Promotor: Prof. dr. Daisy Vanrompay
Department of Molecular Biotechnology
Laboratory of Immunology and Animal Biotechnology

Dean: Prof. dr. Herman Van Langenhove

Rector: Prof. dr. Paul Van Cauwenberge

Taher Harkinezhad

Molecular epidemiology of *Chlamydophila psittaci* in psittacine birds and humans and prevention by DNA vaccination

Thesis submitted in fulfillment of the requirements
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Moleculaire epidemiologisch onderzoek naar het voorkomen van *Chlamydophila psittaci* bij papegaai-achtigen en de mens en preventie door middel van DNA vaccinatie

Cover illustration:
Top; immunofluorescence (FITC) staining of Buffalo Green Monkey cells 6 days after inoculation (bar 20 μm). Negative control (left). Positive parrot sample (right), with characteristic chlamydial inclusions inside the cytoplasm of the host cell.
Midlle; transmission electron microscopic view of *Chlamydophila psittaci* infected Buffalo Green Monkey cell (bar 5 μm). Note the large inclusion with electron dense elementary bodies (EB) and less electron dense reticulate bodies.
Below; immunofluorescence (FITC) staining of frozen tissue sections of the abdominal airsac of budgerigars after infection with *Chlamydophila psittaci* (bar 20 μm). Abdominal airsac of a non-vaccinated bird with characteristic chlamydial inclusions (left) and abdominal airsac of a vaccinated bird without inclusions (right).

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Promotor

Prof. dr. Daisy Vanrompay
Department of Molecular Biotechnology
Ghent University, Belgium

Members of the examination committee

Prof. dr. Els Van Damme (chairman)
Department of Molecular Biotechnology
Ghent University

Prof. dr. Patrick Van Oostveldt (secretary)
Department of Molecular Biotechnology
Ghent University

Prof. dr. Lutgart Braeckman
Department of Public Health
Ghent University

Prof. dr. Patrick Butaye
Department of Bacteriology, Pathology
Center for Research in Veterinary Science
and Agrochemics, Brussels

Dr. ir. Sven Arnouts
Department of Virology, Parasitology
and Immnology, Ghent University

Dr. ir. Marnix Van Loock
HIV virology Department
Tibotec BVBA, Mechelen

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Abbreviations

AA	Amino Acid
ATP	Adenosine TriPhosphate
BGM	Buffalo Green Monkey
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumine
BSL	BioSafety Level
C.	*Chlamydia*
CDC	Centers for Disease Control and prevention
CF test	Complement Fixation test
CI	Confidence Interval
COMC	Chlamydia Outer Membrane Complex
Con A	Concanavalin A
Cop	Chlamydia outer protein
Cp.	*Chlamydophila*
CPAF	Chlamydial Protease-like Activity Factor
CRP	Cysteine Rich Protein
DCF test	Direct Complement Fixation test
DMEM	Dulbecco's Modified Eagle's Medium
DTH	Delayed-Type Hypersensitivity
EAE	Enzootic Abortion of Ewes
EB	Elementary Body
EBA	Elementary Body Agglutination
ELISA	Enzyme Linked ImmunoSorbent Assay
EPEC	Entero Pathogenic *Escherichia coli*
E.coli	*Escherichia coli*
EtBr	EthidiumBromide
FA	Fluorescent Antibody
FCS	Fetal Calf Serum
FCV	Feline CaliciVirus
FISH	Fluorescence In Situ Hybridization
FITC	Fluorescein IsoThioCyanate
FPL	Feline PanLeukopenia
FVR	Feline Viral Rhinotracheitis
GIT	Guanidinium IsoThyosyanate
GIT-HCl	Guanidinium IsoThyosyanate-HydroChloride
GPIC	Guinea Pig Inclusion Conjunctivitis
GTP	Guanosine TriPhosphate
Hc1	Histone 1-like protein
HRP	Horse Radish Peroxidase
hsp	heat shock protein
IB	Intermediate Body
IcM	Inclusion Membrane
IFU	Infection Forming Unit
Ig	Immunoglobulin
Inc	Inclusion membrane protein
LGV	Lymphogranuloma Venereum
LPS	LipoPolySaccharide

