 and Yersinia enterocolitica O:9 vs Brucella spp. Veterinaria italiana 54, 107–114. https://doi.org/10.12834/VetIt.1176.6539.2

Borel, N., Frey, C.F., Gottstein, B., Hilbe, M., Pospischil, A., Franzoso, F.D., Waldvogel, A., 2014. Laboratory diagnosis of ruminant abortion in Europe. Vet J 200, 218–29. https://doi.org/10.1016/j.tvjl.2014.03.015

Botta, C., Pellegrini, G., Hässig, M., Pesch, T., Prähauser, B., Wunderlin, S., Guscetti, F., Schneeberger, M., Schmitt, S., Basso, W., Hilbe, M., Schuler, G., Borel, N., 2019. Bovine Fetal Placenta During Pregnancy and the Postpartum Period. Vet Pathol 56, 248–258. https://doi.org/10.1177/0300985818806453

Brickell, J.S., Mcgowan, M.M., Wathes, D.C., 2010. Association between Neospora caninum seropositivity and perinatal mortality in dairy heifers at first calving. Veterinary Record 167, 82–85. https://doi.org/10.1136/vr.c3583

Bronner, A., Hénaux, V., Fortané, N., Hendrikx, P., Calavas, D., 2014. Why do farmers and veterinarians not report all bovine abortions, as requested by the clinical brucellosis surveillance system in France? BMC Vet Res 10, 93. https://doi.org/10.1186/1746-6148-10-93

Callens, J., Debleecker, K., Stoop, S., Miry, C., 2009. Abortion in cattle: developing an optimal diagnostic approach, in: Espinasse, C. (Ed.), European Buiatrics Forum, 1st, Abstracts. French Buiatrics Association, Marseille, pp. 151–151.

Carpenter, T.E., Chrièl, M., Andersen, M.M., Wulfson, L., Jensen, A.M., Houe, H., Greiner, M., 2006. An epidemiologic study of late-term abortions in dairy cattle in Denmark, July 2000-August 2003. Preventive veterinary medicine 77, 215–29. https://doi.org/10.1016/j.prevetmed.2006.07.005

Clothier, K., Anderson, M., 2016. Evaluation of bovine abortion cases and tissue suitability for identification of infectious agents in California diagnostic laboratory cases from 2007 to 2012. Theriogenology 85, 933–938. https://doi.org/10.1016/j.theriogenology.2015.11.001

De Meerschman, F., Speybroeck, N., Berkvens, D., Rettignera, C., Focant, C., Leclipteux, T., Cassart, D., Losson, B., 2002. Fetal infection with Neospora caninum in dairy and beef cattle in Belgium. Theriogenology 58, 933–45. https://doi.org/10.1016/s0093-691x(02)00934-2

De Vries, A., 2006. Economic value of pregnancy in dairy cattle. Journal of Dairy Science 89, 3876–3885. https://doi.org/10.3168/jds.S0022-0302(06)72430-4

Dubey, J.P., Buxton, D., Wouda, W., 2006. Pathogenesis of Bovine Neosporosis. J Comp Pathol. https://doi.org/10.1016/j.jcpa.2005.11.004

Dubey, J.P., Schares, G., Ortega-Mora, L.M., 2007. Epidemiology and control of neosporosis and Neospora caninum. Clin Microbiol Rev. https://doi.org/10.1128/CMR.00031-06

Evans, C.A., Pinior, B., Larska, M., Graham, D., Schweizer, M., Guidarini, C., Decaro, N., Ridpath, J., Gates, M.C., 2019. Global knowledge gaps in the prevention and control of bovine viral diarrhoea (BVD) virus. Transboundary and Emerging Diseases. https://doi.org/10.1111/tbed.13068

Forar, A.L., Gay, J.M., Hancock, D.D., Gay, C.C., 1996. Fetal loss frequency in ten Holstein dairy herds. Theriogenology 45, 1505–1513.

Fredriksen, B., Sandvik, T., Løken, T., Ødegaard, S.A., 1999. Level and duration of serum antibodies in cattle infected experimentally and naturally with bovine virus diarrhoea virus. Veterinary Record 144, 111–114. https://doi.org/10.1136/vr.144.5.111

Garigliany, M., De Leeuw, I., Kleijnen, D., Vandenbussche, F., Callens, J., Van Loo, H., Lebrun, M., Saulmont, M., Desmecht, D., De Clercq, K., 2011. The presence of bluetongue virus serotype 8 RNA in Belgian cattle since 2008. Transboundary and emerging diseases 58, 503–9. https://doi.org/10.1111/j.1865-1682.2011.01230.x

Givens, M.D., Marley, M.S.D., 2008. Infectious causes of embryonic and fetal mortality. Theriogenology 70, 270–85. https://doi.org/10.1016/j.theriogenology.2008.04.018

Glover, A.D., Rech, R.R., Howerth, E.W., 2011. Pathology in practice. Mycotic abortion. Journal of the American Veterinary Medical Association 239, 319–21. https://doi.org/10.2460/javma.239.3.319

Graham, D.A., Beggs, N., Mawhinney, K., Calvert, V., Cunningham, B., Rowan-Layberry, L., Mclaren, I., 2009. Comparative evaluation of diagnostic techniques for bovine viral diarrhoea virus in aborted and stillborn fetuses. https://doi.org/10.1136/vr.164.2.56

Grooms, D.L., Bolin, C.A., 2005. Diagnosis of fetal loss caused by bovine viral diarrhea virus and Leptospira spp. Veterinary Clinics of North America - Food Animal Practice. https://doi.org/10.1016/j.cvfa.2005.02.010

Hall, G.A., Jones, P.W., 1977. A study of the pathogenesis of experimental Salmonella dublin abortion in cattle. Journal of Comparative Pathology 87, 53–65. https://doi.org/10.1016/0021-9975(77)90079-2

Hansen, T.R., Smirnova, N.P., Van Campen, H., Shoemaker, M.L., Ptitsyn, A.A., Bielefeldt-Ohmann, H., 2010. Maternal and fetal response to fetal persistent infection with bovine viral diarrhea virus. American journal of reproductive immunology (New York, N.Y. : 1989) 64, 295–306. https://doi.org/10.1111/j.1600-0897.2010.00904.x

Hemphill, A., Gottstein, B., 2000. A European perspective on Neospora caninum. International Journal for Parasitology. https://doi.org/10.1016/S0020-7519(00)00072-2

Henker, L.C., Lorenzett, M.P., Fagundes-Moreira, R., Dalto, A.G.C., Sonne, L., Driemeier, D., Soares, J.F., Pavarini, S.P., 2020. Bovine abortion, stillbirth and neonatal death associated with Babesia bovis and Anaplasma sp. infections in southern Brazil. Ticks and Tick-borne Diseases 11, 101443. https://doi.org/10.1016/j.ttbdis.2020.101443

Hovingh, E., 2009. Abortions in Dairy Cattle - II: Diagnosing and Preventing Abortion Problems | VCE Publications | Virginia Tech, Virginia Cooperative Extension.

Jamaluddin, A.A., Case, J.T., Hird, D.W., Blanchard, P.C., Peauroi, J.R., Anderson, M.L., 1996. Dairy cattle abortion in California: Evaluation of diagnostic laboratory data. Journal of Veterinary Diagnostic Investigation. https://doi.org/10.1177/104063879600800211

Kennedy, P.C., Richards, W.P.C., 1964. The Pathology of Abortion Caused by the Virus of Infectious Bovine Rhinotracheitis. Pathol Vet 1, 7–17. https://doi.org/10.1177/030098586400100103

Khodakaram-Tafti, A., Ikede, B.O., 2005. A retrospective study of sporadic bovine abortions, stillbirths, and neonatal abnormalities in Atlantic Canada, from 1990 to 2001. Canadian Veterinary Journal 46, 635–637.

Kiernan, J.A., 2008. Histological and histochemical methods: theory and practice, 4th ed. Scion Publishing Ltd, UK.

Kirkbride, C.A., Gates, C.E., Libal, M.C., 1989. Ovine and bovine abortion associated with Fusobacterium nucleatum. Journal of Veterinary Diagnostic Investigation 1, 272–273. https://doi.org/10.1177/104063878900100318

Kirkbride, C.A., 1993. Bacterial agents detected in a 10-year study of bovine abortions and stillbirths. J Vet Diagn Invest 5, 64–8. https://doi.org/10.1177/104063879300500114

Lanyon, Sasha R., Hill, F.I., Reichel, M.P., Brownlie, J., 2014. Bovine viral diarrhoea: Pathogenesis and diagnosis. Veterinary Journal. https://doi.org/10.1016/j.tvjl.2013.07.024

Lanyon, Sasha R, Sims, S.K., Cockcroft, P.D., Reichel, M.P., 2014. Comparison of serum, ear notches, and nasal and saliva swabs for Bovine viral diarrhea virus antigen detection in colostrum-fed persistently infected (PI) calves and non-PI calves. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 26, 783–7. https://doi.org/10.1177/1040638714550181

Lee, J.I., Kim, I.H., 2007. Pregnancy loss in dairy cows: The contributing factors, the effects on reproductive performance and the economic impact. Journal of Veterinary Science 8, 283–288. https://doi.org/10.4142/jvs.2007.8.3.283

Macías-Rioseco, M., Riet-Correa, F., Miller, M.M., Sondgeroth, K., Fraga, M., Silveira, C., Uzal, F.A., Giannitti, F., 2019. Bovine abortion caused by Coxiella burnetii: report of a cluster of cases in Uruguay and review of the literature. Journal of veterinary diagnostic

investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 31, 634–639. https://doi.org/10.1177/1040638719856394

Marugan-Hernandez, V., 2017. Neospora caninum and Bovine Neosporosis: Current Vaccine Research. Journal of comparative pathology 157, 193–200. https://doi.org/10.1016/j.jcpa.2017.08.001

McCausland, I.P., Slee, K.J., Hirst, F.S., 1987. Mycotic abortion in cattle. Australian veterinary journal 64, 129–132. https://doi.org/10.1111/j.1751-0813.1987.tb09659.x

McClurkin, A.W., Littledike, E.T., Cutlip, R.C., Frank, G.H., Coria, M.F., Bolin, S.R., 1984. Production of cattle immunotolerant to bovine viral diarrhea virus. Canadian Journal of Comparative Medicine 48, 156–161.

Mee, J., 2013. Why Do So Many Calves Die on Modern Dairy Farms and What Can We Do about Calf Welfare in the Future? Animals 3, 1036–1057. https://doi.org/10.3390/ani3041036

Mee, J.F., 2015. Investigating stillbirths in cattle – What should a practitioner do? Cattle Practice 23, 114–121.

Mee, J.F., 2020. Investigation of bovine abortion and stillbirth/perinatal mortality - Similar diagnostic challenges, different approaches. Irish Veterinary Journal. https://doi.org/10.1186/s13620-020-00172-0

Méroc, E., Poskin, A., Van Loo, H., Quinet, C., Van Driessche, E., Delooz, L., Behaeghel, I., Riocreux, F., Hooyberghs, J., De Regge, N., Cay, B., van den Berghe, T., Van Der Stede, Y., 2013. Schmallenberg virus: Results of Belgian cross-sectional serological surveys, in: Epizone Abstracts, 7th Annual Meeting “Nothing Permanent, except Change.” p. 58.

Meyer, C.L., Berger, P.J., Koehler, K.J., Thompson, J.R., Sattler, C.G., 2001. Phenotypic trends in incidence of stillbirth for Holsteins in the United States. Journal of Dairy Science 84, 515–523. https://doi.org/10.3168/jds.S0022-0302(01)74502-X

Noden, D.M., DeLahunta, A., 1985. The embryology of domestic animals: developmental mechanisms and malformations. Williams & Wilkins, Baltimore MD.

Norman, H.D., Miller, R.H., Wright, J.R., Hutchison, J.L., Olson, K.M., 2012. Factors associated with frequency of abortions recorded through Dairy Herd Improvement test plans. J Dairy Sci 95, 4074–4084. https://doi.org/10.3168/jds.2011-4998

Norquay, R., Orr, J., Norquay, B., Ellis, K.A., Mee, J.F., Reeves, A., Scholes, S., Geraghty, T., 2020. Perinatal mortality in 23 beef herds in Orkney: Incidence, risk factors and aetiology. Veterinary Record. https://doi.org/10.1136/vr.105536

Pereira-Bueno, J., Quintanilla-Gozalo, A., Seijas-Carballedo, A., Costas, E., Ortega, L.M., 2000. Observational studies in Neospora caninum infected dairy cattle: pattern of transmission and age-related antibody fluctuations. International Journal of Parasitology 30, 877–924.

Reggiardo, C., Kaeberle, M.L., 1981. Detection of bacteremia in cattle inoculated with bovine viral diarrhea virus. American journal of veterinary research 42, 218–21.

Ribbens, S., De Bleecker, K., Van Crombrugge, J.M., De Meulemeester, L., Mijten, E., Stoop, S., De Kruif, A., Dewulf, J., 2009. Serosurvey of four 'emerging' cattle diseases (Q-fever, Neosporosis, Leptospirosis and Salmonellosis) in Northern-Belgian dairy herds using bulk-milk samples.

Roth, J.A., Kaeberle, M.L., Griffith, R.W., 1981. Effects of bovine viral diarrhea virus infection on bovine polymorphonuclear leukocyte function. American journal of veterinary research 42, 244–50.

Sager, H., Fischer, I., Furrer, K., Strasser, M., Waldvogel, A., Boerlin, P., Audigé, L., Gottstein, B., 2001. A Swiss case-control study to assess Neospora caninum-associated bovine abortions by PCR, histopathology and serology. Veterinary Parasitology 102, 1–15. https://doi.org/10.1016/S0304-4017(01)00524-6

Sánchez-Miguel, C., Crilly, J., Grant, J., Mee, J.F., 2018. Sensitivity, specificity and predictive probability values of serum agglutination test titres for the diagnosis of Salmonella Dublin culture-positive bovine abortion and stillbirth. Transbound Emerg Dis 65, 676–686. https://doi.org/10.1111/tbed.12784

Smyth, J.A., McNamee, P.T., Kennedy, D.G., McCullough, S.J., Logan, E.F., Ellis, W.A., 1992. Stillbirth/perinatal weak calf syndrome: preliminary pathological, microbiological and biochemical findings. The Veterinary record 130, 237–240. https://doi.org/10.1136/vr.130.12.237

Smyth, J.A., Fitzpatrick, D.A., Ellis, W.A., 1999. Stillbirth/perinatal weak calf syndrome: a study of calves infected with Leptospira. Veterinary Record 145, 539–542. https://doi.org/10.1136/vr.145.19.539

Taylor, R.F., Njaa, B.L., 2012. General Approach to fetal and neonatal loss, in: Njaa, Bradley L; (Ed.), Kirkbride's Diagnosis of Abortion and Neonatal Loss in Animals. John Wiley & Sons, Oxford: UK, pp. 1–12.

Thurmond, M.C., Blanchard, P.C., Anderson, M.L., 1994. An example of selection bias in submissions of aborted bovine fetuses to a diagnostic laboratory. Journal of veterinary

diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 6, 269–271. https://doi.org/10.1177/104063879400600224

Thurmond, M.C., Anderson, M.L., Blanchard, P.C., 1995. Secular and seasonal trends of Neospora abortion in California dairy cows. The Journal of parasitology 81, 364–7.

Toussaint, J.F., Sailleau, C., Mast, J., Houdart, P., Czaplicki, G., Demeestere, L., VandenBussche, F., Van Dessel, W., Goris, N., Bréard, E., Bounaadja, L., Thiry, E., Zientara, S., De Clercq, K., 2007. Bluetongue in Belgium, 2006. Emerging Infectious Diseases 13, 614–616. https://doi.org/10.3201/eid1304.061136

Van Loo, H., Méroc, E., Cay, B., Van de Stede, Y., De Bleecker, K., 2013. Use of abortion surveillance in cattle to verify the introduction of Schmallenberg Virus in Flanders., in: Epizone Abstracts, 7th Annual Meeting "Nothing Permanent, except Change." p. 101.

Vangeel, I., Méroc, E., Roelandt, S., Welby, S., Driessche, E. V., Czaplicki, G., Schoubroeck, L. V., Quinet, C., Riocreux, F., Hooyberghs, J., Houdart, P., Stede, Y. V.D., 2012. Seroprevalence of Neospora caninum, paratuberculosis and Q fever in cattle in Belgium in 2009-2010. Veterinary Record 171, 477. https://doi.org/10.1136/vr.101016

Waldner, C.L., Kennedy, R.I., Rosengren, L.B., Pollock, C.M., Clark, E.G., 2010. Article Gross postmortem and histologic examination findings from abortion losses and calf mortalities in western Canadian beef herds, Can Vet J.

Wheelhouse, N., Mearns, R., Willoughby, K., Wright, E., Turnbull, D., Longbottom, D., 2015. Evidence of members of the Chlamydiales in bovine abortions in England and Wales. The Veterinary record 176, 465. https://doi.org/10.1136/vr.103075

Wickham, H., 2009. ggplot2 Elegant Graphics for Data Analysis, 1st ed. Springer-Verlag New York, New York.

Wilson, A., Darpel, K., Mellor, P.S., 2008. Where does bluetongue virus sleep in the winter? PLoS Biology. https://doi.org/10.1371/journal.pbio.0060210

Wolf-Jäckel, G.A., Hansen, M.S., Larsen, G., Holm, E., Agerholm, J.S., Jensen, T.K., 2020. Diagnostic studies of abortion in Danish cattle 2015-2017. Acta Vet Scand 62, 1. https://doi.org/10.1186/s13028-019-0499-4

Wouda, W., Bartels, C.J.M., Moen, A.R., 1999. Characteristics of Neospora caninum-associated abortion storms in dairy herds in the Netherlands (1995 to 1997). Theriogenology 52, 233–245. https://doi.org/10.1016/S0093-691X(99)00125-9

Wouda, W., Moen, A.R., Visser, I.J., van Knapen, F., 1997. Bovine fetal neosporosis: a comparison of epizootic and sporadic abortion cases and different age classes with regard to lesion severity and immunohistochemical identification of organisms in brain, heart, and liver. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc 9, 180–5. https://doi.org/10.1177/104063879700900212

Yaeger, M.J., Holler, L.D., 2007. Bacterial causes of bovine infertility and abortion, in: Youngquist, R.S., Threlfall, W.R. (Eds.), Current Therapy in Large Animal Theriogenology. WB Saunders Co, pp. 389–398.

Chapter 5

Detection of *Anaplasma phagocytophilum* in fetal and placental tissue of bovine abortions and perinatal mortalities

Hans Van Loo[1], Osvaldo Bogado Pascottini[1], Jozef Hooyberghs[2], Koen De Bleecker[3], Stefaan Ribbens[3], Geert Opsomer[1], and Bart Pardon[1]

[1] Department of Internal Medicine, Reproduction and Population Medicine, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium
[2] Federal Agency for the Safety of the Food Chain, Brussels, Belgium
[3] Animal Health Services Flanders (Dierengezondheidszorg Vlaanderen), Torhout, Belgium

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Abstract

Background: *Anaplasma phagocytophilum* (*A. phagocytophilum*) is a tick-borne zoonotic bacterium that is the etiologic pathogen of tick-borne fever (TBF) in ruminants. In clinical bovine cases of TBF, abortion and stillbirth may be observed. However, in this regard, the pathophysiology of TBF is not completely elucidated yet, and no clear guidelines to diagnose *A. phagocytophilum*-related abortions and perinatal mortalities (APM) are available.

Materials & methods: This exploratory study aimed to investigate the presence of *A. phagocytophilum* in bovine cases of APM, and to determine which is the tissue with the greatest sensitivity for *A. phagocytophilum* identification, placental or fetal spleen. The placenta and fetal spleen of 150 late term bovine APM cases were analyzed by real-time PCR for *A. phagoctyophilum*.

Results: Two-point seven percent of sampled placentas were positive for *A. phagocytophilum*, while none of the fetal spleen samples was.

Limitations: No histopathology to detect associated lesions was performed. Consequently, no evidence of causality between the detection of *A. phagocytophilum* and the APM events could be achieved.

Conclusion: The detection of *A. phagocytophilum* suggests a potential role of this pathogen in bovine APM, and placental tissue seems to be the most adequate tissue for its identification.

Keywords: *dairy cow; beef cow; abortion; infection; diagnosis*.