MAb	Monoclonal Antibody
MFA	Mean Fluorescent Area
MHC	Major Histocompatibility Complex
MIF	Micro ImmunoFluorescence
MOI	Multiplicity Of Infection
MOMP	Major Outer Membrane Protein
MTOC	Microtubule Organizing Centre
NASPHV	National Association of State Public Health Veterinarians
OEA	Ovine Enzootic Abortion
ompA	outer membrane protein A gene (encoding MOMP)
OR	Odds Ratio
PAb	Polyclonal Antibody
PBS	Phosphate Buffered Saline
PBV	Post Booster Vaccination
PC	Post Challenge
(q)PCR	(quantitative or real-time) Polymerase Chain Reaction
PCR/EIA	Polymerase Chain Reaction/ Enzyme Immuno Assay
PI	Post Inoculation
PPV	Post Primary Vaccination
RB	Reticulate Body
RFLP	Restriction Fragment Length Polymorphism
RT	Room Temperature
SDS(-PAGE)	Sodium Dodecyl Sulphate(-Poly Acrylamide Gel Electrophoresis)
S.D.	Standard Deviation
SI	Stimulation Index
SPF	Specific Pathogen Free
SPG	Sucrose Phosphate Glutamate
TCA	TriCarboxylic Acid (=Krebs cycle)
TCID50	50% Tissue Culture Infective Dose
TNF	Tumor Necrosis Factor
TTS(S)	Type Three Secretion (System)
USDA	United Stated Department of Agriculture
WIV	Wetenschappelijk Instituut Volksgezondheid

Study objectives

Chlamydophila psittaci (formerly *Chlamydia psittaci*) is found in birds and can be transmitted to humans by inhalation or direct contact causing psittacosis or parrot fever. The disease name dates back to 1893 when transmission of *Cp. psittaci* from parrots (Latin; *psittacus* or parrot) to humans, causing flu-like symptoms, was observed in Paris (Morange, 1895). In the mean time *Cp. psittaci* infections have been found in at least 475 bird species (Kaleta and Taday, 2003; Chahota et al., 2006).

Chapter I gives and overview on the history, taxonomy, and infection biology of chlamydial infections. Moreover, different aspects of avian chlamydiosis such as epidemiology, clinical disease, diagnosis, treatment, public health significance and prevention and control measurements are reviewed.

Cp. psittaci includes 9 known outer membrane gene A (*ompA*) genotypes designated A to F, E/B, M56, and WC (Everett et al., 1999a; Geens et al., 2005a). The 7 first genotypes are known to naturally infect birds. These genotypes are distinct from those associated with chlamydiosis in mammals. Some avian genotypes appear to occur more often in a specific order of birds. Genotype A for instance, is endemic among psittacine birds. Genotype B is endemic in pigeons. Water fowl most frequently seem to be infected with genotype C strains while genotype D strains are often associated with turkeys. However, genotype E, also known as Cal-10, MP, or MN was first isolated during an outbreak of pneumonia in humans during the early 1930s. Later on, genotype E isolates were obtained from a variety of bird species including ducks, pigeons, ostriches, and rheas. Genotype F is represented by the psittacine isolates VS225, Prk Daruma, 84/2334 and 10433-MA, but has also been isolated on a Belgian turkey farm (Van Loock et al., 2005a). The mammalian M56 and WC genotypes were isolated during an outbreak in muskrats and hares and during an outbreak of enteritis in cattle, respectively. All genotypes should be considered to be readily transmissible to humans. In the present study, we focused on the avian *Cp. psittaci* genotypes A to F and E/B causing psittacosis in man. Psittacosis is a notifiable disease in most European countries, the U.S, Australia and Japan (Smith et al., 1973; Williams et al., 1998; Longbottom and Coulter, 2003; Telfer et al., 2005; Matsui et al., 2007)

Over the years, human psittacosis has occurred worldwide and reported cases were mostly related to contact with psittacine birds or poultry, especially turkeys and ducks (reviewed in Vanrompay et al., 1995a; Andersen, 2004). In Belgium, only three psittacosis

cases were reported to the Wetenschappelijk Instituut Volksgezondheid (WIV) last year (http://www.iph.fgov.be/). Reported cases probably represent only the tip of the iceberg as diagnosis of this obligate intracellular, biosafety level (BSL) 3, Gram-negative organism has been extremely difficult due to the lack of sensitive and specific diagnostic tests and the believe that psittacosis was only a rare disease. The latter, was probably due to the fact that only severe infections, involving pneumonia with or without cardiac and/or neurological complications are noticed by medical doctors. Less severe infections, characterized by flu-like symptoms and asymptomatic infections are probably unnoticed.

At present, *Cp. psittaci* diagnostic research has made enormous progress with the development of highly specific and sensitive molecular diagnostic techniques such as a *Cp. psittaci*- specific nested PCR enzyme immunoassay (PCR/EIA), (Van Loock et al., 2005b), a *Cp. psittaci*- specific real-time PCR (Geens et al., 2005b) as well as an *ompA*-based genotype-specific real-time PCR and a recombinant antibody ELISA (Verminnen et al., 2006) allowing both detection and tracing of the pathogen. So far, these techniques have only been used to detect *Cp. psittaci* infections in birds. The first objective of the present study was the implementation of these diagnostic techniques in *Cp. psittaci* molecular epidemiology in man and to get insight in the real prevalence of *Cp. psittaci* infections in Belgium. In Chapter II, we therefore studied the prevalence of psittacosis in a large representative and healthy Flemish population (945 men or women; 35-55 year) using a nested PCR/EIA and a recombinant antibody ELISA. Pharyngeal swabs and serum were taken and supplementary questions concerning contact frequency with different bird species, as well as the presence of respiratory signs were included in the anamnesis. Most human *Cp. psittaci* infections could be traced back to psittacine birds and infections were clearly related to contact frequency. Psittacine birds are popularly kept as pet and considerable numbers of people are involved in breeding and selling these birds.

In chapter III, we studied the prevalence of *Cp. psittaci ompA* genotypes in a human risk population, which we defined in Chapter II, namely psittacine pet bird breeders. Nested PCR/EIA as well as an *ompA* genotype-specific real-time PCR were used to detect and genotype *Cp. psittaci* in samples of both birds and their owners. In chapter III, we additionally document three psittacosis cases related to contact with African Grey parrots (Psittacus erithacus), hereby demonstrating the occupational risk for veterinarians.

Thus, *Cp. psittaci* infected psittacine birds are a major source of infection for humans. Any attempts to prevent chlamydial infections in *Psittacidae* would diminish the risk for human health. Therefore, the third objective of the study was to develop a *Cp. psittaci*

DNA vaccine for psittacine birds as former research has demonstrated the ability of plasmid DNA to significantly reduce clinical signs, macroscopic lesions and bacterial excretion in experimentally infected turkeys (Vanrompay et al., 1999b; Vanrompay et al., 2001a; Van Loock et al., 2004; Verminnen et al., 2005).

In Chapter IV, we therefore evaluate the ability of plasmid DNA (pcDNA1) encoding the major outer membrane protein (MOMP) of a *Cp. psittaci* genotype A strain, the most prevalent genotype among psittacine birds, to produce protective immunity in budgerigars *(Melopsittacus undulatus)*, experimentally infected with the homologous *Cp. psittaci* genotype A strain.

Chapter I

Chlamydophila psittaci infections in birds and its zoonotic consequences

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This chapter is adapted from:

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1. History

In 1879, psittacosis or parrot fever was documented for the first time when Ritter (1880) described an epidemic of unusual pneumonia in seven Swiss humans associated with exposure to tropical pet birds. The disease name "psittacosis" however, dates back to 1893, when transmission of an infectious agent from parrots to humans, causing flu-like symptoms, was observed (Morange, 1895).