Introduction

Anaplasma phagocytophilum (*A. phagoctyophilum*) is a tick-born organism with an important impact on both human and animal health (Chianini et al., 2004; Woldehiwet, 2010; Chochlakis et al., 2020). In ruminants, this gram-negative bacterium is known as the cause of tick-borne fever (TBF). Clinical signs of TBF in cattle may vary in severity, although dullness, anorexia, reduced milk yield, coughing, abortions and stillbirth are common (Atif, 2015). However, many asymptomatic bovine cases have been described (Pusterla et al., 1997). Tick-borne fever is reported worldwide, but in Europe, the majority of clinical cases are observed in summer, when environmental conditions are optimal for tick proliferation, and when cattle are grazing in potentially tick-infested pasture (Pusterla et al., 1998). In Belgium, the first bovine case of TBF was detected in 2005 (Guyot et al., 2011). The vector (*Ixodes ricinus* tick) is present all over the country, but some provinces are at higher risk because of a more suitable habitat for ticks and their reservoir hosts (e.g. roe deers and rodents) (Tavernier et al., 2015; Hing et al., 2018). A longitudinal field study, conducted in southern Belgium, reported a high seroprevalence of *A. phagocytophilum* in cattle with seasonal effect (31 % in spring, 77 % in summer, and 57 % in autumn) (Lempereur et al., 2012). These findings suggest the pathogen is endemic in the country. However, the high prevalence in cattle may be the result of sampling bias, because all samples from the study originated from farms with a confirmed history of TBF. In the northern part of Belgium (Flanders), an *A. phagocytophilum* seroprevalence of 46% was observed in roe deer (Tavernier et al., 2015). Unfortunately, no data about the prevalence in cattle are available for Flanders, nor its association with abortion.

Diagnosis of TBF relies on laboratorial analyses. During the acute phase of TBF, typical inclusions (morulae) may be seen in granulocytes of peripheral blood-stained smears. However, more sensitive techniques such as PCR were developed to detect specific nucleic acids of *A. phagocytophilum* in blood and tissues samples (Courtney et al., 2004). Moreover, serological techniques may be used to demonstrate rising antibody titers for sero-diagnosis (Atif, 2015). Yet, no clear guidelines to diagnose *A. phagocytophilum*-related abortions and perinatal mortalities (APM) are available. Previous studies detected *A. phagocytophilum* in blood samples from aborted cattle (Wilson et al., 1964; Dugat et al., 2017), but the pathophysiology of TBF is not completely elucidated yet. Therefore, it may be interesting to analyze the potential role of *A. phagocytophilum* in cases of bovine APM.

This descriptive, cross-sectional study aims to investigate the presence of *A. phagocytophilum* in placenta and fetal spleen samples from bovine cases of APM in Flanders, Belgium.

Materials and methods

Samples originated from APM cases that were submitted for analysis, as part of the mandatory national brucellosis monitoring program. As *A. phagocytophilum* is transmitted by ticks, cases were selected from a period with high tick activity, between July and September 2012. Sample sets (aborted fetus/calf, placenta, and maternal blood sample) were collected at the farm, individually transported and stored at 4°C, and processed within 24h after arrival at the laboratory facilities. Cases with absence of one of those samples, and twin cases were excluded from the present study. The study population consisted of 150 APM cases originating from 142 cattle herds in Flanders.

At necropsy, gestation length was calculated as described by Noden et al. (1985) (gestation length in days = 68 + 2.25 x crown-rump length [CRL] in cm). Crown-rump length was measured from the most anterior point of the calvarium with the head flexed 90˚ to the spine until the most caudal part of the thigh (tuber ischiadicum). Only cases with a gestation length of > 7 months were included, as *A. phagocytophilum* is thought to cause fetal death in late pregnancy (Wilson et al., 1964; Pusterla et al., 1997). Afterwards, the fetus was cut-opened for sampling. Spleen and placental tissue samples were collected using sterile scalpels. The placental tissue was collected at the central location level of a cotyledon.

Placental and spleen samples were tested by real-time PCR for *A. phagocytophilum* (ID Gene Anaplasma phagocytophilum Duplex, IDvet Genetics, France) after DNA extraction using a commercial kit (IndiMag Pathogen Kit, Indical Bioscience GmbH, Germany). The limit of detection of the real-time PCR was 2.5 copies per PCR-reaction. The diagnostic sensitivity of the test was 96%, and specificity was 100%. Cases with a cycle threshold < 45 were classified as positive for *A. phagocytophilum*. Acute (at the moment of APM) and convalescent (4 weeks later) serum samples of the dams with *A. phagocytophilum* real-time PCR positive cases were analyzed using an immunofluorescence antibody test (VMRD, Pullman, USA) according the instructions of the manufacturer. The diagnostic sensitivity of the test was 98%, and the specificity 100%.

Results

From all the APM cases analyzed, the production type of the dam was dairy in 59% (88/150) and beef in 41% (62/150). Nine percent of the cases (13/150) happened in the 7th month of gestation, 33% (49/150) in the 8th month, and 27% (41/150) in the 9th month. In 31% (47/150) of the cases, gestation length was more than 290 days. Twenty-eight percent (43/150) of the dams were primiparous, while 71% (107/150) were multiparous.

Of the placenta samples, 2.7% (4 of 150) were positive for *A. phagocytophilum*, while none of the spleen samples was. Three of the 4 positive cases were detected in beef (4.8%, 3/62), while 1 in dairy (1.1%, 1/88). All of the *A. phagocytophilum* positive samples were found in the 8th and 9th month of gestation and originated from different farms. In two of the *A. phagocytophilum* positive cases, co-infections were detected (one with BVDv, and one with *Pseudomonas aeruginosa*).

In 2 of the *A. phagocytophilum* real-time PCR positive cases, the dam was seropositive at the moment of APM, and remained positive in the convalescent serum sample. In another PCR positive case, the dam was seronegative in both the acute and convalescent sample. For the fourth PCR positive case, no maternal serology could be performed because of hemolysis of the corresponding serum sample.

Discussion

Although this study was conducted during the period of highest tick activity, only 2.7 % of the cases were positive for this tick-borne pathogen. Nevertheless, care must be taken when handling abortion tissues, because *A. phagoctyphilum* has been identified as a potential zoonosis (Hing et al., 2018).

Beef cattle seem to be over-represented in the *A. phagocytophilum* positive samples, although the small size of the present study population impedes to draw statistically relevant conclusions. This finding may be explained by relevant differences in production system between beef and dairy cattle. In Flanders, zero-grazing is applied in many dairy herds all year round, while most of the Belgian blue beef cattle go on pasture during breeding season, from spring until fall. This may result in a higher chance of tick bites in beef cattle, which includes a higher chance of becoming infected with *A. phagocytophilum*.

The pathophysiology of APM due to *A. phagocytophilum* remains obscure. The rise in body temperature following infection, which usually lasts for about 7 days, was suggested to be involved (Wilson et al., 1964; Woldehiwet, 2010). Based on this hypothesis, a rapid onset of APM after infection could be expected. In the present study, acute and convalescent serum samples of 3 dams of *A. phagocytophilum* positive cases were analyzed for specific antibodies against the pathogen. Two of the dams were seropositive for *A. phagocytophilum* at the moment of APM, and remained positive in the next sample, which indicates the chronic character of these APM events. In these chronic cases, probably another factor (e.g. placental dysfunction) instead of only acute fever might have been involved in the *A. phagocytophilum* APM process. Surprisingly, one of the 3 analyzed dams was seronegative in both the acute and convalescent serum sample. No clear explanation for this finding could be found.

Diagnosing a definite cause of APM is challenging. It remains difficult to achieve causality, even when one single potential cause of APM is detected (Van Loo et al., 2021). *Anaplasma phagocytophilum* has been found in asymptomatic cattle (Ooshiro et al., 2008), meaning that the detection of the organism in APM tissue samples may be without clinical significance. Nevertheless, both transplacental fetal infection and abortion caused by *A. phagocytophilum* are reported in goats (Chochlakis et al., 2020), cattle (Pusterla et al., 1997), and sheep (Reppert et al., 2013). Dugat et al. (2017) described genetic differences in *A. phagocytophilum* strains from cattle that had aborted, compared to strains from cattle that had not aborted. This finding could be used as a potential marker to evidence *A. phagocytophilum* as the cause of a case of

APM. Unfortunately, no genotyping to detect specific alleles related to abortive strains was performed in the present study.

Analysis of APM for the presence of infectious abortifacient agents should be combined with immunohistochemistry, and histopathology from placental or fetal tissue to detect associated lesions. In ovine fetuses, histopathological examination of brain tissue of infected fetuses revealed leukomalacia in the cerebral and cerebellar white matter after transplacental infection with *A. phagocytophilum* (Chianini et al., 2004). According to Loeliger et al. (2003), leukomalacia may be associated with ischemia and/or hypoxia in late pregnancy, which may be caused by placental dysfunction (Chianini et al., 2004). In lambs born after transplacental *A. phagocytophilum* infection, lesions were restricted to the lymphoid system (splenomegaly and lymphomegaly) (Reppert et al., 2013). In the present study, no macroscopic splenomegaly or lymphomegaly could be detected in the cases positive for *A. phagocytophilum*. To the best of our knowledge, no information is available about histopathological lesions in bovine placental and fetal tissue associated with an *A. phagocytophilum* infection. Due to financial limitations, no histopathology was performed in the present study.

In previous studies, *A. phagocytophilum* could be identified by PCR in the blood, spleen, heart, skin, and lymph nodes of transplacental infected lambs after an experimental infection in sheep (Reppert et al., 2013). In aborted goats, only abomasal content and especially placental tissue, but no other tissues, were reported as positive by PCR for the organism (Chochlakis et al., 2020). Unfortunately, no reports are available about the predilection sites of the pathogen after infection during gestation in cattle. Based on the main lesion after intrauterine infection in lambs (splenomegaly) (Reppert et al., 2013), and on the detection of the organism in placental tissue of caprine abortions (Chochlakis et al., 2020), we analyzed only placenta and fetal spleen for the presence of *A. phagocytophilum* in the present study.

Conclusion

The present study suggests a potential role of *A. phagocytophilum* in cases of bovine APM in Belgium. Placental tissue is probably the most preferred tissue for detecting this pathogen, although other tissues, which have not been analyzed until now, could be more suitable. To improve cost-effectiveness of diagnosing APM, a test algorithm based on maternal (month of gestation, and production type) and environmental characteristics (region, season, and access to pasture) could be used to decide if the inclusion of this PCR test may be beneficial. Future studies are warranted to reveal whether placental transmission plays a role in the epidemiology of *A. phagocytophilum* in cases of APM.

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References

Atif, F.A., 2015. Anaplasma marginale and Anaplasma phagocytophilum: Rickettsiales pathogens of veterinary and public health significance. Parasitology Research 2015 114:11 114, 3941–3957. https://doi.org/10.1007/S00436-015-4698-2

Chianini, F., Adams, C., Buxton, D., 2004. Neuropathological changes in ovine fetuse caused by tickborne fever. Vet Rec 155, 805–806.

Chochlakis, D., Giadinis, N., Petridou, E., Filioussis, G., Tselentis, Y., Psaroulaki, A., Ioannidou, E., Papanikolopoulou, V., Karatzias, H., 2020. Molecular evidence of Anaplasma phagocytophilumin aborted goat fetuses and placenta. Vet Ital 56, 301–303. https://doi.org/10.12834/VETIT.1173.6516.2

Courtney, J.W., Kostelnik, L.M., Zeidner, N.S., Massung, R.F., 2004. Multiplex real-time PCR for detection of Anaplasma phagocytophilum and Borrelia burgdorferi. J Clin Microbiol 42, 3164–3168. https://doi.org/10.1128/JCM.42.7.3164-3168.2004/FORMAT/EPUB

Dugat, T., Haciane, D., Durand, B., Lagrée, A.-C., Haddad, N., Boulouis, H.-J., 2017. Short Report: Identification of a Potential Marker of Anaplasma phagocytophilum Associated with Cattle Abortion. Transboundary and emerging diseases 64, e1–e3. https://doi.org/10.1111/tbed.12508

Guyot, H., Ramery, E., O'Grady, L., Sandersen, C., Rollin, F., 2011. Emergence of bovine ehrlichiosis in Belgian cattle herds. Ticks Tick Borne Dis 2, 116–118. https://doi.org/10.1016/J.TTBDIS.2010.12.001

Hing, M., Van Den Bossche, D., Lernout, T., Cochez, C., Pirnay, J.-P., Heuninckx, W., 2018. Prevalence of Anaplasma phagocytophilum in humans in Belgium for the period 2013-2016. Acta Clin Belg 74, 280–285.

Lempereur, L., Lebrun, M., Cuvelier, P., Sépult, G., Caron, Y., Saegerman, C., Shiels, B., Losson, B., 2012. Longitudinal field study on bovine Babesia spp. and Anaplasma phagocytophilum infections during a grazing season in Belgium. Parasitology research 110, 1525–1530. https://doi.org/10.1007/s00436-011-2657-0

Loeliger, M., Watson, C.S., Reynolds, J.D., Penning, D.H., Harding, R., Bocking, A.D., Rees, S.M., 2003. Extracellular glutamate levels and neuropathology in cerebral white matter following repeated umbilical cord occlusion in the near term fetal sheep. Neuroscience 116, 705–714. https://doi.org/10.1016/S0306-4522(02)00756-X

Noden, D.M., DeLahunta, A., 1985. The embryology of domestic animals: developmental mechanisms and malformations. Williams & Wilkins, Baltimore MD.

Ooshiro, M., Zakimi, S., Matsukawa, Y., Katagiri, Y., Inokuma, H., 2008. Detection of Anaplasma bovis and Anaplasma phagocytophilum from cattle on Yonaguni Island, Okinawa, Japan. Veterinary parasitology 154, 360–364. https://doi.org/10.1016/J.VETPAR.2008.03.028

Pusterla, N., Braun, U., 1997. Clinical Findings in Cows after Experimental Infection with Ehrlichia phagocytophila. Zentralblatt fur Veterinarmedizin 44, 385–390. https://doi.org/10.1111/j.1439-0442.1997.tb01123.x

Pusterla, N., Braun, U., Wolfensberger, C., Lutz, H., 1997. Intrauterine infection with Ehrlichia phagocytophila in a cow. Vet Rec 141, 101–102. https://doi.org/10.1136/VR.141.4.101

Pusterla, N., Pusterla, J.B., Braun, U., Lutz, H., 1998. Serological, Hematologic, and PCR Studies of Cattle in an Area of Switzerland in Which Tick-Borne Fever (Caused by Ehrlichia phagocytophila) Is Endemic. Clin Diagn Lab Immunol 5, 325. https://doi.org/10.1128/CDLI.5.3.325-327.1998

Reppert, E., Galindo, R.C., Breshears, M.A., Kocan, K.M., Blouin, E.F., de la Fuente, J., 2013. Demonstration of transplacental transmission of a human isolate of Anaplasma phagocytophilum in an experimentally infected sheep. Transbound Emerg Dis 60 Suppl 2, 93–96. https://doi.org/10.1111/TBED.12120

Tavernier, P., Sys, S.U., De Clercq, K., De Leeuw, I., Caij, A.B., De Baere, M., De Regge, N., Fretin, D., Roupie, V., Govaerts, M., Heyman, P., Vanrompay, D., Yin, L., Kalmar, I., Suin, V., Brochier, B., Dobly, A., De Craeye, S., Roelandt, S., Goossens, E., Roels, S., 2015. Serologic screening for 13 infectious agents in roe deer (Capreolus capreolus) in Flanders. Infect Ecol Epidemiol 5. https://doi.org/10.3402/IEE.V5.29862

Van Loo, H., Pascottini, O.B., Ribbens, S., Hooyberghs, J., Pardon, B., Opsomer, G., 2021. Retrospective study of factors associated with bovine infectious abortion and perinatal mortality. Prev Vet Med 105366. https://doi.org/10.1016/j.prevetmed.2021.105366

Wilson, J.C., Foggie, A., Carmichael, M.A., 1964. Tick-borne fever as a cause of abortion and stillbirhts in cattle. Vet Rec 76, 1081–1081.

Woldehiwet, Z., 2010. The natural history of Anaplasma phagocytophilum. Vet Parasitol 167, 108–122. https://doi.org/10.1016/J.VETPAR.2009.09.013

Chapter 6

Detection of *Chlamydia* and *Chlamydia*-like organisms in bovine placental tissue

Hans Van Loo[1], Osvaldo Bogado Pascottini[1], Stefaan Ribbens[2], Jozef Hooyberghs[3], Marcella Mori[4], Daisy Vanrompay[5], Geert Opsomer[1], and Bart Pardon[1]

[1] Department of Internal Medicine, Reproduction and Population Medicine, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium
[2] Animal Health Services Flanders (Dierengezondheidszorg), Torhout, Belgium
[3] Federal Agency for the Safety of the Food Chain, Brussels, Belgium
[4] Federal Research Institute for Health, Bacterial Zoonoses, Veterinary Bacteriology, Brussels, Belgium
[5] Department of Molecular Biotechnology, Ghent University, Ghent, Belgium

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Abstract

This study aimed to investigate the presence of *Chlamydia* spp. and *Parachlamydia acanthamoebae* in bovine placental tissue originating from abortion and non-abortion cases in Belgium. Placentas of 164 late term bovine abortions (last trimester of gestation) and 41 non-abortion (collected after calving) cases were analyzed by PCR for *Chlamydia* spp., *Chlamydia abortus, Chlamydia psittaci,* and *Parachlamydia acanthamoebae*. Additionally, a subset of 101 (75 abortion and 26 non-abortion cases) of these placenta samples were also analyzed by histopathology to detect possible *Chlamydia*-induced lesions. *Chlamydia* spp. were detected in 5.4% (11/205) of the cases, being 6.1% in the abortion cases (10/164, of which 1 was positive, and 9 were inconclusive), and 2.4% in the non-abortion cases (1/41, inconclusive). Three of those cases were positive for *Chlamydia psittaci*. *Parachlamydia acanthamoebae* was detected in 36% (75/205) of the cases, being 44% in abortions (72/164, of which 18 were positive, and 54 inconclusive), and 7.3% in non-abortion cases (3/41, of which 2 were positive, and 1 inconclusive) ($P < 0.001$). None of the cases was positive for *Chlamydia abortus*. Purulent and/or necrotizing placentitis with or without vasculitis was observed in 18.8% (19/101) of the histopathologically analyzed placenta samples. In 5.9% (6/101) of the cases, placentitis was observed along with vasculitis. In the abortion cases, 24% (18/75) of the samples showed purulent and/or necrotizing placentitis, while purulent and/or necrotizing placentitis was visible in 3.9% (1/26) of the non-abortion cases. Placental lesions of inflammation and/or necrosis were present in 44% (15/34) of the cases where *P. acanthamoebae* was detected, while inflammation and/or necrosis was present in 20.9% (14/67) of the negative cases ($P < 0.05$). The detection of *Chlamydia* spp. and especially *Parachlamydia acanthamoebae*, in combination with correlated histological lesions such as purulent and/or necrotizing placentitis and/or vasculitis in placental tissue following abortion, suggests a potential role of this pathogen in cases of bovine abortion in Belgium. Further in-depth studies are necessary to unravel the role of these species as abortifacient agents in cattle and to include them in bovine abortion monitoring programs.

Keywords: *bovine; abortion; infection; diagnosis; placentitis*.

Introduction

Chlamydia spp. are gram-negative obligate intracellular bacteria that might be involved in a wide range of important diseases in several species (Longbottom et al., 2003). In ruminants, infections with *Chlamydia* (C.) *abortus*, *C. pecorum*, *C. psittaci*, and rarely *C. suis* have been described (Reinhold et al., 2011). In cattle, *C. psittaci* is reported as the most prevalent representative of *Chlamydia* spp., followed by *C. abortus* and *C. pecorum* (Kemmerling et al., 2009). *Chlamydia* spp. are associated with several clinical syndromes like arthritis, encephalomyelitis, conjunctivitis, pneumonia, enteritis, hepatitis, and reproductive disorders (Reinhold et al., 2011). It is also known that *Chlamydia* spp. are able to affect placental function, which may result in abortions and perinatal mortalities (Cavirani et al., 2001; Wang et al., 2001; Kemmerling et al., 2009). In the last decades, novel *Chlamydia*-like organisms (CLO) have also been detected in aborted bovine fetal and placental tissue samples using both molecular and immunohistochemical techniques (Dilbeck et al., 1990; Ruhl et al., 2009; Wheelhouse et al., 2010, 2012). *Chlamydia*-like organisms include members of the *Parachlamydiaceae* (e.g., *P. acanthamoebae*), *Rhabdochlamydiacaea*, and *Waddlia chondrophila*, and are reported to be present in the US, Switzerland, and the UK (Dilbeck et al., 1990; Ruhl et al., 2009; Wheelhouse et al., 2012). In Belgium, epidemiological data on *Chlamydia* spp. and CLO in cattle are scant. In an epidemiological study, a *C. abortus* cow-level seroprevalence of 1.7% and a herd-level seroprevalence of 14.7% were observed in Belgium (Yin et al., 2014). One case report described the detection of *C. psittaci* in Belgian dairy cattle with signs of respiratory disease and milk drop syndrome (Van Loo et al., 2014). To the best of our knowledge, no other data are available about the presence of *Chlamydia* spp. and CLO in the Belgian bovine populations.