In 1907, Halberstaedter and von Prowazek were the first to make drawings of *Chlamydia*-infected conjunctival cells of trachoma (*C. trachomatis*) patients, holding the observed vacuoles for mantled protozoa and conclusively naming them *Chlamydozoa* (after the Greek word for mantle: *chlamys*) (Halberstaedter and von Prowazek, 1907). The following years, similar infective agents causing inclusion conjunctivitis of the newborn or infection of the adult genital tract were described. The inability to grow these, in fact *C. trachomatis* organisms, on artificial media made scientists assume that they were viruses. The relationship with the psittacosis agent detected by Ritter (1880) was not yet made.

During winter of 1929-1930, a pandemic of human psittacosis occurred in the United States and Europe. The disease was attributed to the importation of Green Amazon parrots from Argentina. Shortly after, 174 cases of human psittacosis were reported from the Faroe Islands in the period from 1930 to 1938. The human dead rate was 20% and was especially high (80%) in pregnant women (Rasmussen-Ejde, 1938). Humans contracted the infection while capturing juvenile fulmars (*Fulmarus glacialis*) and preparing them for cooking. Herrmann et al., (2006), presented phylogenetic data on the outer membrane protein A (*ompA)* gene supporting the speculation that fulmars in the Faroes acquired psittacosis from infected and dead parrots thrown overboard during shipment from Argentina to Europe in 1930.

The psittacosis agent was finally identified in humans by (Bedson et al., 1930) and was classified together with the simultaneously identified, causative agent of lymphogranuloma venereum (LGV), as viruses of the psittacosis-LGV group. Bedson et al., (1930) defined the unique biphasic developmental cycle of 'these viruses' and the following years, the organisms became referred to as *Bedsoniae*. Hereafter, Thygeson described the resemblance between the developmental cycle of the trachoma and psittacosis agent, and suggested that lymphogranuloma venereum (LGV) strains and mouse pneumonitis strain also belonged to the same unique group of viruses (Thygeson,

1963). The next years, the "viruses" were propaged in chorio-allantoic chick embryo membranes, monkey brains and mice. In 1965, with the development of electron microscopy, scientists could actually see that the chlamydial agent was not a virus, but a bacterium. Page (1966) proposed to gather all organisms of the psittacosis-LGV-trachoma group in one genus *Chlamydia.* For some time *Chlamydia* were then considered among the *Rickettsiae*, but later on they have been differentiated from *Rickettsiae* because they lack an electron transport system, have no cytochromes and apparently could not synthesise ATP and GTP in the same way as *Rickettsiae*. Finally, *Chlamydiae* were classified as Gram-negative intracellular bacteria because they divided by binary fission, had cell walls comparable in structure to those of Gram-negative bacteria, possessed DNA containing nucleoids without membranes between the nucleoid and the cytoplasm and had ribosomes with antibiotic susceptibilities characteristic for prokaryotic ribosomes. Shortly afterwards, the genus *Chlamydia* was placed in the order *Chlamydiales*, family *Chlamydiaceae*. In the late sixties, two species were identified within the genus *Chlamydia*: *Chlamydia trachomatis* (Capponi and Haider, 1969) and *Chlamydia psittaci* (Tamura et al., 1971), to which the species *Chlamydia pneumoniae* (Grayston et al., 1989) and *Chlamydia pecorum* (Fukushi and Hirai, 1992) were added in the nineties.

2. Taxonomy

2.1. A controversial new taxonomy

Until 1999, taxonomic classification of *Chlamydia* was based on limited phenotypic, morphologic and genetic criteria. This old classification did not take into account recent analysis of the ribosomal operon or recently identified obligate intracellular organisms with a *Chlamydia*-like developmental cycle or replication. Neither did it provide a systematic rational for identifying new strains. In 1999, Everett et al. (1999a) presented data on phylogenetic analyses of the 16S and 23S rRNA genes with corroborating genetic and phenotypic information, showing that the order *Chlamydiales* contained at least four distinct groups at the family level, and that two distinct lineages, *Chlamydia* and *Chlamydophila*, branch into nine separate clusters within the *Chlamydiaceae* family (Fig 1.1).

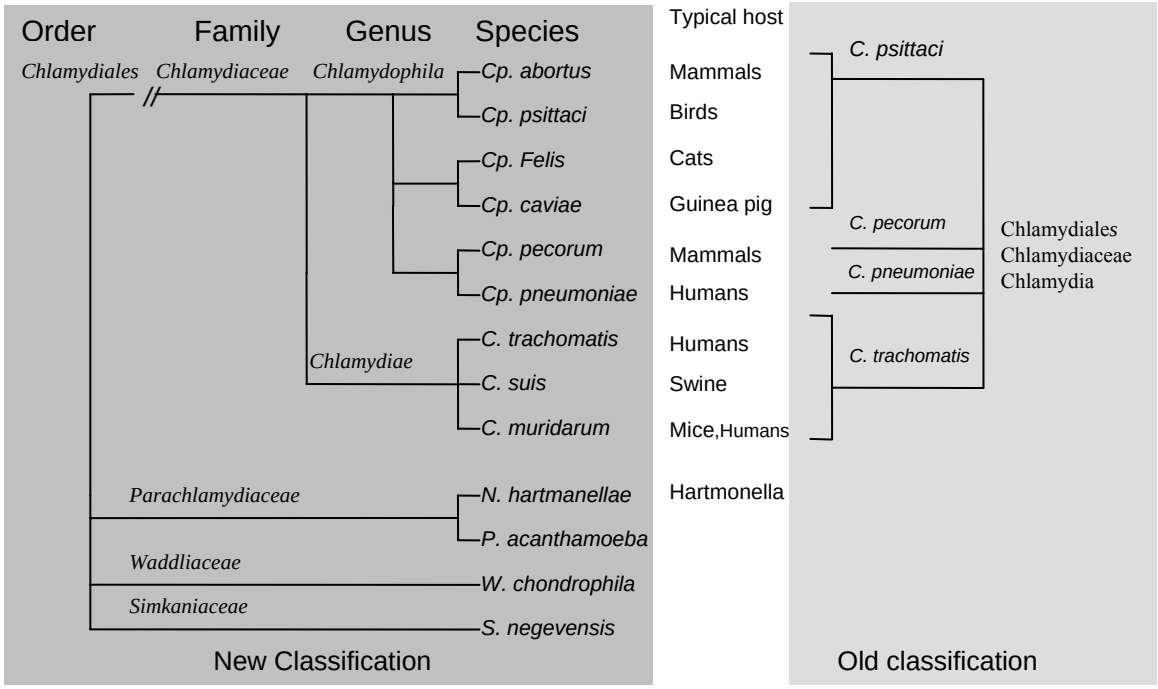

Fig. 1.1. The new taxonomic classification introduced in 1999 versus the old one. Three new families were added to the order *Chlamydiales* and the new genus *Chlamydophila* was introduced in the family *Chlamydiaceae*. The new genus comprises the old *Cp. psittaci* strains specifically infecting birds, as well as three new species, each having a specific mammalian host (Adapted from Bush and Everett, 2001)

There has been a lot of controversy concerning this taxonomy (Schachter et al., 2001) and the debate is still ongoing. The main criticisms were that there was (i) insufficient reason to divide the *Chlamydiaceae* into 2 new genera and that this was counterintuitive, (ii) it had taken a long time to get clinicians used to *Chlamydia* and that it would confuse them, (iii) there is too much emphasis on limited nucleotide sequence data and insufficient consideration of basic biological characteristics, and (iv) the evidence for some species and families, notably the *Waddliaceae* and the *Simkaniaceae*, rests on limited data derived from a single isolate. Some objected to the genus name *Chlamydophila,* which literally means "like *Chlamydia*". Some groups keep using the old classification, especially in human medicine (Longbottom and Coulter, 2003), while others use the new species names within the *Chlamydiaceae* but proceed with the old genus name *Chlamydia* for all nine species (Subtil et al., 2004). A third group chooses to use the new taxonomy (http://www.chlamydiae.com), which in the meantime has been adopted in the second edition of Bergey's Manual of Systematic Bacteriology, then considering the chlamydiae