Diagnosing infections with *Chlamydia* spp. or CLO in cattle requires laboratory assays, as signs of chlamydial infections are not specific. Since most chlamydial infections do not induce high antibody responses, serological diagnosis of a recent infection is generally inappropriate (Griffiths et al., 1996; Reinhold et al., 2011). Culturing *Chlamydia* spp. and CLO may be challenging, but more sensitive techniques such as polymerase chain reaction (PCR) testing have been developed to detect specific nucleic acids of the pathogens (Kaltenboeck et al., 2005; Ruhl et al., 2009; Pantchev et al., 2010). However, infections with *Chlamydia* spp. and CLO may be subclinical (Kemmerling et al., 2009; Reinhold et al., 2011; Wheelhouse et al., 2022), which makes it difficult to achieve causality in case of detection of the organisms. Therefore, histopathology or immunohistochemistry are essential diagnostic tools to establish a conclusive

diagnosis. In cases of chlamydial abortion in cattle, purulent and/or necrotizing placentitis has been described (Borel et al., 2006; Ruhl et al., 2009).

Since *Chlamydia* spp. and CLO are potentially zoonotic (Baud et al., 2008; Longbottom et al., 2003), greater attention should be given to monitor these organisms. Therefore, this cross-sectional study aims to investigate, 1) the presence of *Chlamydia* spp. (*C. abortus* and *C. psittaci*) and *P. acanthamoebae* in bovine placental tissue in the northern part of Belgium (Flanders), 2) the presence of correlated histopathologic lesions, and 3) if there is a difference in prevalence of *Chlamydia*/CLO and correlated histopathologic lesions in abortion versus non-abortion cases.

Materials and methods

Sample selection and processing

Samples originated from abortion cases that were submitted for analysis, as part of the mandatory national brucellosis monitoring program, between August and December 2013. Sample sets consisted of the aborted fetus, the placenta, and a maternal blood sample. Cases lacking one of those samples, and twin cases were excluded. Samples were collected at the farm, individually transported, stored at 4°C, and processed within 24 h after arrival at the laboratory facilities (DGZ Vlaanderen, Animal Health Services Flanders, Torhout, Belgium). The study population consisted of a total of 164 abortions, randomly selected from 150 different cattle herds from the northern part of Belgium (Flanders). At necropsy, length of gestation was estimated as described by Noden and DeLahunta (1985) (gestation length in days = 68 + 2.25 × fetal crown-rump length [CRL] in cm). Fetal CRL was measured from the most anterior point of the calvarium with the head flexed 90˚ to the spine until the most caudal part of the thigh (tuber ischiadicum). Only cases with a gestation length of > 7 months were included, as it is known that chlamydial abortion occurs beyond the sixth month of gestation (Givens and Marley, 2008). As control group, placental tissue samples originating from 41 at-term calving (non-aborting) events were randomly collected at 35 cattle farms, different from the abortion cases (except 1 farm), in Flanders.

Both abortion and non-abortion placental samples were processed in the same way. Two cotyledonary tissue samples per case were collected from random locations using sterile scalpels. Per case, one placental tissue sample was analysed by PCR for the presence of *Chlamydia* spp, *C. abortus*, *C. psittaci*, and *P. acanthamoebae* as described below. Subsequently, the second placental tissue sample was sliced and fixed in 4% formalin for histopathological examination.

The age of the dams (in months) at the moment of abortion (abortion group), or at the moment of sampling (non-abortion group) was calculated by subtracting the date of birth of the dam from the date of sampling. The date of birth of the dams was extracted from the national identification and registration system (Sanitel). Dams with an age < 30 months were classified as primiparous, and dams with an age ≥ 30 months as multiparous. Production type of the dams (dairy, beef, double purpose) was also extracted from Sanitel.

Fifty-five percent (113/205) of the analyzed samples originated from beef cattle (> 90% Belgian Blue), while 38% (78/205) were from dairy cattle (> 90% Holstein Friesian). Six samples (3%) were from double purpose cattle, and for 8 cases (4%) the production type was unknown. Production type of the abortion samples was beef (54%; 75/164) and dairy (46%; 76/164). Of the non-abortion samples, 93% (38/41) originated from beef cattle, 5% (2/41) were from dairy, and 2% (1/41) from double purpose cattle.

Twenty percent (41/205) of the analyzed samples were from primiparous cows, while 71% (146/205) originated from multiparous animals. The parity was unknown for 9% (18/205) of the cases. The parity of the dam of the abortion samples was primiparous in 20% (32/164) of the cases, while this was multiparous in 75% (124/164) of the cases. In 5% (8/164) of the abortion samples, parity was unknown. Parity of the dam of the non-abortion samples was primiparous and multiparous in 22% (9/41) and 54% (22/41), respectively, but this was unknown for 24% (10/41) of the samples.

DNA extraction and PCR

DNA was extracted from 200 µL of mashed placental tissue samples (max. 1 g in 1 mL MilliQ water) using the the MagMax™ Isolation Kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) following manufacturer's instructions. Briefly, the homogenized sample was lyzed by incubation for 15 min under vortex shaking conditions with lysis buffer, nucleic acid carrier, and zirconium beads. The lysate was then transferred to 96-plate wells and mixed with magnetic beads before processing within the automated instrument. The automated method consisted of capturing the DNA-coated beads on magnetic columns, four successive washing steps, and elution (50 µL) in pre-warmed (65°C) elution buffer.

Pan-Chlamydia *real-time PCR*

All placental samples were analyzed for the presence of *Chlamydia* spp. by using a pan-*Chlamydia* real-time PCR. Amplification and PCR product detection of *Chlamydia* spp. were performed by using an internal design based on the 23S ribosomal gene.

Chlamydia abortus *real-time PCR*

To detect the presence of *C. abortus*, all placental samples were analysed with a *C. abortus* real-time PCR. The *C. abortus* primers and fluorescent probe were selected following analysis of the ompA gene, which encodes the major outer membrane protein (MOMP) (GenBank accession number X51859), using the ABI Primer Express software (Applied Biosystems, Warrington, UK), and synthesized by Eurogentec (Seraing, Belgium). The specificity of the

primer set was confirmed following alignment of the ompA genes of all chlamydial species using an alignment software (Geneious, Biomatters Ltd., New Zealand). The sequences of the selected primers and the TaqMan® probe were as follows: forward primer, 5′-GCGGCATTCAACCTCGTT-3′; reverse primer, 5′-CCTTGAGTGATGCCTACATTGG-3′; and TaqMan® probe, 5′-TGTTAAAGGATCCTCCATAGCAGCTGATCAG-3′. The TaqMan probe was fluorescently labelled with a 6-carboxy-fluorescein (FAM) reporter molecule attached at the 5′-end and 6-carboxytetramethylrhodamine (TAMRA) as the 3′-end quencher molecule. The size of the amplification product was 86 bp.

Chlamydia psittaci *nested PCR*

Placental samples that turned out positive or inconclusive for the pan-*Chlamydia* PCR, were analysed by a nested PCR specific for *C. psittaci* as described by Van Loock et al. (Van Loock et al., 2005). This PCR is able to detect the ompA gene of all *C. psittaci* genotypes, with a sensitivity of 1 inclusion forming unit.

Parachlamydia acanthamoebae *real-time PCR*

The presence of *P. acanthamoebae* was analysed in all placental samples with a *P. acanthamoebae* specific real-time PCR. Primers were used as described by Lienard et al. (Lienard et al., 2011). For amplification, the following program was used: 15 min at 95 °C, followed by 50 cycles of 15 s at 95 °C, 15 s at 67 °C and 15 s at 72 °C. All qPCRs were performed using Mix ABsolute (Biorad, Temse, Belgium) and were carried out on an iQTM5 Real-time PCR cycler (Biorad, Temse, Belgium).

PCR result classification

Samples were considered negative if no amplification was detected following more than 45 cycles (Ct-value). Samples with a Ct-value between 35 and 45 were considered inconclusive, and samples with a Ct-value ≤ 35 were considered positive. Cases with more than one positive/inconclusive test result were classified as co-infections.

Histopathology

Haematoxylin and eosin (HE) stained histological sections were analysed as described by Kiernan (Kiernan, 2008) to identify the eventual type and degree of placentitis (purulent and/or necrotizing), and/or the presence of vasculitis. For the histopathological analyses, a random subset of 101 cases (75 abortion and 26 non-abortion cases) were used. Evaluation of placental samples for gross pathological lesions was not performed.

Statistics were performed with a chi-square test in R version 4.0.5 (R Core Team, Vienna, Austria).

Results

Chlamydia spp.

One of the 205 placental tissue samples (0.5%) was positive for *Chlamydia* spp., and 4.9% of the samples (10/205) tested as inconclusive. Six percent of the placentas of the abortion group were positive (1/164) or inconclusive (9/164). All of the positive/inconclusive abortion samples were from dairy cattle (3/10 primiparous, and 7/10 multiparous), while none of them originated from beef. In the non-abortion group, 2.4% (1/41) of the placenta samples were inconclusive while none of them was positive, and the inconclusive sample originated from a multiparous beef cow.

Chlamydia abortus

None of the 205 samples were positive or inconclusive for *C. abortus*.

Chlamydia psittaci

Three of the 11 (27.3%) placental tissue samples that were positive or inconclusive for *Chlamydia* spp. tested also positive for *C. psittaci*. All of the *C. psittaci* positive samples originated from dairy abortion cases. Two of them were from multiparous cows, while one was from a primiparous cow.

Parachlamydia acanthamoebae

Nine-point seven percent of placental tissue samples (20/205), were positive for *P. acanthamoebae*, and 26.8% of the samples (55/205) tested inconclusive. In the placentas of the abortion group, 10.9% of the cases were positive (18/164) and 32.9% (54/164) were inconclusive. In the placentas of the non-abortion group, 7.3% of the cases were positive or inconclusive (2/41 samples were positive, and 1/41 was inconclusive).

From the abortion samples that originated from dairy cattle, 44.7% (34/76) was positive/ inconclusive (7/76 positive, and 27/76 inconclusive), and this was 46.7% (35/75) for the beef abortion samples (10/75 positive, and 25/75 inconclusive) ($P > 0.05$). For the primiparous animals, 43.8% (14/32) were positive/inconclusive (6/32 positive, and 8/32 inconclusive) for *P. acanthamoebae*, while this was 45.2% (56/124) for the multiparous cows (11/124 positive, and 45/124 inconclusive) ($P > 0.05$).

Co-infections

In 1.5% of the analyzed placental samples (3/205), a co-infection was detected. These samples were positive/inconclusive for both *C. psittaci*. and *P. acanthamoebae,* in the following combinations: 2 samples were positive for both *C. psittaci* and *P. acanthamoebae*; 1 sample was positive for *C. psittaci* and inconclusive for *P. acanthamoebae*. All the samples with a co-infection originated from abortion cases.

Histopathology

Purulent and/or necrotizing placentitis with or without vasculitis was observed in 18.8% (19/101) of the histologically analyzed placenta samples. In 5.9% (6/101) of the samples, placentitis was observed along with vasculitis. In the placentas of the abortion group, 24% (18/75) of the samples showed purulent and/or necrotizing placentitis, while this was in 3.9% (1/26) of the samples in the non-abortion group. Vasculitis could not be detected in any of the non-abortion samples, while this histopathologic finding was present in 8% (6/75) of the samples of the abortion group. Autolysis was present in 13.9% (14/101) of the samples. Placental inflammatory lesions and/or necrosis were present in 44% (15/34) of the samples that were positive/inconclusive for *P. acanthamoebae*, while this was 20.9% (14/67) in the negative samples ($P = 0.02$). In 11.8% (4/34) of the samples where *P. acanthamoebae* was detected, vasculitis was present, while this was only 3% (2/67) in the negative samples.

In **Table 6.1.**, the percentual distribution of PCR results is depicted. The histopathological findings are summarized in **Table 6.2.** Histopathological lesions detected in a *P. acanthamoebae* positive placenta sample are shown in **Figure 6.1.**

Table 6.1. Percentual distribution of positive and inconclusive PCR results for *Chlamydia* spp., *Chlamydia abortus*, and *P. acanthamoebae*, stratified for case type (164 abortion cases and 41 non-abortion cases).

	Abortions[1] (n=164)	**Non-abortions[1] (n=41)**	
Agent	% ($n_{positive,}$ $n_{inconclusive}$)	% ($n_{positive,}$ $n_{inconclusive}$)	***P*-value**
***Chlamydia* spp.**	6.1 (1, 9)	2.4 (0, 1)	0.2
Chlamydia abortus	0 (0, 0)	0 (0, 0)	–
P. acanthamoebae	43.9 (18, 54)	7.3 (2, 1)	< 0.001

P. acanthamoebae = Parachlamydia acanthamoebae
[1] Percentage positives and/or inconclusives from the analyzed cases
[*] Positive = Ct-values ≤ 35; Inconclusive = Ct-values 36-45

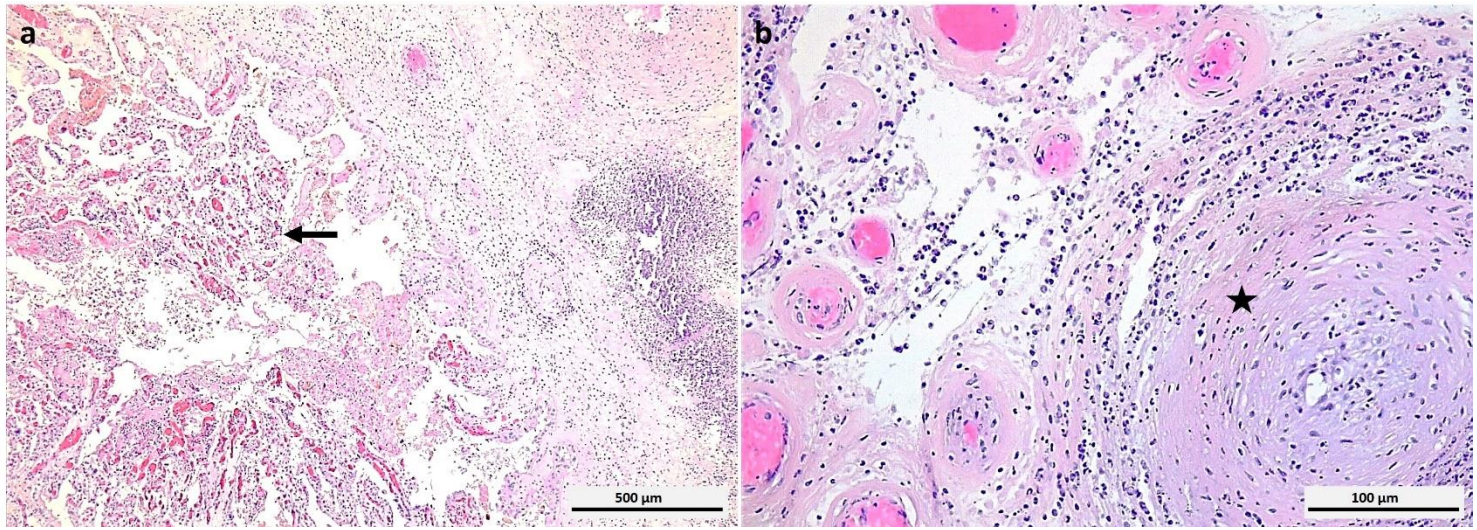

Figure 6.1.
Placenta; Histopathology of a placental sample positive for *Parachlamydia acanthamoebae* by real-time PCR showing (a) purulent necrotizing placentitis (arrow) (× 50; scale bar 500 µm), and (b) vasculitis (asterisk) (× 200; scale bar 100 µm). Hematoxylin and eosin staining. (Courtesy of Dr. Nermin Caliskan, DGZ Vlaanderen)

Table 6.2. Distribution of histopathologic findings (purulent placentitis, necrotizing placentitis, vasculitis; number (%)) for each agent identified via PCR of 101 histopathologic analyses (75 abortions, 26 non-abortions).

		Abortions (n = 75)				Non-abortions (n = 26)			
Agent	**PCR result***	**n**	**PP**	**NP**	**V**	**n**	**PP**	**NP**	**V**
***Chlamydia* spp.**	Positive	1	1 (100)	0 (0)	1 (100)	0	0 (0)	0 (0)	0 (0)
	Inconclusive	5	0 (0)	0 (0)	0 (0)	1	0 (0)	0 (0)	0 (0)
	Negative	69	16 (23.2)	10 (14.5)	5 (7.2)	25	1 (4.0)	1 (4.0)	0 (0)
Chlamydia abortus	Positive	0	0 (0)	0 (0)	0 (0)	0	0 (0)	0 (0)	0 (0)
	Inconclusive	0	0 (0)	0 (0)	0 (0)	0	0 (0)	0 (0)	0 (0)
	Negative	75	17 (22.7)	10 (13.3)	6 (8.0)	26	1 (3.8)	1 (3.8)	0 (0)
P. acanthamoebae	Positive	11	4 (36.4)	3 (27.3)	1 (9.1)	1	0 (0)	0 (0)	0 (0)
	Inconclusive	22	5 (22.7)	3 (13.6)	3 (13.6)	0	0 (0)	0 (0)	0 (0)
	Negative	42	8 (19.0)	4 (9.5)	2 (4.8)	25	1 (4.0)	1 (4.0)	0 (0)

PP = purulent placentitis; NP = necrotizing placentitis; V = vasculitis; *P. acanthamoebae* = *Parachlamydia acanthamoebae*
*Positive = Ct-value ≤ 35; Inconclusive = Ct-value 36-45; Negative = Ct-value > 45

Discussion

In this study, the detection of *Chlamydia* spp. and *P. acanthamoebae* and their associated lesions in bovine placental tissue was described. The occurrence of *P. acanthamoebae* was greater than the one of *Chlamydia* spp. and the overall occurrence of both *Chlamydia* and CLO were higher in abortion versus non-abortion cases. No differences in occurrence of *P. acanthamoebae* between dairy and beef cattle could be observed, nor was there a difference in occurrence between primiparous and multiparous animals. More histopathological placental lesions were present in cases that were diagnosed as positive for *P. acanthamoebae*. No previous studies describing the difference in occurrence of these organisms in bovine abortion versus non-abortion cases are available.

In the present study, a low number of the analyzed cases was positive/inconclusive for *Chlamydia* spp., but *P. acanthamoebae* could be detected in more than 35% of the total cases. Interestingly, a higher occurrence of *P. acanthamoebae* could be detected in abortion versus non-abortion cases, emphasizing the abortifacient potential of this pathogen in cattle. However, diagnosing a definitive cause of abortion is challenging (Van Loo et al., 2021). It remains difficult to achieve causality, even when one single potential abortifacient agent is detected. *Chlamydia* spp. and CLO have been found in subclinical infections in cattle (Kemmerling et al., 2009; Wheelhouse et al., 2022), suggesting that the detection of these organisms in aborted tissue samples may be without clinical significance. Nevertheless, the detection of *Chlamydia* spp. and especially *P. acanthamoebae* within corresponding placental lesions in bovine cases of abortion was previously described (Ruhl et al., 2009), which suggests a relation between infection and abortion. The main route of chlamydial infections in cattle is through the respiratory or reproductive tract. *Chlamydia* spp. and CLO are obligate intracellular bacteria that may infect a wide range of host cells, including epithelial and immune cells. After an initial infection, affected cells may reach the pregnant uterus, which may lead to placental and fetal infection. Chlamydial infection of the placenta causes inflammation (purulent and/or necrotizing placentitis with vasculitis), gradually progressing to placental insufficiency, which may turn out in fetal death and abortion (Borel et al., 2018). Our results reveal an occurrence of histopathologic findings linked to a *P. acanthamoebae* infection comparable to a previous, similar study (Ruhl et al., 2009) where inflammation and placental necrosis were present in almost 60% of the *P. acanthamoebae* positive cases. However, in the present study,

histopathologic lesions corresponding with purulent and/or necrotic placentitis could also be detected in some of the negative cases, and even in the non-abortion samples. This may have been caused by an infection of the placenta by another pathogen that was not included in our analyses. Besides, Botta and others (2019) reported that histological lesions in bovine placental tissue (purulent and/or necrotic placentitis, with or without vasculitis) must be interpreted with caution. Placental lesions are not necessarily linked to an infection of the placenta, but can be part of physiological processes during pregnancy (e.g., apoptotic processes, or regulation of cell proliferation and regression as key for placental maturation), or placental expulsion (Botta et al., 2019). Therefore, it could be interesting to perform immunohistochemistry in other future studies to establish a more accurate diagnosis in cases of bovine chlamydial and CLO abortions.