as a novel (eu)bacterial phylum: *Chlamydiae* phylum B-XVI (Garrity and Holt, 2001). Throughout the present study, the official *Chlamydophila* genus designation will be used, and in abbreviations, *Cp.* will be used for the reader's comfort. When members of the *Chlamydiales* order are referred to, the widely trivial name "chlamydiae" will be used.

2.2. Nine chlamydial species and their social and/or economical importance

Chlamydia trachomatis comprises two biovars (trachoma and LGV) and multiple serovars (A to K, L1, L2 and L3). Serotyping is based on the presence of serovar-specific epitopes on the major outer membrane protein (MOMP). Serovars A, B and C cause trachoma, worldwide the major cause of preventable infectious blindness. Serovars D–K are sexually transmitted and cause both ocular and genital infections (urethritis, acute epidymitis and proctitis in men; cervicitis, salpingitis, tubal infertility, ectopic pregnancy and pelvic inflammatory disease in women). Other serovars (L1-L3) are responsible for the development of lymphogranuloma venereum (LGV). About 17% of the neonates of *C. trachomatis*-infected mothers develop conjunctivitis and 20% develop pneumonia. *C. trachomatis* may also trigger reactive arthritis. Some of the strains have an extrachromosomal plasmid and most strains are sensitive to sulfadiazine and tetracyclines. *C. trachomatis* infections have an enormous impact on human health. *C. trachomatis* is the most common cause of sexually transmitted infections throughout the world. Six hundred million people are estimated to be infected (Gerbase et al., 1998). Additionally, there are between five and six million cases of infectious blindness in man due to a chlamydial infection (Thylefors et al., 1995). Genital infections are asymptomatic in most females (>75%) and can result in scarring and fibrosis of urogenital tissues, resulting in chronic pelvic pain, ectopic pregnancy, and pelvic inflammatory disease, which can lead to infertility. Acute *C. trachomatis* infections can be treated effectively with antibiotics, although once infection and pathology are established, treatment may not prevent complications. Therefore, treating only symptomatic patients will never control the spread of infection. Asymptomatic individuals can be identified through screening programs, but this approach is likely to be too costly for developing countries. A vaccination program would be much cheaper and have a greater impact on controlling *C. trachomatis* infections worldwide. The reference strain for the trachoma biovar is C/PK-2 while the reference strains for the LGV biovar is L2/434/BU (ATCC VR-902B).

Chlamydia suis has only been isolated from swine, where it causes conjunctivitis, enteritis, pneumonia, pericarditis, perinatal mortality and reproductive disorders, although the infection can also be asymptomatic (Rogers and Andersen, 1996; 1999; 2000). Limited epidemiological evidence suggests that *C. suis* infections in pigs are both common and wide spread, but under-diagnosed, although there appears to be a high prevalence of mixed infections with *Cp. abortus* (Hoelzle et al., 2000), making it difficult to attribute clinical pathology to *C. suis* alone. The 16S rRNA genes that have been sequenced to date differ by < 1.1%. The *ompA* sequence diversity is larger than for other chlamydial species. As for *C. trachomatis*, glycogen has been detected in *C. suis* inclusions. *Chlamydia suis* strains carry a plasmid called pCS and some of the strains are resistant to sulfadiazine and tetracyclines. The type strain for *C. suis* is S45 (ATCC VR-1474).

Chlamydia muridarum strains MoPn and SFPD have been isolated from mice and hamsters, respectively. MoPn can cause pneumonia in mice, whereas SFPD was obtained from a hamster, concurrent with a causative agent of proliferative ileitis. The 16S rRNA genes that have been sequenced to date differ by 0.13% and their 16S-23S intergenic spacers and 23S domain I segments are identical. Analysis of the *ompA* gene can also identify this species. The species is sulfadiazine sensitive and glycogen production is difficult to detect in MoPn. The type strain for *C. muridarum* MoPn (ATCC VR-123) carries a pMoPn plasmid.