In the present study, around 15% of the cases that were positive or inconclusive for *P. acanthamoebae* were also positive/inconclusive for *Chlamydia* spp., which is comparable with the results of a previous study (Ruhl et al., 2009), where 21% of the *Parachlamydia* real-time PCR positive samples were also identified as *Chlamydia* spp. positive using a broad-range PCR. This finding reflects that the specific real-time PCR for the detection of *P. acanthamoebae* is probably more sensitive than the pan-*Chlamydia* real-time PCR that was used to identify *Chlamydia* spp. Although it was low (1.5%), co-infections of *C. psittaci* and *P. acanthamoebae* were detected in the present study. The finding that several samples contained genetic material of two chlamydial agents, corresponds with the observations of previous studies (Borel et al., 2008; Pantchev et al., 2010). Therefore, the combined use of several diagnostic tests specific for different chlamydial agents may be essential to increase the diagnostic accuracy of an analytical protocol for cases of bovine abortion. Future research to reveal possible interactions or synergies between the co-infecting agents would be worthwhile.

Both *Chlamydia* spp. and CLO have zoonotic implications, and have been isolated from human cases of respiratory disease, as well as from fetal loss or premature delivery (Longbottom et al., 2003; Baud et al., 2008). From the *Chlamydia* spp. found in cattle, mainly *C. psittaci* and *C. abortus* are involved in human chlamydial infections. *Chlamydia psittaci* mainly affects birds but it may also cause human psittacosis when contaminated aerosols from infected birds are inhaled. The pathogen can be found in several tissues including blood and fecal material of infected birds. Consequently, individuals having contact with birds are more at risk to become infected with *C. psittaci* (Dai et al., 2022). In contrast to *C. psittaci* infections, transmission of *C. abortus* from livestock to men is relatively rare, although it is mainly related to direct exposure to infected small ruminants, especially during lambing season (Longbottom

et al., 2003; Turin et al., 2022). *Chlamydia abortus* is the causative agent of enzootic ovine abortion, which is one of the most common causes of infectious abortion in sheep and goats worldwide. For *Parachlamydia* spp., interaction with farm animals is described as a risk factor for seropositivity in human, and the pathogen may be involved in fetal loss in pregnant women (Baud et al., 2009; Ammerdorffer et al., 2017). In the present study, both *C. psittaci* and *P. acanthamoebae*, but no *C. abortus*, could be detected in bovine cases of abortion. Interestingly, *P. acanthamoebae* was also detected in non-abortion placental samples, although in lower proportions. Consequently, it needs to be emphasized that handling of bovine placental tissue should be done with caution to prevent transversal spread of the disease to humans.

Conclusion

The present study suggests a potential role of *Chlamydia* spp. and *P. acanthamoebae* in cases of bovine abortion in Belgium. Based on these findings, it could be concluded that the implementation of a PCR test to detect *Chlamydia* spp. and CLO in placental tissue, preferably in combination with histopathology or immunohistochemistry, may increase the success of laboratory investigations to diagnose cases of bovine abortion. Bearing in mind the zoonotic potential of *Chlamydia* spp. and *P. acanthamoebae*, handling of cattle and especially of bovine placental tissue should be done with vigilance.

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References

Ammerdorffer, A., Stojanov, M., Greub, G., Baud, D., 2017. Chlamydia trachomatis and Chlamydia-like bacteria: New enemies of human pregnancies. Curr Opin Infect Dis 30, 289–296. https://doi.org/10.1097/QCO.0000000000000369

Baud, D., Regan, L., Diseases, G.G.-C. opinion in infectious, 2008, U., 2008. Emerging role of Chlamydia and Chlamydia-like organisms in adverse pregnancy outcomes. Current Opinion in Infectious Diseass 21, 70–76.

Baud, D., Kebbi, C., Külling, J.P., Greub, G., 2009. Seroprevalence of different Chlamydia-like organisms in an asymptomatic population. Clin Microbiol Infect 15 Suppl 2, 213–215. https://doi.org/10.1111/J.1469-0691.2008.02134.X

Borel, N., Thoma, R., Spaeni, P., Weilenmann, R., Teankum, K., Brugnera, E., Zimmermann, D.R., Vaughan, L., Pospischil, A., 2006. Chlamydia-related abortions in cattle from Graubunden, Switzerland. Vet Pathol 43, 702–708. https://doi.org/10.1354/VP.43-5-702

Borel, N., Kempf, E., Hotzel, H., Schubert, E., Torgerson, P., Slickers, P., Ehricht, R., Tasara, T., Pospischil, A., Sachse, K., 2008. Direct identification of chlamydiae from clinical samples using a DNA microarray assay-A validation study. Molecular and Cellular Probes 22, 55–64. https://doi.org/10.1016/J.MCP.2007.06.003

Borel, N., Polkinghorne, A., Pospischil, A., 2018. A Review on Chlamydial Diseases in Animals: Still a Challenge for Pathologists? Veterinary Pathology 55, 374–390. https://doi.org/10.1177/0300985817751218/FORMAT/EPUB

Botta, C., Pellegrini, G., Hässig, M., Pesch, T., Prähauser, B., Wunderlin, S., Guscetti, F., Schneeberger, M., Schmitt, S., Basso, W., Hilbe, M., Schuler, G., Borel, N., 2019. Bovine Fetal Placenta During Pregnancy and the Postpartum Period. Vet Pathol 56, 248–258. https://doi.org/10.1177/0300985818806453

Cavirani, S., Cabassi, C.S., Donofrio, G., De Iaco, B., Taddei, S., Flammini, C.F., 2001. Association between Chlamydia psittaci seropositivity and abortion in italian dairy cows. Prev Vet Med 50, 145–151. https://doi.org/10.1016/S0167-5877(01)00197-0

Dai, N., Li, Q., Geng, J., Guo, W., Yan, W., 2022. Severe pneumonia caused by Chlamydia psittaci: report of two cases and literature review. J Infect Dev Ctries 16, 1101–1112. https://doi.org/10.3855/JIDC.16166

Dilbeck, P.M., Evermann, J.F., Crawford, T.B., Ward, A.C.S., Leathers, C.W., Holland, C.J., Mebus, C.A., Logan, L.L., Rurangirwa, F.R., McGuire, T.C., 1990. Isolation of a

previously undescribed rickettsia from an aborted bovine fetus. J Clin Microbiol 28, 814–816. https://doi.org/10.1128/JCM.28.4.814-816.1990

Givens, M.D., Marley, M.S.D., 2008. Infectious causes of embryonic and fetal mortality. Theriogenology 70, 270–85. https://doi.org/10.1016/j.theriogenology.2008.04.018

Griffiths, P.C., Plater, J.M., Horigan, M.W., Rose, M.P.M., Venables, C., Dawson, M., 1996. Serological Diagnosis of Ovine Enzootic Abortion by Comparative Inclusion Immunofluorescence Assay, Recombinant Lipopolysaccharide Enzyme-Linked Immunosorbent Assay, and Complement Fixation Test. J Clin Microbiol 34, 1512–1518.

Kaltenboeck, B., Hehnen, H.R., Vaglenov, A., 2005. Bovine Chlamydophila spp. infection: Do we underestimate the impact on fertility? Vet Res Commun 29, 1–15. https://doi.org/10.1007/S11259-005-0832-4/METRICS

Kemmerling, K., Müller, U., Mielenz, M., Science, H.S.-J. of dairy, 2009, U., 2009. Chlamydophila species in dairy farms: polymerase chain reaction prevalence, disease association, and risk factors identified in a cross-sectional study in. J Dairy Sci 92, 4747–4354.

Kiernan, J.A., 2008. Histological and histochemical methods: theory and practice, 4th ed. Scion Publishing Ltd, UK.

Lienard, J., Croxatto, A., Aeby, S., Jaton, K., Posfay-Barbe, K., Gervaix, A., Greub, G., 2011. Development of a new chlamydiales-specific real-time PCR and its application to respiratory clinical samples. Journal of clinical microbiology 49, 2637–2642. https://doi.org/10.1128/JCM.00114-11

Longbottom, D., Pathology, L.C.-J. of comparative, 2003, U., 2003. Animal chlamydioses and zoonotic implications. J Comp Pathol 128, 217–244.

Noden, D.M., DeLahunta, A., 1985. The embryology of domestic animals: developmental mechanisms and malformations. Williams & Wilkins, Baltimore MD.

Pantchev, A., Sting, R., Bauerfeind, R., Tyczka, J., Sachse, K., 2010. Detection of all Chlamydophila and Chlamydia spp. of veterinary interest using species-specific real-time PCR assays. Comparative Immunology, Microbiology and Infectious Diseases 33, 473–484. https://doi.org/10.1016/J.CIMID.2009.08.002

Reinhold, P., Sachse, K., Kaltenboeck, B., 2011. Chlamydiaceae in cattle: Commensals, trigger organisms, or pathogens? The Veterinary Journal 189, 257–267. https://doi.org/10.1016/J.TVJL.2010.09.003

Ruhl, S., Casson, N., Kaiser, C., Thoma, R., Pospischil, A., Greub, G., Borel, N., 2009. Evidence for Parachlamydia in bovine abortion. Vet Microbiol 135, 169–174. https://doi.org/10.1016/J.VETMIC.2008.09.049

Turin, L., Surini, S., Wheelhouse, N., Rocchi, M.S., 2022. Recent advances and public health implications for environmental exposure to Chlamydia abortus: from enzootic to zoonotic disease. Veterinary Research 2022 53:1 53, 1–17. https://doi.org/10.1186/S13567-022-01052-X

Van Loo, H., Pardon, B., De Schutter, P., De Bleecker, K., Vanrompay, D., Deprez, P., Maris, J., 2014. Detection of Chlamydia psittaci in Belgian cattle with signs of respiratory disease and milk drop syndrome. Veterinary Record 175, 562. https://doi.org/10.1136/VR.102527

Van Loo, H., Pascottini, O.B., Ribbens, S., Hooyberghs, J., Pardon, B., Opsomer, G., 2021. Retrospective study of factors associated with bovine infectious abortion and perinatal mortality. Prev Vet Med 105366. https://doi.org/10.1016/j.prevetmed.2021.105366

Van Loock, M., Verminnen, K., Messmer, T.O., Volckaert, G., Goddeeris, B.M., Vanrompay, D., 2005. Use of a nested PCR-enzyme immunoassay with an internal control to detect Chlamydophila psittaci in turkeys. BMC Infectious Diseases 5, 1–9. https://doi.org/10.1186/1471-2334-5-76/TABLES/3

Wang, F.I., Shieh, H., Liao, Y.K., 2001. Prevalence of Chlamydophila abortus infection in domesticated ruminants in Taiwan. J Vet Med Sci 63, 1215–1220. https://doi.org/10.1292/JVMS.63.1215

Wheelhouse, N., Katzer, F., Wright, F., Longbottom, D., 2010. Novel chlamydia-like organisms as cause of bovine abortions, UK. Emerg Infect Dis 16, 1323–1324.

Wheelhouse, N., Howie, F., Gidlow, J., Greub, G., Dagleish, M., Longbottom, D., 2012. Involvement of Parachlamydia in bovine abortions in Scotland. The Veterinary Journal 193, 586–588. https://doi.org/10.1016/J.TVJL.2012.01.008

Wheelhouse, N., Hearn, J., Livingstone, M., Flockhart, A., Dagleish, M., Longbottom, D., 2022. Identification of Parachlamydiaceae DNA in nasal and rectal passages of healthy dairy cattle. J Appl Microbiol 132, 2642–2648. https://doi.org/10.1111/JAM.15422

Yin, L., Schautteet, K., Kalmar, I.D., Bertels, G., Van Driessche, E., Czaplicki, G., Borel, N., Longbottom, D., Frétin, D., Dispas, M., Vanrompay, D., 2014. Prevalentie van Chlamydia abortus bij herkauwers in België. Vlaams Diergeneeskd Tijdschr 83, 164–170. https://doi.org/10.21825/VDT.V83I4.16642

Chapter 7

General Discussion

Introduction

Pregnancy success remains a central driver for sustainable milk and meat production in cattle industry. Unfortunately, a relatively large number of bovine pregnancies is interrupted before the expected calving date, with the majority of losses occurring in the embryonic period during the first weeks after conception. However, the economic impact of pregnancy losses during the fetal period is much greater. When pregnancy loss happens during the fetal period, beyond 45 days of gestation, it is referred to as abortion, and from 265 days of gestation as perinatal mortality, including stillbirths and calves that died within 48 h after birth. In dairy cattle, the expected incidence of EFL (between 45 and 60 d of gestation) is around 7%, while LFL (between 60 and 260 d of gestation) ranges from 1 to 3% (Wiltbank et al., 2016; Albaaj et al., 2023). Beyond 120 d of gestation, a fetal loss rate threshold of 2 to 5% is considered acceptable (Mee, 2020), although opinions in this area vary. Specific abortion thresholds for beef cattle have not been determined until now. Perinatal mortality has been defined as the loss of a non-viable fetus beyond 265 d of gestation, and deaths of full-term calves up to 48 h of age (Mee, 2013). In dairy cattle, the perinatal mortality rate varies between 2.4 and 9.7%, with a median of 6.6% (Cuttance and Laven, 2019), while also this rate remains largely unknown for beef cattle. Abortion and perinatal mortality are often a result of an infection, although non-infectious causes are also common (Mee, 2013; Clothier & Anderson, 2016; Wolf-Jäckel et al., 2020). In most laboratories, the primary emphasis is placed on investigating for infectious agents that are most relevant and prevalent in a particular region when studying cases of APM. Depending on the region, numerous infectious agents may be involved in bovine APM, including bacteria, yeasts/molds, viruses, and parasites. Several infectious causes of APM have also a zoonotic impact (e.g., *Brucella abortus*, *A. phagocytophilum*, *Chlamydia* spp.), which makes monitoring of infectious causes of APM crucial for both animal and human health. As for all diseases, the monitoring of APM relies on the notification and reporting of suspected cases (Bronner et al., 2013, 2014). However, under-reporting of detected bovine APM is a major issue worldwide, although it is notifiable in many countries. This under-reporting may hamper the early detection of zoonotic and other infectious causes of APM. Therefore, it is crucial to identify factors which may influence the motivation of farmers to submit cases of APM. In Belgium, an extended abortion surveillance program was installed by the government to stimulate farmers to submit APM cases, and to monitor the prevalence of the most relevant

infectious causes of APM for the region. Due to this new APM monitoring program, an overview of the most relevant pathogens involved in bovine APM, and related risk factors became available. In the current thesis, the main aim was to broaden the knowledge on the epidemiology of infectious bovine pregnancy loss (abortion and perinatal mortality) in Flanders. In **Chapter 3** and **Chapter 4**, data of the regional abortion monitoring program were evaluated to: 1) analyse how successful the implementation of a new abortion monitoring program was, 2) identify factors which may influence the number of submitted APM cases, 3) determine the prevalence of infectious causes of bovine APM, and 4) identify factors that are associated with the prevalence of the different pathogens. However, multiple other infectious agents are reported in literature to be potentially involved in APM, but analyses for these pathogens are not included in the official Belgian APM monitoring program. Therefore, in **Chapter 5** and **Chapter 6** the present thesis focused on the potential involvement of *A. phagocytophilum*, *Chlamydia* spp. and *Chlamydia*-like organisms, zoonotic and abortifacient agents that were not included in the extensive APM monitoring program, in bovine APM cases in Flanders.

Trends in the number of APM submissions

In **Chapter 3**, it was described that a significant increase of the number of submitted APM cases was associated with the implementation of a more extensive APM monitoring program and the provision of free on-farm sample pick-up services. The submission of APM cases nearly doubled in dairy and nearly tripled in beef cattle. Prior to the introduction of the extensive analytical panel, APM cases were solely analysed for brucellosis, even though the country had been officially declared free of bovine brucellosis since 2003. This may have led to a diminished interest among farmers in participating in the former abortion monitoring program. Hence, farmers are more likely to be aware of a disease and tend to prioritize biosecurity measures when faced with an outbreak situation, whereas diseases that are absent in the country are generally regarded as posing minimal risk (Ekboir, 1999; Bronner et al., 2014). This became apparent in 2012 following the emergence of SBv in the country, which resulted in an increase in the number of submitted APM cases. Nevertheless, it is also possible that this upsurge in APM submissions could have been attributed to a higher APM rate because of the introduction of this previously undetected virus.

Despite the higher number of submitted APM cases since the introduction of the extended panel of analyses, its believed that there is still an under-reporting of APM cases in Flanders. Assuming that when only observed abortions are considered, normal abortion rates in dairy cattle appear to be about 2-5% (Kinsel, 1999; Hovingh, 2009), while perinatal mortality rates in dairy cattle have been reported to vary between 2 to 10% (Cuttance and Laven, 2019). In the present thesis, the number of submitted APM cases was analyzed in commercial herds with at least 25 reproductive females per year, which implicates that at least one APM event every 2 years on each commercial herd should have been reported, assuming a minimal APM rate of 2%. In this context, it could be questioned if the mentioned thresholds for pregnancy loss in cattle are applicable for each region in the world, and also for each cattle breed. For instance, thresholds are generally established for dairy cattle (primarily Holstein Friesian), but such thresholds are lacking for beef cattle, the main population of our study. Extrapolation of this threshold to other breeds might also be incorrect, but no data on this issue are available. Additionally, farmers and veterinarians typically acknowledge a certain level of fetal loss as 'normal', and this ‘accepted’ abortion rate may vary across different enterprise types (dairy/beef) and countries. For instance, a recent benchmarking study in North America (beef herds in Canada) revealed intervention thresholds for abortion at approximately 6% in heifers and 3% in cows (Waldner et al., 2019). In Europe (dairy and beef herds in the UK and Ireland), the investigation thresholds for abortion were 4%, 2%, and 1% for veterinarians, dairy farmers, and beef farmers, respectively (Clothier et al., 2020). These European thresholds seem low when compared to reported losses in US dairy herds after ~day 30 pregnancy diagnosis (11% pluriparae and 3% primiparae) (Santos et al., 2009). In the US, higher thresholds have been recommended for dairy herds, with a goal of < 5% and an intervention level of 10% (Heersche, 2023). The reason accepted abortion rates are often lower than actual rates is because the former refers to observed abortions, while the latter includes the much higher early pregnancy abortion rates (~90% of fetal loss after day 42 occurs before day 150, Sigdel et al., 2022), (Mee, 2021a). In areas where the underlying true/observed/detected abortion rate is high and early (~30d) pregnancy detection is common practice (e.g., high-producing dairy herds in North America or Israel), accepted/normal abortion rates will also be high. Despite farmers stating their investigation thresholds, only a minority (e.g., 38% in a recent Canadian study) actually submit cases for analysis when the abortion rate exceeds their threshold (>5%) (Denis-Robichaud et al., 2019).

Based on the fact that 14% of the herds that were included in the present thesis never submitted any APM case over a period of 13 years, it can be concluded that many APM cases remain unreported. However, when comparing the results of the present thesis with international data, it can be assumed that the Belgian abortion monitoring is relatively successful in terms of the number of cases submitted. For instance, in France and the UK, only 10% of the cattle farmers seem to be motivated to submit APM cases for analysis (Bronner et al., 2014; Clothier et al., 2020). Furthermore, the minimum required number of 4,000 submitted APM cases per year in Flanders, calculated by Welby et al., (2009) to ensure freedom of brucellosis with a 99% confidence level, was achieved during the study period. In this context, it is important to question whether the threshold of 4,000 APM cases remains relevant, as it was originally based on the regional cattle population in 2007 and 2008. Considering the gradual decline in the number of Flemish cattle herds and the cattle population over the years, it may be concluded that the current number of submitted APM cases remains sufficient for brucellosis monitoring in the region.