Chlamydophila pneumoniae has been isolated from humans worldwide. In addition, a number of isolates obtained from koalas and a horse have genetic and antigenic characteristics very similar to the human isolates. *Cp. pneumoniae* has three known biovars, TWAR, Koala and Equine and the coherence of this taxon has been demonstrated by DNA sequence analysis of rRNA, *ompA*, *kdtA* (KDO-transferase) and the 60 kDa cysteine-rich gene. The TWAR biovar contains strains that are primarily pathogens of the human respiratory tract where they predominantly cause acute or chronic bronchitis and pneumonia and in some cases otitis media, obstructive pulmonary disease and pulmonary exacerbation of cystic fibrosis. The TWAR biovar is responsible for one tenth of the pneumonia cases in industrialized countries (Grayston, 1999). The name TWAR was formed by merging the first two letters of isolates TW-183 and AR-39 (Grayston et al., 1989). TWAR has also been associated with Alzheimer's, atherosclerosis, asthma, erythema nodosum, reactive airway disease, Reiter's syndrome and sarcoidosis (Kuo et al., 1995; Jackson et al., 1997; Ellis, 1997; Braun et al., 1997; Balin et al., 1998). TWAR strains are genetically almost indistinguishable. The Koala biovar has only been found in

the marsupial *Phascolarctos cinereus* and it was present in all five Australian free-ranging koala populations that have been tested, including a colony that was suffering from respiratory disease. Isolates are commonly obtained from ocular and urogenital sites, where they do not appear to be highly pathogenic. The reference strain for the Koala biovar is LPCon. The Equine biovar currently includes only one strain (N16), isolated from the respiratory tract of a horse (Wills et al., 1990). Inoculation of horses with N16 leads to asymptomatic infections. The equine strain was the only *Cp. pneumoniae* strain carrying a plasmid (pCpnE1). The type strain for *Cp. pneumoniae* is TW-183 (ATCC VR-2282).

Chlamydophila pecorum strains characterized so far have been limited to mammals, but not to a specific host family. They are serologically and pathologically diverse. The organism has been isolated from ruminants, a marsupial and swine. In koalas, *Cp. pecorum* causes reproductive disease, infertility and urinary tract disease. It has been a major treat to the Australian koala population. In other animals *Cp. pecorum* causes abortion, conjunctivitis, encephalomyelitis, enteritis, pneumonia and polyarthritis (Kaltenboeck et al., 1993; Salinas et al., 1996). The *Cp. pecorum* strains have a high degree of ribosomal sequence conservation. *Chlamydophila pecorum* 16S rRNA genes that have been sequenced differ by < 0.6%. Analysis of the domain I signature sequence in the 23S rRNA gene or of other genes like the *ompA* gene and the 16S rRNA gene sequence can help to identify this species. Two strains, E58 (ATCC VR-628) and Koala II, have been shown to have extrachromosomal plasmids.

Chlamydophila felis is endemic among house cats worldwide. It causes primarily conjunctivitis and rhinitis (Gaillard et al., 1984; TerWee et al., 1998). *Cp. felis* strains are characterized by a high degree of rRNA and *ompA* conservation and have a distinctive serotype (Andersen, 1991a; Sayada et al., 1994; Pudjiatmoko et al., 1997). Strain FP Pring and FP Cello have an extrachromosomal plasmid while the FP Baker strain does not. Zoonotic infection of humans has been reported (Schachter et al., 1969; Yan et al., 2000; Hartley et al., 2001). The type strain for *Cp. felis* is FP Baker (ATCC VR-120).