The present thesis identified some factors that were associated with the number of submitted APM cases. Farmers tended to submit more APM cases from beef compared to dairy cattle, and also more in the winter compared to fall, spring, and summer. Additionally, if an APM event happened in a smaller herd, a higher chance of submission for analysis was observed compared to APMs that happened on larger herds. Also herds that had submitted an APM in the previous year had a higher proportion of submitted APM cases in the next year compared to herds without an APM submission. Furthermore, herds for which there was evidence for the presence of BVDv had a higher proportion of submitted cases compared to herds without this evidence. These results suggest that there is still room for improvement in increasing the awareness among farmers and their veterinarians regarding the importance of reporting and submitting cases of APM. Therefore, it could be helpful to highlight that in APM outbreaks, the initial occurrence of APM cases is often sporadic and irregular, and the first single case may mark the start of an outbreak that becomes apparent only after several weeks. Moreover, an observed APM case may also indicate a more extensive underlying herd problem, emphasizing the need to investigate every single APM case (Caldow and Gray, 2004). Accurate diagnosis remains the key to effectively controlling diseases (Baumgartner, 2015), meaning that thoroughly investigating APM cases is an essential aspect of good herd management. Its significance cannot be overstated, although many farmers become motivated to submit samples only when an increase in abortion incidence happens (Clothier et al., 2020). To increase the number of

submitted APM cases in specific herds, it would be beneficial to identify the specific reasons of why farmers might not be interested in utilizing a fully funded analytical and on-farm sample pick up service, as offered in Belgium. Several potential reasons are already described in literature, although it should be noted that these reasons may vary based on the region, management system (dairy versus beef), and breed (e.g., Belgian Blue versus other beef breeds). Unfortunately, no studies on this issue are available for the cattle industry in Belgium. In a previous study in Canada, the laboratory cost was mentioned as a potential barrier for reporting and submitting APM cases (Denis-Robichaud et al., 2019). However, in Belgium, sampling of aborted dams, collection of samples at the involved herd by the regional accredited laboratory, as well as the complete laboratory analyses on both maternal and fetal samples are completely funded by the government. This signifies that the laboratory costs may not be appointed as one of the obstacles for a well-respected obligation to report and submit each case of APM. Regional variations, as well as differences between farmers to adopt diagnostics for APM may also partly be attributed to variations in communication with their veterinarians (Clothier et al., 2020). Guidance from their veterinarian is perceived as one of the main drivers for farmers to submit samples for APM investigations. However, it is important to note that certain veterinarians are more motivated to submit cases compared to others (Bronner et al., 2014). Although a veterinarian's decision not to submit samples for necropsy is mainly based on the decision of the involved farmer, some other influencing factors are described (Bronner et al., 2014). It is reported that some veterinarians are less inclined to submit samples for necropsy because they are convinced that a diagnosis would not change their treatment options (Salopek et al., 2016). Indeed, treatment of APM is not possible (the calf is lost) and implementing measures to prevent APM during the actual calving season can also be challenging or even impossible. However, in the majority of cases, it may be feasible to reassess or implement improved biosecurity protocols, eradication programs, vaccinations, feeding strategies, and other measures to prevent future cases. Furthermore, the lack of confidence in their necropsy interpretation has also been identified as a significant factor that discourages veterinarians from submitting samples (Salopek et al., 2016). Therefore, it is crucial to initiate comprehensive education on necropsies at the veterinary school level and ensure lifelong learning opportunities. Additionally, organizing information campaigns by diagnostic laboratories can play a key role in enhancing the knowledge of bovine practitioners on APM. In this context, it should be mentioned that during the introduction of the extensive abortion monitoring program in Belgium, an intensive communication campaign was implemented. This campaign included distributing information

leaflets on APM, but also employing two veterinarians (one per region, Flanders and Wallonia) in the diagnostic regional accredited laboratories to visit cattle herds facing APM problems and educate farmers and their veterinarians through dedicated meetings around the topic. As a result of this information campaign, the awareness of farmers and veterinarians regarding the importance of submitting cases of APM, and the knowledge of involved pathogens, increased. However, since 2015, there has been a lack of dedicated veterinarians specifically supporting the abortion monitoring program in Flanders, which may have contributed to the declining interest of farmers and their vets in submitting APM cases, as revealed in our study. Consequently, it may be concluded that an effective abortion monitoring program requires continuing support and information provided by experts on the topic.

One of the reasons why veterinarians may be less motivated to submit cases of APM is the lack of diagnosis from previous submissions (Salopek et al., 2016). Farmers as well recognize identifying the underlying cause of APM as the main motivator to submit cases, while not the legally mandatory requirement (Clothier et al., 2020). However, we found that, despite the inclusion of an extensive diagnostic workout, no diagnosis could be reached in about 60% of the cases (**Chapter 4**). Trying to improve the diagnostic rate is therefore crucial to increase the motivation of both farmers and veterinarians for submitting new cases, and inclusion of analyses to detect newly recognized abortifacient pathogens like *A. phagocytophilum*, *Chlamydia* spp. and CLO may increase the diagnostic rate (**Chapter 5** and **Chapter 6**), and consequently the number of APM submissions. On the other hand, it should be emphasized that also a negative diagnostic outcome may be relevant for some diseases. For example, a maternal seronegative result for *N. caninum* may be used to rule out this pathogen as the underlying cause of APM (Pereira-Bueno et al., 2000; Mee, 2020).

The primary objective of the presented abortion monitoring program was to monitor bovine brucellosis in Belgium. Throughout the entire study period, no cases positive for *B. abortus* were detected in Flanders. However, as mentioned in the general introduction, a re-emergence of *Brucella* outbreaks was observed in Wallonia between 2010 and 2013. This outbreak was identified following the detection of *B. abortus* in aborted fetuses. This observation can be seen as evidence of the effectiveness of the abortion monitoring program at that time. However, it is worth questioning whether the reduction in the number of included analyses that occurred during the last years of the program may have had a negative impact on the number of submitted cases, which could potentially hamper the monitoring of brucellosis in the country. Hence, it is

crucial to continuously monitor the number of submitted cases to enable timely intervention and ensure that the effectiveness of brucellosis monitoring can be maintained.

It is essential to recognize that certain aspects of the presented APM monitoring program are specific for our region. This means that not all findings may be directly applicable to other countries or regions globally. Therefore, it is essential to interpret and apply the results of our monitoring program within the context of these regional variations. For instance, the presence of Belgian Blue cattle, a breed with significantly higher market value compared to dairy or other beef breeds, may influence the willingness of beef farmers to report and submit APM cases. In regions where the number of Belgian Blue cattle is minimal, e.g. France, studies suggest that beef farmers are less likely to contact a veterinarian to report APM cases compared to their dairy counterparts (Bronner et al., 2013). Conversely, in the UK, research has identified a higher motivation among beef farmers to report APM cases (Clothier et al., 2020). These findings highlight the existence of regional differences in the motivators and barriers for farmers when it comes to reporting cases of APM. Also the presence of disease eradication programs may significantly influence the motivation of farmers and veterinarians to report APM cases. The introduction of such programs accompanied by information campaigns can raise awareness among farmers and veterinarians regarding specific diseases. This increased awareness often results in an increase in reported cases related to the targeted disease, as observed in our study following the implementation of the BVDv eradication program (**Chapter 3**). Conversely, when diseases are either absent, under control or eradicated, farmers may exhibit reduced interest in participating in such programs. The same principle applies to emerging diseases with known abortifacient potential, such as SBv. In this case, there might be an increase in the number of submitted APM cases due to both the actual prevalence of the disease and related APM cases, and the heightened interest of farmers and veterinarians in determining if the specific disease is involved in the APM case. However, it is important to note that the presence of specific diseases can also act as a demotivator to submitting APM cases. This occurs when the detection of such diseases results in severe consequences, including mandatory culling of infected dams. The fear of these drastic outcomes may discourage farmers and veterinarians from reporting cases, presenting a potential obstacle to effective disease monitoring. The free on-farm sample pick up service and access to a fully funded broad analytical panel, as highlighted in this study, are unparalleled globally. Farmers have identified laboratory accessibility and the time cost associated with submitting APM samples as significant barriers (Clothier et al., 2020). In regions where laboratories are situated far from farms, and on-farm

sample pick-up services are unavailable, there might be a reduced willingness/interest among farmers to submit APM cases. The provision of free diagnostic services serve as a strong incentive for farmers to submit each APM, although individual responses may vary as observed in **Chapter 3**. This approach can lead to the earlier detection of disease outbreaks. In contrast, in countries where APM analyses come with a cost, farmers may postpone submissions until a threshold of APM cases is reached, potentially hindering the timely detection of disease outbreaks. Another factor that may impact the external validity of our study is the varied management practices of cattle worldwide. For instance, depending on the region, an increasing number of dairy farms are adopting zero-grazing, which theoretically enhances the likelihood of detecting cases of APM. This is because the detection of abortions tends to decrease during the grazing season (Bronner et al., 2014). Consequently, in regions where cattle are managed more extensively, a lower submission rate might be expected.

Infectious causes of APM, and related risk factors

Establishing a definitive causative diagnosis for APM is frequently a frustrating and unrewarding exercise. Submitted aborted fetuses often exhibit autolysis, hampering the potential to isolate significant pathogens or detect diagnostically relevant gross or histological abnormalities. Even in cases where the fetus and related membranes are in good condition, pinpointing a diagnosis with a specific cause is rarely attained. The infectious diagnostic rate of bovine abortion varies between 23 to 58% with an average of 42% of cases being diagnosed as infectious (Kirkbride, 1993; Jamaluddin et al., 1996; Khodakaram-Tafti & Ikede, 2005; Wolf-Jäckel et al., 2020; Mee, 2023). As for bovine perinatal mortality, the proportion caused by infections shows significant variability across studies, ranging from 5 to 35%, with an average of 15% (Mee et al., 2021). In our study, both abortions and perinatal mortalities were included, and analysed as one category. Therefore, it needs to be mentioned that conclusions on prevalence of infectious abortifacients must be drawn with caution. The utilization of the analytical panel within the Belgian abortion monitoring program led to a diagnostic rate of up to 39% in Flanders (**Chapter 4**), aligning well with findings from previously published studies. Notably, the pathogens most frequently identified in cases of perinatal mortality or abortions include *N. caninum*, BVDv, bacteria (*T. pyogenes* in abortions, and *Bacillus* spp. as well as

Leptospira spp. in perinatal mortalities), along with fungal agents (Mee et al., 2021; Mee, 2023). In **Chapter 4**, a detailed overview of the most relevant pathogens involved in APM within the northern region of Belgium is presented. Of the infectious causes of APM, *N. caninum* was the most commonly diagnosed abortifacient, corresponding with global data (Mee, 2023). Fifteen percent of the submitted cases were positive for *N. caninum*, which is consistent with findings from other studies (Dubey et al., 2007; Wolf-Jäckel et al., 2020). Neosporosis was followed by *T. pyogenes*, BVDv, *E. coli*, and *A. fumigatus*. Previous studies indicated a prevalence range of 3 to 17% for *T. pyogenes*-induced APM (Mee, 2023), while we found this pathogen in 3 (primiparous dairy) to 7% (beef primi- and multiparous) of the analyzed cases. Our study results (4%), originating from a time period before the initiation of the national BVDv eradication program, correspond with current data indicating that BVDv contributes to 2 to 6% of APM cases in regions where the virus has not been eradicated (Mee, 2023). Furthermore, *Bacillus* spp. have been found in a low percentage (< 1%) in our study, while this bacterium has been detected in 2% of perinatal mortalities in the UK (Murray et al., 2008). Notably, our analysis did not encompass the detection of *Leptospira* spp.; our focus for this disease was solely on serological examination of maternal blood samples. In literature, fungi are associated with less than 10% of cases of APM (Mee et al., 2021; Mee, 2023), with *A. fumigatus* being the most commonly diagnosed fungal organism (Knudtson and Kirkbride, 1992). In our study, *A. fumigatus* has been found in 1.5 to 3.5% of the cases. Variations in prevalence of infectious causes of bovine APM are described in literature, mainly because of regional and managerial differences (e.g., dairy versus beef), the variability in presence of abortifacient agents (e.g., in countries where *B. abortus* has not yet been eradicated, it remains one of the most important causes of bovine APM), but also because of differences in study design, analytical methodology, and parameter definitions. It is worth noting that the findings of our study are based on data from earlier years, specifically 2010 and 2011. It could be possible that since these years, the infectious profile of APM has already changed in Flanders. Although very few studies examined temporal trends in the occurrence of infectious abortions, it is known that the rate of abortions caused by specific pathogens may vary over time in a specific region, for example due to the implementation of eradication programs or increasing vaccination rates (Crilly, 2004; Cantón et al., 2022). For instance, in Belgium, a government-led mandatory eradication program for BVDv was established in the year 2015. As a result, the percentage of APM cases positive for BVDv decreased from 4% in the period between January 2010 and December 2011 to only 0.06% in 2021 (**Figure 7.1.**) (Deschuytere et al., 2016; De Graef et al.,

2022). Also, the prevalence of *N. caninum* seropositive dams that aborted decreased between 2010 and 2021 from 20 to 13% (**Figure 7.1.**). No clear explanation for this decrease is available, but it is possible that farmers and their veterinarians have become more aware of the negative impact of *N. caninum* on bovine herd health. This increased awareness may be attributed to the introduction of free analyses for this pathogen as part of the in 2009 introduced extended abortion monitoring program, and the information about the disease provided during the communication campaign. Furthermore, in 2012 neosporosis has become an officially recognized disease for which farmers can legally request a purchase annulment if a cow they purchased tests seropositive for *N. caninum* at the moment of purchase. This measure helps to reduce the risk of introducing the disease to their herd. These examples emphasize the significance of ongoing regional monitoring of infectious causes of APM at the national, regional and herd level. Monitoring ensures access to an up-to-date understanding of the epidemiological situation regarding the most relevant infectious causes of APM.

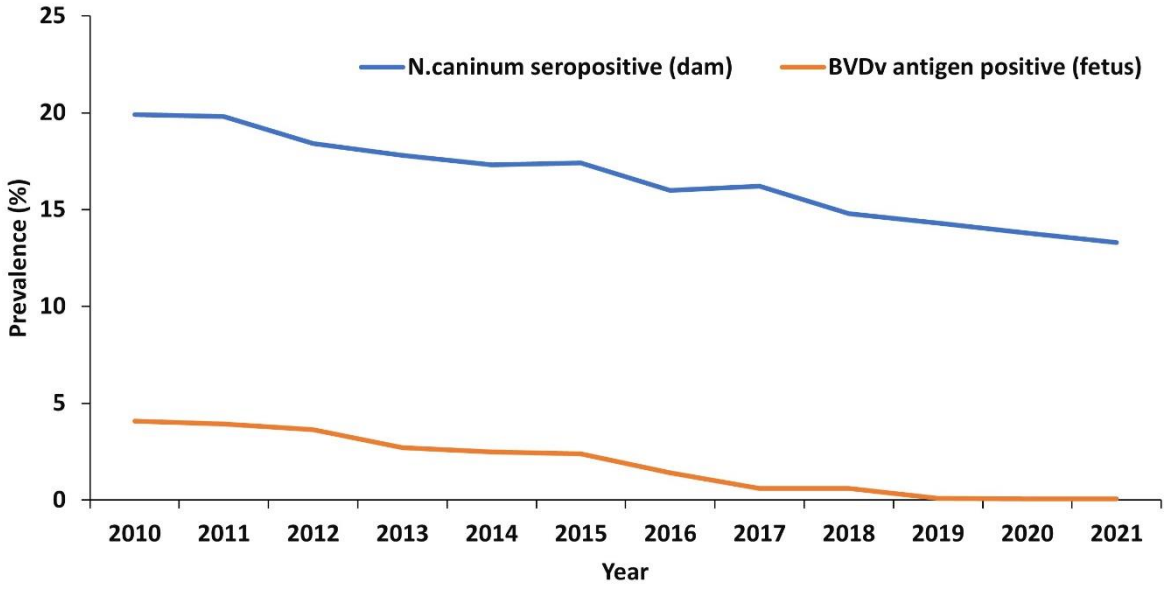

Figure 7.1. Prevalence of *N. caninum* seropositive aborted dams and BVDv antigen positive fetuses between 2010 and 2021.

The finding that herds with smaller sizes exhibited higher rates of submissions for APM cases to undergo laboratory analysis (**Chapter 3**) could potentially influence the prevalence of the diagnosed infectious causes of APM, as the occurrence of specific pathogens might differ in these types of herds compared to larger herds. In this context, it was found in a previous study that larger herds were significantly more likely to be BVDv seropositive (Sarrazin et al., 2013), a finding that was supported by a recent meta-analysis. This meta-analysis concluded that for every additional 10 animals, the odds of a BVDv infection in a herd increase by 1.04

(van Roon et al., 2020). Given this information and considering that larger herds are less likely to submit APM cases for laboratory analysis (**Chapter 3**), one might infer that the prevalence of APM cases attributed to BVDv, which we identified as approximately 4% between 2010 and 2011 (**Chapter 4**), might actually be an underestimation of the true prevalence, although herds showing evidence of BVDv presence are more likely to submit a case of APM (**Chapter 3**).

Due to financial constraints, the focus of the Belgian monitoring program was primarily on infectious causes of APM that are most relevant to the country, as well as brucellosis. Other abortifacient pathogens with low prevalence in Belgium were not included. Nevertheless, the actual prevalence of certain agents, such as the venereally-transmitted pathogens *Campylobacter fetus* subsp. *venerealis* and *Tritrichomonas foetus*, remains unknown in the country. Although a significant number of Belgian cattle are bred through artificial insemination using certified and well controlled bulls, many farms, particularly in the beef industry, still utilize breeding bulls. Given that the majority (55%) of submitted APM cases in our study originated from beef cattle, a more rigorous monitoring of these venereally-transmitted pathogens through the national abortion monitoring program could offer valuable insights for the Belgian (beef) cattle industry. Such efforts might also contribute to an increased diagnostic rate of the submitted APM cases.

To prevent infectious causes of APM, it is crucial to identify the most prevalent infectious abortifacients and associated risk factors. In our study (**Chapter 4**), risk factors for *N. caninum*, *A. fumigatus* and BVDv could be defined. We found that *N. caninum* positive cases were more common during the 3rd to 6th month of pregnancy compared to the 6th to 9th month. Fetal infection with *N. caninum* prior to fetal immunocompetence has been established, has a higher likelihood of causing abortion compared to an infection occurring in later stages of pregnancy. As the pregnancy advances, the fetus gains the ability to initiate an immune response against *N. caninum* infection, potentially offering protection against fetal mortality (Dubey et al., 2006). Additionally, we observed fewer *N. caninum* positive cases in winter compared to other seasons, which is apparent to the findings of Wouda et al. (1999). Season also played a role in BVDv positive cases being lower in winter in comparison to warmer seasons, although no clear explanation for this finding is available. Regarding *A. fumigatus*, we detected more positive cases during the 3rd to 6th month of gestation, and it appears that beef cattle are significantly more sensitive to experience an *A. fumigatus* positive case of APM compared to dairy cattle. This difference may be attributed to variations in management systems between beef and dairy cattle. In Belgium, beef cattle are traditionally housed during winter, on straw bedding or in tie-

stalls, while most dairy cattle are housed in comfortable free-stalls with mattresses. Fungal growth is known to occur in preserved forage and other feedstuffs, but also in bedding materials such as straw. Therefore, in APM cases where fungi are detected, it is essential to review the management system and identify possible sources of these organisms (Caldow and Gray, 2004). The results of our study also revealed that opportunistic bacteria are more frequently involved in APM cases in beef compared to dairy cattle. While these opportunistic bacteria are typically associated with sporadic abortions, multiple animals on the same farm may be affected within a short time period when specific risk factors are present (Holler, 2012). Yaeger and Holler, (2007) mentioned poor feed quality and external lesions as possible risk factors for abortions caused by opportunistic bacteria. One hypothesis could be that feeding quality may be lower towards the end of the housing period. Additionally, external lesions may be more present at the end of winter in cattle housed in tie-stalls due to reduced housing comfort. External skin lesions may also be caused by external parasites. In this regard, Belgian Blue cattle are particularly susceptible to mange infections (Sarre et al., 2012), and the lesions may create an entrance for opportunistic bacteria (such as *T. pyogenes* and *E. coli*) to the maternal bloodstream, subsequently leading to transit to the fetoplacental unit, and ultimately causing APM. However, further research is needed to investigate this hypothesis more in detail.

In the present study, we analyzed the distribution of APM cases across various diagnostic outcomes, categorized by gestation length. The gestation length was estimated using the CRL measurement taken at the time of necropsy (GA in days = 68 + 2.25 × CRL in cm). However, it raises the question whether this formula accurately estimates the gestational age for APM cases originating from different breeds and specific environmental circumstances. After all, fetal growth and development is a complex process related to interactions among genetic potential, environmental factors, and nutrient supply (Mao et al., 2008). For instance, notable differences in fetal development might be expected between dairy and beef cattle, particularly in cases of double muscled breeds (such as the Belgian Blue), as well as between singleton and twin pregnancies. Unfortunately, to the best of our knowledge, no adequate breed and plurality-adjusted gestational age body norm reference data are available.

In **Chapter 5**, the detection of *A. phagocytophilum* in bovine cases of APM is described. This pathogen has been detected in placental tissue, but not in fetal spleen tissue. This finding may shape future research strategies to optimize our understanding of the pathogenesis and diagnosis of *A. phagocytophilum* infections and associated cases of APM. However, it is important to note that the pathogen might also be present in asymptomatic cattle, indicating that

the mere detection of *A. phagocytophilum* in placental tissue may not be sufficient to conclusively diagnose APM. Transplacental transmission of the pathogen has been described in cattle under natural conditions (Henniger et al., 2013), and observed experimentally in sheep (Reppert et al., 2013). However, comprehensively assessing the significance of this transmission route in the dissemination of *A. phagocytophilum* infection has proven challenging due to data scarcity. In France, one study noted *A. phagocytophilum* DNA in 5.2% aborted bovine fetuses, as well as in placental and vaginal swabs from cows that had aborted (Guatteo et al., 2014). Yet, the presence of DNA alone does not definitively establish the existence of viable bacteria or their direct contribution to abortions. Therefore, additional studies are necessary before definitive conclusions can be drawn. To establish causality, histology or confirmatory specific immunohistochemistry should be conducted on placental and/or fetal tissue samples from positive cases. However, to the best of our knowledge, there is no information available regarding histopathological lesions in bovine placental and fetal tissue associated with an *A. phagocytophilum* infection. In *A. phagocytophilum*-infected ovine fetuses, histopathological examination of brain tissue revealed leukomalacia in the cerebral and cerebellar white matter (Chianini et al., 2004). According to Loeliger et al., (2003), leukomalacia might be correlated with ischemia and/or hypoxia in late pregnancy, which may be caused by placental dysfunction (Chianini et al., 2004). In lambs born following a transplacental *A. phagocytophilum* infection, lesions were restricted to the lymphoid system, leading to spleno- and lymphomegaly (Reppert et al., 2013).

In our study, *A. phagocytophilum*-specific DNA was detected in 2.7% of the analyzed cases of APM. Our results suggest a lower prevalence of the pathogen in Flanders compared to Wallonia, where an average yearly prevalence of APM cases positive for *A. phagocytophilum* ranged from 3.6 to 7.6% between 2014 and 2017 (Delooz et al., 2018). This finding is consistent with the results of a recent seroprevalence study, which demonstrated a significantly higher *A. phagocytophilum* seroprevalence in Wallonia compared to Flanders (Adjadj et al., 2023). The variation between the regions could be attributed to multiple factors, including distinctions in vegetation, tick-favorable habitats, and the density of wildlife reservoir hosts. In this context, Rousseau et al. (2021), highlighted the influence of forests, fragmented landscapes, and wild hosts as environmental determinants of bovine *A. phagocytophilum* infections. The presence of a favorable environment for ticks and their hosts would likely lead to a greater tick population, and subsequently a higher likelihood of infection. The main vector for *A. phagocytophilum* is *I. ricinus*, the most prevalent tick species in Belgium, primarily inhabiting woodlands and

forests. According to Tack et al. (2012), *I. ricinus* is more abundant in oak than in pine forests. While Flanders has approximately 11% forest coverage, this is over 30% in Wallonia (Vandekerkhove, 2013). Furthermore, forest coverage in Flanders contains a mere 8% of oak forests compared to Wallonia's 18%, while there are 30% pine plantations in Flanders versus 3% in Wallonia (Vandekerkhove, 2013). This information suggests that *I. ricinus* might be more abundant in Wallonia than in Flanders, though this remains speculative and warrants further investigation. Moreover, the variation in red and roe deer populations between both regions, with a higher prevalence of these *A. phagocytophilum* reservoir hosts in Wallonia (Prévot and Licoppe, 2013), could contribute to the elevated occurrence of *A. phagocytophilum* in Wallonia as compared to Flanders. The discrepancy in prevalence of *A. phagocytophilum* observed in our study compared to the study of Delooz et al. (2018), may also be related to differences in sample procedures. We analyzed placental and fetal spleen tissue, while Delooz et al. (2018), used a pooled set of abortion samples, including placenta, and fetal spleen, kidney and liver tissue samples. This raises the possibility that the pathogen could be present in tissues other than placenta and fetal spleen, potentially leading to a higher detection rate when analyzing different tissue samples from cases of APM caused by TBF. For example, in previous studies, *A. phagocytophilum*-specific DNA could be identified by PCR in the blood, spleen, heart, skin and lymph nodes of lambs infected transplacentally during experimental infections in sheep (Reppert et al., 2013). In the case of *A. phagocytophilum* infected and aborted goats, only abomasal content and placental tissue, but no other tissues, tested positive for the organism (Chochlakis et al., 2020).

The results of our data originate from cases that occurred over more than 10 years ago (2012). It may be worth considering whether the observed prevalence remains unchanged. As demonstrated by Delooz et al. (2018), the prevalence of *A. phagocytophilum* may vary depending on the year due to fluctuations in tick activity influenced by diverse weather characteristics. Additionally, there is more and more evidence suggesting that climate change (changes in temperature and weather patterns) may alter tick presence and increase the risk of transmission of tick-borne diseases (Tsoumani et al., 2023). Research has shown that warmer temperatures accelerate oviposition rates, egg development, and interstadial development, and lead to a higher activity of *I. ricinus* ticks (Gilbert, 2021). Consequently, building up a more intensive surveillance program to monitor the prevalence of ticks and tick-borne diseases in animals (e.g., *A. phagocytophilum*) would be beneficial for both human and animal health.

Implementing laboratory analyses for *A. phagocytophilum* in bovine cases of APM could be a key part of such a surveillance program.

The detection of *Chlamydia* spp. and *P. acanthamoebae* along with their associated lesions in bovine placental tissue was described in **Chapter 6**. In 50% of the analyzed APM cases, DNA of *Chlamydia* spp. (6%) or *P. acanthamoebae* (44%) was detected in either low or high quantities. Our results are comparable with a study from Switzerland, where 1.8% of placental tissues of bovine abortions were positive by PCR for *Chlamydia* spp., while 18.2% were positive for *Parachlamydia* (Ruhl et al., 2009). However, as already mentioned, the detection of an abortifacient agent alone is not conclusive. The diagnostic investigation of APM is a stepwise process, which includes appropriate sample collection, recording of a complete case history documentation, macroscopic evaluation, microbiological examinations, as well as histopathology and molecular analyses. Establishing associations between molecular results and macroscopic and/or microscopic findings is crucial to confirm the cause of APM, and to prevent false positives. This comprehensive workflow, integrating molecular and histological assessments, is important to avoid misinterpretation of molecular findings. Therefore, histological examination was performed in our study on a random subset of the initial sample set. The number of *Chlamydia* spp. positive cases analyzed by histology was too limited for drawing conclusions. However, among the histologically analyzed cases that tested positive for *P. acanthamoebae*, corresponding histological lesions such as purulent and/or necrotizing placentitis and/or vasculitis in placental tissue were observed in 44% of the cases. It is known that, in chlamydial abortion in sheep, severe placentitis will result in placental insufficiency and subsequent abortion. A similar mechanism could trigger chlamydial and parachlamydial APM in cattle. Based on this statement, our observations suggest that nearly 20% of bovine APM cases in Flanders might be associated with a *P. acanthamoebae* infection. Nevertheless, even when histological lesions are found alongside the detection of specific pathogens, cautious evaluation remains necessary. Botta et al. (2019) warned that interpreting histological lesions in bovine placental tissue (such as purulent and/or necrotic placentitis, with or without vasculitis) requires care. These placental lesions are not necessarily linked to placental infection; they might be part of physiological processes during pregnancy (e.g. apoptotic processes, or regulation of cell proliferation and regression as key for placental maturation), or placental expulsion. Consequently, considering future studies, incorporating immunohistochemistry to detect specific pathogens into associated histological lesions could

be valuable in establishing a more precise diagnosis for cases of bovine chlamydial and CLO related APM.

Given that the APM diagnostic rate was 39% using the extended panel of analyses outlined in **Chapter 4**, the inclusion of analyses to detect *P. acanthamoebae* as well as *A. phagocytophilum* would likely lead to a substantial increase in diagnostic rate, which could be helpful to stimulate farmers and veterinarians to submit cases of APM. It is important to note that both *A. phagocytophilum* and *P. acanthamoebae* have zoonotic implications as described in **Chapter 1**. Therefore, the detection of these pathogens underscores the importance of handling bovine placental tissue with caution to prevent the potential transmission of these diseases to humans, as well as for other zoonotic abortifacients. When assisting with births or APM, wearing gloves, boots and farm clothing, and changing clothes afterwards, reduce the likelihood to become infected with zoonotic diseases (Schimmer et al., 2014). Additionally, it is recommended to establish regular training programs regarding hygiene principles and disease prevention for farmers, farm personnel, and veterinarians. Limit visitor access to the farm, and post warning signs to keep out unauthorized people. To prevent zoonotic infections during necropsy procedures of APM cases at the laboratory, ensure that laboratory personnel are well trained and educated about zoonotic diseases. Use appropriate personal protective equipment, including lab coats, gloves, face shields, and respiratory protection if needed, and practice thorough hand hygiene. Furthermore, necropsies should be performed in well-ventilated laboratory spaces, equipped with safety cabinets or hoods to protect personnel.

Conclusions and opportunities for further research

The present thesis offers an overview of infectious abortion and perinatal mortality in cattle in Flanders. Although our results may contribute to a better understanding of the epidemiology of APM in the region, there were also some limitations in our study as mentioned above. These limitations provide opportunities for future research.

To ensure a more precise monitoring of the APM submission count, and to verify the proportion of identified APM cases undergoing laboratory analysis, it is essential to establish APM rate thresholds specific for both dairy and beef cattle in Flanders. Presently, only international thresholds for dairy cattle exist, making it challenging to assess the true proportion of APM cases that are submitted.

In order to effectively translate recommendations and facilitate the adoption of diagnostics for cases of APM, it is essential to conduct socio-psychological research. There is a need to gain a more detailed understanding of the factors that influence the Belgian farmers' behavior when they are faced with a case of APM. This understanding is necessary to optimize communication strategies, enhance compliance with legislation, and improve adherence to specialists' advice. It is also important to include bovine veterinary practitioners in this research, as many of the farmers' decisions are driven by advice provided by their veterinarian.

While it is important to thoroughly investigate all cases of APM, it can also be costly. Therefore, it would be beneficial to develop a decision tree, that guides veterinarians and lab technicians in prioritizing specific pathogenic and non-pathogenic analyses based on anamnesis, various maternal factors (such as production type, gestation length, and parity) and environmental circumstances (such as season and region where the APM happened) when faced with a new APM case. Understanding the most influential predictors for a specific diagnostic outcome in advance may greatly enhance the cost-effectiveness of laboratory analysis for any given APM case.

To increase the diagnostic rate, it is worth considering alternative or novel approaches to diagnose causes of APM. New techniques like at-random nanopore sequencing of pathogen genetic material may reveal the involvement of previously unknown agents in cases of APM.

On the other hand, given the limitations in detecting (infectious) agents in APM cases, an alternative approach could be to use biomarkers of infection, rather than solely focusing on detecting the infectious agents themselves. This approach could be particularly valuable in cases of unexplained fetal/calf mortality. Detection of fetal humoral immune response, specifically the detection of antibodies against specific pathogens, is an important diagnostic parameter in confirming intrauterine infection (Wernike et al., 2014; Jawor et al., 2017). Jawor et al., (2021) reported that focusing on the fetal antibody response increases the likelihood to detect pathogen exposure during pregnancy. Despite fetal serology being an established technique, it is not included in the current abortion monitoring program. Apart from immunoglobulins, measurement of acute phase proteins like serum amyloid A may be promising in detecting an acute inflammatory response in perinatal mortalities caused by a bacterial infection (Jawor et al., 2021). In the future, new methods like metabolomics, which investigate metabolites that represent the functioning of an organism, should help in detecting fetuses/calves infected in utero, by analyzing their specific metabolic profile, or the metabolic profile of the dam, although research on this topic is scarce (Jawor et al., 2021). To the best of our knowledge, only one paper describes distinct metabolite profiles in perinatal mortality cases (Jawor et al., 2019), while it focused solely on metabolic variations in calves at different times of death, irrespective of the cause. Another recent study demonstrated encouraging outcomes in using metabolic profiles to aid in the diagnosis and treatment of calves with diarrhea (Huang et al., 2020). Additionally, in the context of bovine respiratory diseases, metabolic profiles hold potential for accurately identifying sick animals in the majority of cases (Blakebrough-Hall et al., 2020). In human medicine, studies have shown that the determination of fetal fibronectin in maternal plasma may be helpful to detect the risk of intra-amniotic infection/inflammation and preterm delivery (Oh et al., 2019). Based on their research on this topic in cattle, Jawor et al. (2020) assumed that in the case of bovine intrauterine fetal death, fibronectin-fibrin complexes may appear in maternal blood plasma, which could be used as a biomarker of intrauterine death. However, it requires further research to observe when those complexes appear in the plasma, and whether this could be associated with specific infectious or non-infectious causes of APM.

Achieving a clearer understanding of the role of *A. phagocytophilum*, *Chlamydia* spp. and CLO in bovine APM remains a significant research challenge. Enhancing our comprehension of the underlying pathogenesis could yield innovative strategies for preventing APM caused by these intracellular bacteria. In this context, conducting comparative genomic studies to ascertain

the pathogenic potential of various strains of *Chlamydia* spp. and *P. acanthamoebae* could be of interest. Previous research has already described genetic differences in *A. phagocytophilum* strains from cattle that had aborted compared with strains from cattle that had not aborted (Dugat et al., 2017).

In the present study, data from abortions and perinatal mortalities were combined. However, in cases of perinatal mortality, non-infectious causes of death (e.g., dystocia and asphyxia due to dystocia) are more important than infectious causes (Jawor et al., 2017). Therefore, while one of the main objectives of this thesis was to determine the prevalence of the most relevant infectious causes of APM, it is worth considering putting more emphasis on non-infectious causes of death, in particular in cases of perinatal mortality. This broader focus would not only help to improve the diagnostic rate, but also raise awareness about animal welfare at both the national as well as the individual herd level, as perinatal mortality is considered an important indicator of welfare.

References

Adjadj, N. R., Cargnel, M., Ribbens, S., Quinet, C., Malandrin, L., Mignon, B., & Mori, M. (2023). Prevalence of Anaplasma phagocytophilum, Borrelia burgdorferi sensu lato, Rickettsia spp. and Babesia spp. in cattle serum and questing ticks from Belgium. Ticks and Tick-Borne Diseases, 14(4), 102146. https://doi.org/10.1016/J.TTBDIS.2023.102146

Albaaj, A., Durocher, J., Leblanc, S. J., & Dufour, S. (2023). Meta-analysis of the incidence of pregnancy losses in dairy cows at different stages to 90 days of gestation. JDSC, 4, 144–148. https://doi.org/10.3168/jdsc.2022-0278

Baumgartner, W. (2015). Fetal disease and abortion: diagnosis and causes. In R. M. Hopper (Ed.), Bovine Reproduction (1st ed., p. 496). Wiley-Blackwell.

Blakebrough-Hall, C., Dona, A., D'occhio, M.J., McMeniman, J., González, L.A. (2020). Diagnosis of bovine respiratory disease in feedlot cattle using blood 1H NMR metabolomics. Sci Rep 10, 115.

Botta, C., Pellegrini, G., Hässig, M., Pesch, T., Prähauser, B., Wunderlin, S., Guscetti, F., Schneeberger, M., Schmitt, S., Basso, W., Hilbe, M., Schuler, G., & Borel, N. (2019). Bovine Fetal Placenta During Pregnancy and the Postpartum Period. Veterinary Pathology, 56(2), 248–258. https://doi.org/10.1177/0300985818806453

Bronner, A., Hénaux, V., Fortané, N., Hendrikx, P., & Calavas, D. (2014). Why do farmers and veterinarians not report all bovine abortions, as requested by the clinical brucellosis surveillance system in France? BMC Veterinary Research, 10, 93. https://doi.org/10.1186/1746-6148-10-93

Bronner, A., Hénaux, V., Vergne, T., Vinard, J.-L., Morignat, E., Hendrikx, P., Calavas, D., & Gay, E. (2013). Assessing the mandatory bovine abortion notification system in France using unilist capture-recapture approach. PloS One, 8(5), e63246. https://doi.org/10.1371/journal.pone.0063246

Caldow, G., & Gray, D. (2004). Fetal loss. In A. H. Andrews, R. W. Blowey, H. Boyd, & R. G. Eddy (Eds.), Bovine Medicine: Diseases and Husbandry of Cattle (2e ed., pp. 577–593). Blackwell Publishing.

Cantón, G. J., Moreno, F., Fiorentino, M. A., Hecker, Y. P., Spetter, M., Fiorani, F., Monterubbianesi, M. G., García, J. A., Altamiranda, E. G., Cirone, K. M., Louge Uriarte,

E. L., Verna, A. E., Marin, M., Cheuquepán, F., Malena, R., Morsella, C., Paolicchi, F. A., Morrell, E. L., & Moore, D. P. (2022). Spatial-temporal trends and economic losses associated with bovine abortifacients in central Argentina. Tropical Animal Health and Production, 54(4), 242. https://doi.org/10.1007/S11250-022-03237-0/FIGURES/3

Chianini, F., Adams, C., & Buxton, D. (2004). Neuropathological changes in ovine fetuse caused by tickborne fever. The Veterinary Record, 155(25), 805–806. https://pubmed.ncbi.nlm.nih.gov/15651549/

Chochlakis, D., Giadinis, N., Petridou, E., Filioussis, G., Tselentis, Y., Psaroulaki, A., Ioannidou, E., Papanikolopoulou, V., & Karatzias, H. (2020). Molecular evidence of Anaplasma phagocytophilumin aborted goat fetuses and placenta. Veterinaria Italiana, 56(4), 301–303. https://doi.org/10.12834/VETIT.1173.6516.2

Clothier, G., Wapenaar, W., Kenny, E., & Windham, E. (2020). Farmers' and veterinary surgeons' knowledge, perceptions and attitudes towards cattle abortion investigations in the UK. Veterinary Record, 187(11). https://doi.org/10.1136/vr.105921

Clothier, K., & Anderson, M. (2016). Evaluation of bovine abortion cases and tissue suitability for identification of infectious agents in California diagnostic laboratory cases from 2007 to 2012. Theriogenology, 85(5), 933–938. https://doi.org/10.1016/j.theriogenology.2015.11.001

Crilly, J. (2004). The epidemiology of bovine salmonellosis in Cork and Kerry. End of Project Report, Teagasc, Project MKDC-0506-4992.

Cuttance, E., & Laven, R. (2019). Estimation of perinatal mortality in dairy calves: A review. In Veterinary Journal (Vol. 252). Bailliere Tindall Ltd. https://doi.org/10.1016/j.tvjl.2019.105356

De Graef, E., Van Mael, E., Ryckaert, V., Van Praet, W., Lipkens, Z., De Bleecker, K., Ribbens, S., Brossé, C., Coysman, A., Gillis, M., & Vanblaere, T. (2022). Activiteitenverslag 2021 - Diergeneeskundige begeleiding Herkauwers.

Delooz, L., Gregoire, F., & Saegerman, C. (2018). Bovine abortion caused by Anaplasma phagocytophilum in southern Belgium, evidence of an outbreak in summer 2017. First PhD Student Day - Liège. https://www.researchgate.net/publication/333080452

Denis-Robichaud, J., Kelton, D. F., Bauman, C. A., Barkema, H. W., Keefe, G. P., & Dubuc, J. (2019). Biosecurity and herd health management practices on Canadian dairy farms. Journal of Dairy Science, 102(10), 9536–9547. https://doi.org/10.3168/jds.2018-15921

Deschuytere, H., Callens, J., De Bleecker, K., Pierré, E., Ribbens, S., Ryckaert, V., Van de Wouwer, E., Van Mael, E., Van Praet, W., & Van Schoubroeck, L. (2016). Activiteitenverslag 2015 - Diergeneeskundige begeleiding Herkauwers.

Dubey, J. P., Buxton, D., & Wouda, W. (2006). Pathogenesis of Bovine Neosporosis. In Journal of Comparative Pathology (Vol. 134, Issue 4, pp. 267–289). https://doi.org/10.1016/j.jcpa.2005.11.004

Dubey, J. P., Schares, G., & Ortega-Mora, L. M. (2007). Epidemiology and control of neosporosis and Neospora caninum. In Clinical Microbiology Reviews (Vol. 20, Issue 2, pp. 323–367). https://doi.org/10.1128/CMR.00031-06

Dugat, T., Haciane, D., Durand, B., Lagrée, A. C., Haddad, N., & Boulouis, H. J. (2017). Short Report: Identification of a Potential Marker of Anaplasma phagocytophilum Associated with Cattle Abortion. Transboundary and Emerging Diseases, 64(5), e1–e3. https://doi.org/10.1111/TBED.12508

Ekboir, J. M. (1999). The role of the public sector in the development and implementation of animal health policies. Preventive Veterinary Medicine, 40(2), 101–115. https://doi.org/10.1016/S0167-5877(99)00015-X

Gilbert, L. (2021). The Impacts of Climate Change on Ticks and Tick-Borne Disease Risk. https://doi.org/10.1146/annurev-ento-052720

Guatteo, R., Nicolet, P., LeDréan, E., Ninio, C., & Joly, A. (2014). Diagnostic différentiel des causes infectieuses d'avortements chez les bovins intérêt d'une démarche harmonisée et d'une technique PCR multi-agents. Journées Nationales GTV.

Heersche, G., 2023. Benchmarks for evaluating the reproductive performance of the dairy herd. Cooperative Extension Service Bulletin, University of Kentucky, College of Agriculture. (https://afs.ca.uky.edu/files/benchmarks_for_evaluating_the_reproductive_performance_of_the_dairy_herd.pdf); accessed December 1, 2023.

Henniger, T., Henniger, P., Grossmann, T., Distl, O., Ganter, M., & Von Loewenich, F. D. (2013). Congenital infection with Anaplasma phagocytophilum in a calf in northern Germany. http://www.actavetscand.com/content/55/1/38

Holler, L. D. (2012). Ruminant Abortion Diagnostics. Veterinary Clinics of North America: Food Animal Practice, 28(3), 407–418. https://doi.org/10.1016/J.CVFA.2012.07.007

Hovingh, E. (2009). Abortions in Dairy Cattle - II: Diagnosing and Preventing Abortion Problems | VCE Publications | Virginia Tech. In Virginia Cooperative Extension (Vols. 404–289).

Huang, M.Z., Cui, D.A., Wu, X.H., Hui, W., Yan, Z.T., Ding, X.Z., Wang, S.Y. (2020). Serum metabolomics revealed the differential metabolic pathways in calves with severe clinical diarrhea symptoms. Animals 10, 769.

Jamaluddin, A. A., Case, J. T., Hird, D. W., Blanchard, P. C., Peauroi, J. R., & Anderson, M. L. (1996). Dairy cattle abortion in California: Evaluation of diagnostic laboratory data. In Journal of Veterinary Diagnostic Investigation (Vol. 8, Issue 2, pp. 210–218). American Assoc. of Veterinary Laboratory Diagnosticians. https://doi.org/10.1177/104063879600800211

Jawor, P., Król, D., Mee, J. F., Sołtysiak, Z., Dzimira, S., Larska, M., & Stefaniak, T. (2017). Infection exposure, detection and causes of death in perinatal mortalities in Polish dairy herds. Theriogenology, 103, 130–136. https://doi.org/10.1016/j.theriogenology.2017.07.044

Jawor, P., Zabek, A., Wojtowicz, W., Król, D., Stefaniak, T., Mlynarz, P. (2019). Metabolomic studies as a tool for determining the post-mortem interval (PMI) in stillborn calves. BMC Vet. Res., 15, 189.

Jawor, P., Krzyzanowska-Golab, D., Bajzert, J., Stefaniak, T., Katnik-Prastowska, I. (2020). Changes of plasma fibronectin and fibronectin-fibrin complexes in dams of stillborn dairy calves. Ir Vet J, 73, 17.

Jawor, P., Mee, J. F., & Stefaniak, T. (2021). Role of Infection and Immunity in Bovine Perinatal Mortality: Part 2. Fetomaternal Response to Infection and Novel Diagnostic Perspectives. Animals 2021, Vol. 11, Page 2102, 11(7), 2102. https://doi.org/10.3390/ANI11072102

Khodakaram-Tafti, A., & Ikede, B. O. (2005). A retrospective study of sporadic bovine abortions, stillbirths, and neonatal abnormalities in Atlantic Canada, from 1990 to 2001. Canadian Veterinary Journal, 46(7), 635–637.

Kinsel, M. L. (1999). An Epidemiologic Approach to Investigating Abortion Problems in Dairy Herds. The Bovine Proceedings, 32, 152–160.

Kirkbride, C. A. (1993). Bacterial agents detected in a 10-year study of bovine abortions and stillbirths. Journal of Veterinary Diagnostic Investigation : Official Publication of the

American Association of Veterinary Laboratory Diagnosticians, Inc, 5(1), 64–68. https://doi.org/10.1177/104063879300500114

Knudtson, W. U., & Kirkbride, C. A. (1992). Fungi associated with bovine abortion in the northern plains states (USA). Journal of Veterinary Diagnostic Investigation, 4(2), 181–185. https://doi.org/10.1177/104063879200400211

Loeliger, M., Watson, C. S., Reynolds, J. D., Penning, D. H., Harding, R., Bocking, A. D., & Rees, S. M. (2003). Extracellular glutamate levels and neuropathology in cerebral white matter following repeated umbilical cord occlusion in the near term fetal sheep. Neuroscience, 116(3), 705–714. https://doi.org/10.1016/S0306-4522(02)00756-X

Mao, W. H., Albrecht, E., Teuscher, F., Yang, Q., Zhao, R. Q., & Wegner, J. (2008). Growth- and breed-related changes of fetal development in cattle. Asian-Australasian Journal of Animal Sciences, 21(5), 640–647. https://doi.org/10.5713/ajas.2008.70293

Mee, J. (2013). Why Do So Many Calves Die on Modern Dairy Farms and What Can We Do about Calf Welfare in the Future? Animals, 3(4), 1036–1057. https://doi.org/10.3390/ani3041036

Mee, J. F. (2020). Investigation of bovine abortion and stillbirth/perinatal mortality - Similar diagnostic challenges, different approaches. In Irish Veterinary Journal (Vol. 73, Issue 1, pp. 1–13). BioMed Central Ltd. https://doi.org/10.1186/s13620-020-00172-0

Mee, J.F., (2021a). Bovine abortion. In Bovine Prenatal, Perinatal and Neonatal Medicine (pp. 58-66). Hungarian Association for Buiatrics.

Mee, J. F., Jawor, P., & Stefaniak, T. (2021). Role of Infection and Immunity in Bovine Perinatal Mortality: Part 1. Causes and Current Diagnostic Approaches. Animals 2021, Vol. 11, Page 1033, 11(4), 1033. https://doi.org/10.3390/ANI11041033

Mee, J. F. (2023). Invited review: Bovine abortion—Incidence, risk factors and causes. Reproduction in Domestic Animals. https://doi.org/10.1111/RDA.14366

Murray, R. D., Williams, A. J., & Sheldon, I. M. (2008). Field investigation of perinatal mortality in Friesian cattle associated with myocardial degeneration and necrosis. Reproduction in Domestic Animals, 43(3), 339–345. https://doi.org/10.1111/j.1439-0531.2007.00911.x

Oh, K.J., Romero, R., Park, J.Y., Kang, J., Hong, J.-S., Yoon, B.H. (2019). A high concentration of fetal fibronectin in cervical secretions increases the risk of intra-amniotic infection and inflammation in patients with preterm labor and intact membranes. J Perinat Med 47, 288-303.

Pereira-Bueno, J., Quintanilla-Gozalo, A., Seijas-Carballedo, A., Costas, E., & Ortega, L. M. (2000). Observational studies in Neospora caninum infected dairy cattle: pattern of transmission and age-related antibody fluctuations. International Journal of Parasitology, 30, 877–924.

Prévot, C., & Licoppe, A. (2013). Comparing red deer (Cervus elaphus L.) and wild boar (Sus scrofa L.) dispersal patterns in southern Belgium. European Journal of Wildlife Research, 59, 795–803.

Reppert, E., Galindo, R. C., Breshears, M. A., Kocan, K. M., Blouin, E. F., & de la Fuente, J. (2013). Demonstration of transplacental transmission of a human isolate of Anaplasma phagocytophilum in an experimentally infected sheep. Transboundary and Emerging Diseases, 60 Suppl 2(SUPPL.2), 93–96. https://doi.org/10.1111/TBED.12120

Rousseau, R., Delooz, L., Dion, E., Quinet, C., & Vanwambeke, S. O. (2021). Environmental determinants of Anaplasma phagocytophilum infection in cattle using a kernel density function. Ticks and Tick-Borne Diseases, 12(6). https://doi.org/10.1016/j.ttbdis.2021.101814

Ruhl, S., Casson, N., Kaiser, C., Thoma, R., Pospischil, A., Greub, G., & Borel, N. (2009). Evidence for Parachlamydia in bovine abortion. Veterinary Microbiology, 135(1–2), 169–174. https://doi.org/10.1016/J.VETMIC.2008.09.049

Salopek, S., Nelson, D., & McConnel, C. (2016). Factors influencing dairy veterinarian necropsy practices and their use of diagnostic laboratories. The Bovine Practitioner, 50(2), 140–152.

Santos, J.E.P., Rutigliano, H.M., Sa Filho, M.F., 2009. Risk factors for resumption of postpartum estrous cycles and embryonic survival in lactating dairy cows. Animal Repro Sci 110, 207-221.

Sarrazin, S., Veldhuis, A., Méroc, E., Vangeel, I., Laureyns, J., Dewulf, J., Caij, A. B., Piepers, S., Hooyberghs, J., Ribbens, S., & Van Der Stede, Y. (2013). Serological and virological BVDV prevalence and risk factor analysis for herds to be BVDV seropositive in Belgian cattle herds. Preventive Veterinary Medicine, 108(1), 28–37. https://doi.org/10.1016/j.prevetmed.2012.07.005

Sarre, C., De Bleecker, K., Deprez, P., Levecke, B., Charlier, J., Vercruysse, J., & Claerebout, E. (2012). Risk factors for Psoroptes ovis mange on Belgian Blue farms in Northern Belgium. Veterinary Parasitology, 190(1–2), 216–221. https://doi.org/10.1016/J.VETPAR.2012.05.026

Schimmer, B., Schotten, N., van Engelen, E., Hautvast, J. L. A., Schneeberger, P. M., & van Duijnhoven, Y. T. H. P. (2014). Coxiella burnetii seroprevalence and risk for humans on dairy cattle farms, the Netherlands, 2010-2011. Emerging Infectious Diseases, 20(3), 417–425. https://doi.org/10.3201/eid2003.131111

Sigdel, A. , Bisinotto, R., & Peñagaricano, F., 2022. Genetic analysis of fetal loss in Holstein cattle, Journal of Dairy Sci 105, 9012-9020.

Tack, W., Madder, M., Baeten, L., De Frenne, P., & Verheyen, K. (2012). The abundance of Ixodes ricinus ticks depends on tree species composition and shrub cover. Parasitology, 139(10), 1273–1281.

Tsoumani, M. E., Papailia, S. I., Papageorgiou, E. G., & Voyiatzaki, C. (2023). Climate Change Impacts on the Prevalence of Tick-Borne Diseases in Europe. COMECAP 2023, 18. https://doi.org/10.3390/environsciproc2023026018

van Roon, A. M., Mercat, M., van Schaik, G., Nielen, M., Graham, D. A., More, S. J., Guelbenzu-Gonzalo, M., Fourichon, C., Madouasse, A., & Santman-Berends, I. M. G. A. (2020). Quantification of risk factors for bovine viral diarrhea virus in cattle herds: A systematic search and meta-analysis of observational studies. Journal of Dairy Science, 103(10), 9446–9463. https://doi.org/10.3168/jds.2020-18193

Vandekerkhove, K. (2013). Integration of Nature Protection in Forest Policy in Flanders (Belgium).

Waldner, C.L., Parker, S., Campbel, J.R., 2019. Identifying performance benchmarks and determinants for reproductive performance and calf survival using a longitudinal field study of cow-calf herds in western Canada. PLoS One 14, e0219901.

Welby, S., Letellier, C., Fretin, D., Hooyberghs, J., Vanholme, L., Godfroid, J., Vanderstede, Y. (2009). Evaluation du programme de surveillance pour la brucellose et la leucose bovine enzootique en Belgique. Epidémiologie et Santé Animale, 55, 1–6.

Wernike, K., Holsteg, M., Schirrmeier, H., Hoffmann, B., & Beer, M. (2014). Natural Infection of Pregnant Cows with Schmallenberg Virus – A Follow-Up Study. PLOS ONE, 9(5), e98223. https://doi.org/10.1371/JOURNAL.PONE.0098223

Wiltbank, M. C., Baez, G. M., Garcia-Guerra, A., Toledo, M. Z., Monteiro, P. L. J., Melo, L. F., Ochoa, J. C., Santos, J. E. P., & Sartori, R. (2016). Pivotal periods for pregnancy loss during the first trimester of gestation in lactating dairy cows. Theriogenology, 86(1), 239–253. https://doi.org/10.1016/J.THERIOGENOLOGY.2016.04.037

Wolf-Jäckel, G. A., Hansen, M. S., Larsen, G., Holm, E., Agerholm, J. S., & Jensen, T. K. (2020). Diagnostic studies of abortion in Danish cattle 2015-2017. Acta Veterinaria Scandinavica, 62(1), 1. https://doi.org/10.1186/s13028-019-0499-4

Wouda, W., Bartels, C. J. M., & Moen, A. R. (1999). Characteristics of Neospora caninum-associated abortion storms in dairy herds in the Netherlands (1995 to 1997). Theriogenology, 52(2), 233–245. https://doi.org/10.1016/S0093-691X(99)00125-9

Yaeger, M. J., & Holler, L. D. (2007). Bacterial causes of bovine infertility and abortion. In R. S. Youngquist & W. R. Threlfall (Eds.), Current therapy in large animal theriogenology (2nd ed., pp. 389–398). WB Saunders Co.

Summary

To ensure the economic viability of the dairy and beef industry, reproductive efficiency plays a pivotal role. In dairy herds, reproduction is essential for generating the next generation of females and initiating milk production after calving, while in beef herds, profitability can be optimized by maximizing the number of cows that produce marketable calves each year. Consequently, events like abortion and perinatal mortality (APM) that interrupt the pregnancy or lead to the birth of a non-viable calf have a substantial economic impact on both milk and meat production. Various factors, both infectious and non-infectious, have been associated with bovine APM, but prevalence may vary depending on the region. Some of these infectious causes may pose a zoonotic risk, which underscores the importance of reporting cases, and regional monitoring of infectious causes of APM for the benefit of both animal and human health, and to safeguard international trade (**Chapter 1**).

The present PhD thesis aimed to investigate the epidemiology of bovine APM in Belgium, more specifically Flanders. Our research consisted three main objectives: evaluating farmer compliance with the national abortion monitoring program (**Chapter 3**), studying the prevalence of infectious causes and related risk factors (**Chapter 4**), and exploring zoonotic pathogens like *Anaplasma phagocytophilum* (*A. phagocytophilum*) and *Chlamydia* spp. in relation to bovine APM (**Chapters 5 and 6**). The overall objective was to unravel the epidemiology of bovine APM in Flanders.

In **Chapter 3**, we described the global issue of underreporting of APM cases, which hinders early detection of infectious and zoonotic causes of APM. Our study evaluated farmer compliance with the Belgian mandatory abortion monitoring program, part of the national brucellosis monitoring program. Focusing on the northern region of Belgium (Flanders), we examined 63,221 APM case submissions between 2006 and 2021. Based on the proportion of APM submissions versus the proportion of bovine reproductive females, an APM proportion (APM_{PR}) was calculated, and influential factors at both animal and herd level were explored. Our study revealed a substantial increase in the APM_{PR} from 0.44 to 0.94% following the implementation of an extensive abortifacient agent investigation panel and free on-farm sample collection at the end of 2009. Additionally, an increase of the APM_{PR} was associated with the emergence of a new pathogen (schmallenbergvirus) (1.23%), and the introduction of a mandatory eradication program for bovine viral diarrhea virus (BVDv) (1.20%). Notably, APM submission was higher in beef cattle, during winter, in smaller herds, among herds with a previous APM history, and in herds with evidence of BVDv presence. Despite mandatory reporting, underreporting remained an issue, emphasizing the need for further research to

motivate farmers to submit APM cases, enhance reporting rates, and strengthen APM and zoonotic pathogen monitoring.

In a second study (**Chapter 4**), we analyzed the prevalence of infectious agents linked to APM in Flanders, and we also identified variations related to production type (dairy versus beef), gestation length, parity, and seasonality. Therefore, we used 4,006 APM cases that were submitted between 2010 and 2011, as in this time period the most extensive panel of analyses for 10 different pathogens was performed. Results showed that at least one potential causal agent was identified in 39% of cases, with *Neospora caninum* (*N. caninum*) being the most common (15.7%), followed by *Trueperella pyogenes* (5.6%), BVDv (4.5%), *Escherichia coli* (3.3%), and *Aspergillus fumigatus* (*A. fumigatus*) (2.7%). Notably, *N. caninum* and *A. fumigatus* were less frequently found in late gestation, while *A. fumigatus* was less common in dairy compared to beef abortion cases. Additionally, winter was associated with lower positivity for *N. caninum* and BVDv compared to warmer seasons. However, a definitive causative diagnosis was not reached in 61% of cases despite extensive testing, underscoring the need for more extensive (both infectious and non-infectious) disease testing or improved diagnostic methods.

In the following studies, the diagnostic panel was extended with analyses for *A. phagocytophilum, Chlamydia* spp., and *Parachlamydia acanthamoebae* on a selected number of APM cases.

In **Chapter 5**, the potential involvement of *Anaplasma phagocytophilum* (*A. phagocytophilum*) in cases of bovine APM was investigated. To address this, an exploratory study analyzing late-term APM cases was conducted, in order to determine the presence of *A. phagocytophilum*. To assess whether placental or fetal spleen tissue is more sensitive for detection of the pathogen, we examined both tissue types using real-time PCR. Our findings revealed that 2.7% of 150 sampled placentas tested positive for *A. phagocytophilum*, while none of the fetal spleen samples did. However, it is important to note that we did not perform histopathology to identify associated lesions, limiting our ability to establish a direct causal relationship between *A. phagocytophilum* presence and APM events. Nevertheless, the detection of *A. phagocytophilum* suggests its potential role in bovine APM, with placental tissue appearing as the more suitable tissue for identification.

In the final study (**Chapter 6**), we aimed to investigate the presence of *Chlamydia* spp. and *Parachlamydia acanthamoebae* (*P. acanthamoebae*) in bovine placental tissue from late-term APM and non-APM cases. Placental samples were tested by PCR for *Chlamydia* spp., *Chlamydia abortus* (*C. abortus*), *Chlamydia psittaci* (*C. pisttaci*), and *P. acanthamoebae*.

Additionally, a subset of samples underwent histopathological analysis to detect *Chlamydia*-induced lesions. *Chlamydia* spp. were detected in 5.4% of 205 analyzed cases, with only 3 of them being positive for *C. psittaci*. *Parachlamydia acanthamoebae* was found in 36% of cases, which was significantly higher in APM (44%) than non-APM cases (7.3%). None of the cases tested positive for *C. abortus*. Histopathological analysis showed purulent and/or necrotizing placentitis in 18.8% of samples, with 5.9% having vasculitis. These lesions were more common in APM (24%) than in non-APM cases (3.9%). The presence of *Chlamydia* spp., and especially *P. acanthamoebae*, along with related histological lesions, suggests their potential role in bovine APM in Flanders.

This PhD thesis sheds light on the epidemiology of infectious causes of bovine APM in Flanders, and provides new insights into the involvement of zoonotic agents such as *A. phagocytophilum* and *Chlamydiae*. It also reveals multiple factors influencing farmers' compliance with mandatory APM reporting and case submission for laboratory analysis. The information in this thesis can be valuable for developing similar APM surveillance programs worldwide, and may serve as a useful resource for bovine farmers and veterinary practitioners seeking to enhance management practices that may affect the prevalence of specific abortifacient pathogens at the herd level. The detection of several pathogens with zoonotic potential emphasizes the importance of caution when handling APM tissues for farmers, and their veterinarians, as well as laboratory personnel. In this context, promoting proper education for these individuals is crucial. Additionally, policy makers may use the findings of this study to support further research into the factors influencing farmers' compliance with mandatory disease monitoring programs.

Summary

Do farmers submit cases of bovine abortion and perinatal mortality?

FARM **LABORATORY**

Increased submissions associated with

- implementation extensive analytical panel + on-farm sample collection
- emergence schmallenbergvirus
- introduction BVDv eradication program
- beef cattle
- winter
- smaller herds
- herds with previous APM cases
- herds with evidence of BVDv presence

Which pathogens are involved in bovine abortion and perinatal mortality?

LABORATORY

Diagnostic rate 39%

Identified abortifacients

N. caninum 16%
T. pyogenes 6%
BVDv 5%
E. coli 3%
A. fumigatus 3%

N. caninum* and *A. fumigatus

- less common in late gestation

A. fumigatus

- less common in dairy cattle

BVDv and *N. caninum*

- less comon in winter

What about *A. phagocytophilum*, *Chlamydia* spp. and *Chlamydia*-like organisms?

LABORATORY

***A. phagocytophilum* in APM**

- placenta 2.7%
- fetal spleen 0%
- no histopathology

***Chlamydiae* and CLO in placenta APM vs non-APM**

- *Chlamydia* spp. 6 vs 2%
- *P. acanthamoebae* 44 vs 7%

Histopathology

- placentitis in 44% of cases positive for *P. acanthamoebae*
- placentitis in 21% of negative cases

Samenvatting

Om de economische duurzaamheid van de rundveeteelt te waarborgen, blijft een efficiënte reproductie een erg belangrijke factor. Zo is de geboorte van een kalf op een melkveebedrijf essentieel om de volgende generatie vrouwelijke dieren te garanderen en de melkproductie na de kalving op gang te brengen, terwijl op vleesveebedrijven de rendabiliteit kan geoptimaliseerd worden door er voor te zorgen dat zoveel mogelijk koeien jaarlijks een gezond kalf voortbrengen. Gebeurtenissen zoals abortus en perinatale sterfte (APM) die de dracht onderbreken of leiden tot doodgeboren kalveren, hebben dan ook een aanzienlijke economische impact op zowel de melk- als vleesvee-industrie. Verschillende infectieuze en niet-infectieuze factoren zijn geassocieerd met APM bij het rund, maar de prevalentie ervan kan variëren afhankelijk van de regio. Sommige van deze infectieuze oorzaken hebben bovendien een zoönotisch karakter, wat het belang benadrukt van regionale monitoring van infectieuze oorzaken van APM, en dit voor de gezondheid van zowel mens als dier, alsook voor het behoud van de internationale handel (**Hoofdstuk 1**).

Dit proefschrift had als doel de epidemiologie van APM bij het rund in Vlaanderen (België) te onderzoeken. Ons onderzoek had 3 objectieven: we evalueerden welke factoren een invloed hadden op het al dan niet inzenden van APM gevallen binnen het verplichte nationale abortusmonitoringsprogramma (**Hoofdstuk 3**), vervolgens werd de prevalentie van de belangrijkste infectieuze oorzaken van APM en gerelateerde risicofactoren bestudeerd (**Hoofdstuk 4**) en finaal onderzochten we het belang van zoönotische pathogenen zoals *Anaplasma phagocytophilum* (*A. phagocytophilum*) en *Chlamydiae* in relatie tot boviene APM in Vlaanderen (**Hoofdstukken 5** en **6**). De algemene doelstelling was om een beter inzicht in de epidemiologie van APM bij het rund in Vlaanderen te krijgen.

In **Hoofdstuk 3** werd het wereldwijde probleem van onderrapportering van APM gevallen beschreven, wat de vroege detectie van infectieuze en zoönotische oorzaken van APM kan belemmeren. Aan de hand van ons onderzoek hebben we geëvalueerd in hoeverre Vlaamse rundveehouders het verplichte abortusmonitoringsprogramma, dat deel uitmaakt van het nationale brucellose monitoringsprogramma, naleven, en daarnaast werden beïnvloedende factoren op zowel dier- als bedrijfsniveau geanalyseerd. We onderzochten 63,221 APM gevallen die tussen 2006 en 2021 werden gemeld en ingezonden voor onderzoek in het laboratorium. Hiervoor werd een APM proportie (APM_{PR}) berekend, gebaseerd op de proportie van APM inzendingen ten opzichte van de proportie van reproductieve vrouwelijke runderen. Onze studie toonde aan dat er een aanzienlijke stijging van APM_{PR} was van 0.44 naar 0.94% na de implementatie van een uitgebreid analytisch panel voor mogelijke infectieuze oorzaken

en de gratis ophaling van APM stalen op rundveebedrijven eind 2009. Ook het opduiken van een nieuwe pathogeen (het Schmallenbergvirus) (1.23%), en de introductie van een nationaal verplicht bestrijdingsprogramma voor het boviene virale diarreevirus (BVDv) (1.20%) zorgden voor een stijging van de APM_{PR}. Opmerkelijk was dat er meer APM gevallen afkomstig van vleesvee werden ingezonden, voornamelijk tijdens de winter, op kleinere rundveebedrijven, op bedrijven met een voorgeschiedenis van APM en op bedrijven met een recent aangetoonde aanwezigheid van BVDv. Ondanks de verplichte rapportering blijft onderrapportering een probleem, wat het belang benadrukt van verder onderzoek om veehouders te stimuleren om APM gevallen in te zenden, het meldingspercentage te verhogen en de monitoring van APM en zoönotische pathogenen te verzekeren.

In een tweede studie (**Hoofdstuk 4**) van dit doctoraat analyseerden we de prevalentie van de meest voorkomende infectieuze oorzaken van APM bij het rund in Vlaanderen. Aanvullend werden verschillen in prevalentie geïdentificeerd, op basis van productietype (melkvee versus vleesvee), drachtduur, pariteit en seizoen waarin de APM plaatsvond. We gebruikten hiervoor 4,006 APM gevallen die waren ingezonden tussen 2010 en 2011, aangezien in deze periode het meest uitgebreide panel van analyses voor 10 verschillende pathogenen werd uitgevoerd. Uit de resultaten bleek dat ten minste één potentieel causatief agens gedetecteerd kon worden in 39% van de geanalyseerde gevallen, waarbij *Neospora caninum* (*N. caninum*) het meeste voorkwam (15.7%), gevolgd door *Trueperella pyogenes* (5.6%), BVDv (4.5%), *Escherichia coli* (3.3%) en *Aspergillus fumigatus* (*A. fumigatus*) (2.7%). Opmerkelijk was dat *N. caninum* en *A. fumigatus* minder vaak voorkwamen in de late dracht, en dat *A. fumigatus* minder vaak gevonden werd bij melkvee in vergelijking met vleesvee. Daarnaast waren er in vergelijking met andere seizoenen minder gevallen positief voor *N. caninum* en BVDv in de winter. Toch kon bij 61% van de gevallen ondanks uitgebreid onderzoek geen definitieve oorzakelijke diagnose worden gesteld, wat benadrukt dat er meer uitgebreide tests en verbeterde diagnostische methodes nodig zijn voor de identificatie van zowel infectieuze als niet-infectieuze oorzaken van APM.

In **Hoofdstuk 5** onderzochten we de mogelijke betrokkenheid van *Anaplasma phagocytophilum* (*A. phagocytophilum*) bij APM in Vlaanderen. Hiervoor werden gevallen van APM in de late dracht geanalyseerd op de aanwezigheid van *A. phagocytophilum*. We onderzochten zowel placenta als foetaal miltweefsel met behulp van real-time PCR om te beoordelen welk weefsel gevoeliger is voor detectie van de pathogeen. Onze bevindingen toonden aan dat 2,7% van de 150 bemonsterde placenta's positief testte op *A. phagocytophilum*,

terwijl geen van de foetale miltmonsters dat deed. Het is echter belangrijk om op te merken dat er geen histopathologie werd uitgevoerd om geassocieerde letsels te identificeren, waardoor het onmogelijk was om een direct causaal verband tussen de aanwezigheid van *A. phagocytophilum* en APM vast te stellen. Desalniettemin suggereert de detectie van *A. phagocytophilum* een potentiële rol van deze pathogeen bij APM bij runderen in Vlaanderen, waarbij placenta het meest geschikte weefsel voor detectie lijkt te zijn.

In de laatste studie (**Hoofdstuk 6**) hebben we de aanwezigheid van *Chlamydia* spp. en *Parachlamydia acanthamoebae* (*P. acanthamoebae*) in placenta weefsel van abortus en niet-abortus gevallen onderzocht. Weefselstalen werden getest op *Chlamydia* spp., *Chlamydia abortus* (*C. abortus*), *Chlamydia psittaci* (*C. pisttaci*) en *P. acanthamoebae* via PCR, en vervolgens histopathologisch onderzocht om door *Chlamydiae* veroorzaakte laesies op te sporen. *Chlamydia* spp. werden gedetecteerd in 5,4% van de 205 gevallen, waarvan 3 positief waren voor *C. psittaci*. *Parachlamydia acanthamoebae* werd gevonden in 36% van de onderzochte stalen, en dit aanzienlijk hoger bij APM (44%) in vergelijking met niet-APM gevallen (7,3%). Geen van de gevallen testte positief voor *C. abortus*. Histopathologische analyse toonde purulente en/of necrotiserende placentitis in 18,8% van de monsters, waarvan 5,9% vasculitis vertoonde. Deze laesies kwamen vaker voor bij APM (24%) dan bij niet-APM gevallen (3,9%). De aanwezigheid van *Chlamydia* spp., en vooral *P. acanthamoebae*, in combinatie met gerelateerde histologische laesies, suggereert hun potentiële rol bij APM bij het rund in Vlaanderen.

Dit doctoraatsonderzoek belicht de epidemiologie van de meest voorkomende infectieuze oorzaken van APM bij het rund in Vlaanderen en biedt inzicht in de mogelijke betrokkenheid van zoönotische agentia zoals *A. phagocytophilum* en *Chlamydiae*. Daarnaast zijn verschillende factoren aangetoond, die mogelijks een invloed hebben op het al dan niet rapporteren en inzenden voor labo-onderzoek van APM gevallen door Vlaamse rundveehouders. De informatie in deze thesis kan waardevol zijn voor de ontwikkeling van soortgelijke APM surveillance programma's in andere landen. Bovendien kan het een nuttige bron van informatie zijn voor rundveehouders en hun dierenartsen, die hun bedrijfsmanagement willen verbeteren om specifieke oorzaken van APM op hun rundveebedrijf te bestrijden. De detectie van verschillende pathogenen met zoönotisch potentieel onderstreept het belang van voorzichtigheid wanneer weefsels van APM worden gemanipuleerd, zowel voor veehouders en dierenartsen als voor laboratoriumpersoneel. In deze context is een goede opleiding en

regelmatige training voor deze beroepsgroepen uiteraard van essentieel belang. Tenslotte kunnen beleidsmakers de bevindingen van deze studie gebruiken om verder onderzoek te ondersteunen naar de factoren die van invloed zijn op de naleving van verplichte monitoringprogramma's door veehouders.

Curriculum Vitae and Bibliography

Curriculum Vitae

Hans Van Loo was born on June 22, 1981, in Jette. In 2005, he obtained the degree of Master in Veterinary Medicine (focus Ruminants) at the Faculty of Veterinary Medicine, Ghent University. After working as an intern (2005-2006) and then as an assistant in the Ambulatory Clinic of the Department of Reproduction, Obstetrics, and Herd Health at the same faculty, he worked in two private practices for large animals between 2007 and 2011. Between 2011 and 2016, Hans worked at Dierengezondheidszorg Vlaanderen, initially within the abortion monitoring project, and later within the Veepeiler Rund project. Since January 2016, he joined the Ambulatory/Herd Health Clinic of the Department of Internal Medicine, Reproduction, and Population Medicine as an educational supervisor. Within this team he contributes to the day, evening, and weekend services as part of the students clinical education. In 2019, he successfully completed the training Cattle Veterinary Specialist (Vakdierenarts Rund) at Ghent University, and in 2021 he became a European Board of Veterinary Specialisation certified specialist in bovine health management (diplomate of the European College of Bovine Health Management). Alongside his clinical and teaching activities, Hans conducted his doctoral research on the epidemiology of abortion and perinatal mortality in cattle in Flanders, and extended it to the possible contribution of 2 zoonotic pathogens, Anaplasma phagocytophilum and Chlamydiae. This doctoral research was supervised by Prof. Dr. Bart Pardon, Prof. Dr. Geert Opsomer, and Dr. Osvaldo Bogado Pascottini (DI08 - UGent), and was made possible thanks to the Federal Agency for the Safety of the Food Chain.

Hans is the author and co-author of several scientific and popular publications and reports in national and international journals. He has been a speaker at various symposia and actively participated in multiple national and international congresses. He is a co-organizer and frequent speaker in postgraduate education courses for ruminant veterinarians at the Academy of Veterinary Medicine at Ghent University, and he has also supervised several master thesis students.

Publications in national and international peer-reviewed journals

Van Loo H., Verberckmoes S., Cox E. Passieve en actieve immuniteit en vaccinatie bij lam en ooi. Vlaams Diergeneeskundig Tijdschrift (2006), 6, 140-152.

Van Loo H., De Vlieger S., Piepers S., Passchyn P., de Kruif A., Opsomer G. Mastitis bij melkvee veroorzaakt door coliformen, met nadruk op Klebsiella spp. Vlaams Diergeneeskundig Tijdschrift (2007), 76, 272-282.

Bossaert P., Leroy J., Cools S., Van Loo H., De Kruif A., Opsomer G. De metabole adaptatiemechanismen bij hoogproductieve melkkoeien. Vlaams Diergeneeskundig Tijdschrift (2008), 77, 207-218.

Bossaert P., Cools S., Van Loo H., Leroy J., De Kruif A., Opsomer G. De pathogenese en kliniek van ketonemie en leververvetting bij hoogproductieve melkkoeien. Vlaams Diergeneeskundig Tijdschrift (2008), 77, 283-290.

Cools S., Bossaert P., Van Loo H., De Kruif A., Opsomer G. De behandeling en preventie van ketonemie en leververvetting bij hoogproductieve melkkoeien. Vlaams Diergeneeskundig Tijdschrift (2008), 77, 363-375.

Van Loo H., Govaere J., Chiers K., Hoogewijs M., Opsomer G. ,De Kruif A. Hydrops uteri bij een Belgisch Witblauwe vaars gepaard gaand met een mandibulair vasculair hamartoma bij het kalf. Vlaams Diergeneeskundig Tijdschrift (2010), 79, 139-142.

Garigliany M., De Leeuw I., Kleijnen D., Vandenbussche F., Callens J., Van Loo H., Lebrun M., Saulmont M., Desmecht D., De Clercq K. The presence of bluetongue virus serotype 8 RNA in Belgian cattle since 2008. Transboundary and Emerging Diseases (2011), 58, 503-509.

Van Loo H., Maris J., Strubbe M., De Bleecker K., De Regge N., Caij AB., Van den Berg T. Schmallenberg virus – Belgium: newborn calves rRT-PCR positive without clinical signs. ProMED-mail; 10 May 2012.

Méroc E., Poskin A., Van Loo H., Quinet C., Van Driessche E., Delooz L., Behaeghel I., Riocreux F., Hooyberghs J., De Regge N., Caij A.B., Van den Berg T., Van der Stede Y. Large-scale cross-

sectional serological survey of Schmallenberg virus in Belgian cattle at the end of the first vector season. Transboundary and Emerging Diseases (2013), 60, 4-8.

Van Loo H., Pardon B., De Schutter P., De Bleecker K., Vanrompay D., Deprez P., Maris J. Detection of Chlamydia psittaci in Belgian cattle with signs of respiratory disease and milk drop syndrome. Veterinary Record (2014), 175, 562.

Méroc E., Poskin A., Van Loo H., Van Driessche E., Czaplicki G., Quinet C., Riocreux F., De Regge N., Caij A.B., Van den Berg T., Hooyberghs J., Van der Stede Y. Follow-up of the Schmallenberg Virus Seroprevalence in Belgian Cattle. Transboundary and Emerging Diseases (2015), 62, e80-e84.

Malrait K., Verschave S., Skuce P., Van Loo H., Vercruysse J., Charlier J. Novel insights into the pathogenic importance, diagnosis and treatment of the rumen fluke (Calicophoron daubneyi) in cattle. Veterinary Parasitology (2015), 207, 134-139.

Roelandts S., Van der Stede Y., Czaplicki G., Van Loo H., Van Driessche E., Dewulf J., Hooyberghs J., Faes C. Serological diagnosis of bovine neosporosis: a Bayesian evaluation of two antibody ELISA tests for in vivo diagnosis in purchased and abortion cattle. Veterinary Record (2015), 176, 598.

Vande Velde F., Claerebout E., Cauberghe V., Hudders L., Van Loo H., Vercruysse J., Charlier J. Diagnosis before treatment: Identifying dairy farmers' determinants for the adoption of sustainable practices in gastrointestinal nematode control. Veterinary Parasitology (2015), 212, 308-317.

Poskin A., Méroc E., Behaeghel I., Riocreux F., Couche M., Van Loo H., Bertels G., Delooz L., Quinet C., Dispas M., Van der Stede Y. Schmallenberg Virus in Belgium: Estimation of Impact in Cattle and Sheep Herds. Transboundary and Emerging Diseases (2015), 64, 264-274.

Gille L., Pilo P., Valgaeren B., Van Driessche L., Van Loo H., Bodmer M., Bürki S., Boyen F., Haesebrouck F., Deprez P., Pardon B. A new predilection site of Mycoplasma bovis: Postsurgical seromas in beef cattle. Veterinary Microbiology (2016), 186, 67-70.

Poskin A., Méroc E., Behaeghel I., Riocreux F. Couche M., Van Loo H., Bertels G., Delooz L., Quinet C., Dispas M., Van der Stede Y. Schmallenberg virus in Belgium: estimation of impact in cattle and sheep herds. Transboundary and Emerging Diseases (2017), 64, 264-274.

De Bie J., Proost K., Van Loo H., Callens J., Bols P.E.J., Fransen E., Leroy J.L.M.R. β-carotene and vitamin E in the dairy industry: blood levels and influencing factors – a case study in Flanders. Vlaams Diergeneeskundig Tijdschrift (2019), 88, 137-149.

Van Loo H., Bogado Pascottini O., Ribbens S., Hooyberghs J., Pardon B., Opsomer G. Retrospective study of factors associated with bovine infectious abortion and perinatal mortality. Preventive Veterinary Medicine (2021), 191.

Kemel C., Salamone M., Van Loo H., Latour C., Vandeputte S., Callens J., Hostens M., Opsomer G. Unaffected semen quality parameters in *Neospora caninum* seropositive Belgian Blue bulls. Theriogenology (2022), 191, 10-15.

Saey V., Van Loo H., Gryspeerdt A., Pronost S., Gasthuys F., Rosiers K., Cassart D., Govaere J. Detection of infectious agents in equine pregnancy loss, stillbirth and neonatal death. Vlaams Diergeneeskundig Tijdschrift (2023), 92, 58-65.

Van Loo H., Bogado Pascottini O., Hooyberghs J., De Bleecker K., Ribbens S., Opsomer G., Pardon B. Detection of *Anaplasma phagocytophilum* in fetal and placental tissue of bovine abortions and perinatal mortalities. Veterinary Record (2023)

Van Loo H., Bogado Pascottini O., Ribbens S., Hooyberghs J., Mori M., Vanrompay D., Opsomer G., Pardon B. Detection of *Chlamydia* and Chlamydia-like organisms in bovine placental tissue. Reproduction in Domestic Animals (2023), 58, 1080-1086.

Van Loo H., Bogado Pascottini O., Ribbens S., Hooyberghs J., Opsomer G., Pardon B. Enhancing bovine abortion surveillance: a learning experience. Journal of Dairy Science (2023).

Oral presentations

Van Loo H., Bogado Pascottini O., Ribbens S., Hooyberghs J., Pardon B., Opsomer G. Retrospective study of factors associated with bovine infectious abortion and perinatal mortality. 27th World Buiatrics Congress, June 2012, Lisbon, Portugal.

Van Loo H., Méroc E., Caij B., Van der Stede Y., De Bleecker K. Use of abortion surveillance in cattle to verify the introduction of Schmallenberg Virus in Flanders. EPIZONE, 7th Annual Meeting, October 2013, Brussels, Belgium.

Méroc E., Poskin A., Van Loo H., Quinet C., Van Driessche E., Delooz L., Behaeghel I., Riocreux F., Hooyberghs J., De Regge N., Caij B., van den Berghe T., Van der Stede Y. Schmallenberg virus: Results of Belgian cross-sectional serological surveys. EPIZONE, 7th Annual Meeting, October 2013, Brussels, Belgium.

Van Loo H. Bovine abortion, Diagnostic approach of a herd problem. National Buiatrics Congress, October 2015, Ieper, Belgium.

Van Loo H., Bogado Pascottini O., Ribbens S., Hooyberghs J., Mori M., Vanrompay D., Opsomer G., Pardon B. Detection of *Chlamydia* and Chlamydia-like organisms in bovine placental tissue. 29th World Buiatrics Congress, July 2016, Dublin, Ireland.

Gille L., Pilo P., Van Driessche L., Van Loo H., Bodmer M., Bürki S., Boyen F., Haesebrouck F., Deprez P., Pardon B. A new prediliction site of Mycoplasma bovis: outbreaks of postsurgical infected seromas in beef cattle. 29th World Buiatrics Congress, July 2016, Dublin, Ireland.

Van Loo H., Bogado Pascottini O., Hooyberghs J., De Bleecker K., Ribbens S., Opsomer G., Pardon B. Detection of *Anaplasma phagocytophilum* in fetal and placental tissue of bovine abortions and perinatal mortalities. 31st World Buiatrics Congress, August 2022, Madrid, Spain.

Kemel C., Salamone M., Van Loo H., Latour C., Vandeputte S., Callens J., Hostens M., Opsomer G. Unaffected semen quality parameters in *Neospora caninum* seropositive Belgian Blue bulls. 31st World Buiatrics Congress, August 2022, Madrid, Spain.

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