Chlamydophila caviae comprises five known isolates, all isolated from guinea pigs. The natural infection site is the mucosal epithelium of the conjunctiva where a non-invasive infection is established. However, it is possible to infect the genital tract of guinea pigs eliciting a disease that is very similar to human genital infection. *Cp. caviae* is highly specific for guinea pigs, as attempts to infect other animals failed, with the exception of one experiment infecting gerbils. Analysis of the domain I signature sequence in the 23S

rRNA gene or other genes sequences including *ompA* and 16S rRNA can assist in identifying this species (Everett and Andersen, 1999). The type strain for *Cp. caviae* is the guinea pig inclusion conjunctivitis (GPIC) strain (ATCC VR-813) containing the extrachromosomal plasmid pCpGP1.

Chlamydophila abortus strains are endemic among ruminants and efficiently colonize the placenta (Rodolakis and Souriau, 1989). They have a distinctive serotype and nearly 100% conservation of ribosomal and *ompA* sequences. Typical isolates include strains B577, EBA, OSP, S26/3 and A22 (Storz et al., 1960; Denamur et al., 1991; Andersen, 1991a). Extrachromosomal plasmids were never found in *Cp. abortus* strains. *Chlamydophila abortus* is the most common infectious cause of abortion in sheep, where the disease is known as ovine enzootic abortion (OEA) or enzootic abortion of ewes (EAE) in countries of Northern, Central and Western Europe. In the UK alone, the disease, which accounts for around 50% of all diagnosed abortions, is estimated to cost £20 (€30) million per annum to agricultural industry. Enzootic abortion in goats is similar in severity to that occurring in sheep, although the spread and economical impact across Europe is less clear because of the lack of epidemiological data. The disease can also affect cattle, swine and horses but this is thought to occur to a much lesser extent (Longbottom, 2004). Sporadic zoonotic abortion due to *Cp. abortus* has been confirmed by analysis of isolates from women who work with sheep (Herring et al., 1987; Jorgensen, 1997; Longbottom and Coulter, 2003) and goats (Pospischil et al., 2002).

Chlamydophila psittaci produces avian respiratory infections and is a serious threat to industrial poultry production. Additionally, it causes epizootic outbreaks in mammals and respiratory psittacosis in humans. The infection is often systemic and can be inapparent, severe, acute or chronic with intermittent shedding. *Cp. psittaci* was first classified in to serovars using monoclonal antibodies to the major outer membrane protein (MOMP) (Andersen, 1991b; Vanrompay et al., 1997). Seven of the nine known serovars (A to F and E/B) solely occur in birds. The other two -M56 and WC- were isolated from mammals. All serovars should be considered to be readily transmissible to humans. *Cp. psittaci* 16S rRNA genes differ by < 0.8%. Several strains have an extrachromosomal plasmid and many strains are susceptible to bacteriophage Chp1 (Storey et al., 1989a; 1989b). Analysis of the MOMP encoding outer membrane protein A *(ompA)* gene, can also identify this species. Recently, (Geens et al., 2005b) developed genotype-specific real-time PCR for typing avian *Cp. psittaci* strains. The type strain for *Cp. psittaci* is 6BC (ATCC VR-125).

3. Infection biology

3.1. Morphology

Morphologically distinct forms of *Chlamydophila* are termed elementary body (EB), reticulate body (RB), and intermediate body (IB). The EB is a small, electron dense, spherical body, about 0.2-0.3 μm in diameter (Costerton et al., 1976; Carter et al., 1991; Miyashita et al., 1993) (Fig 1.2). The EB is the infectious form of the organism, which attaches to the target cell and gains entry. The EBs are characterized by a highly electron-dense nucleoid, located at the periphery of the EB and clearly separated from an electron-dense cytoplasm. The chromatin is maintained condense by the histon H1-like protein, Hc1. Following entry into the host cell, the EB expands in size to form the RB (Fig 1.2), which is the intracellular metabolically active form. Differentiation of EBs into RBs is accompanied by chromatin dispersal as chlamydiae become transcriptionally active. The RB is larger, measuring approximately 0.5-2.0 μm in diameter. The inner and outer bacterial membranes of RBs are relatively closely opposed. The RB divides by binary fission and thereafter matures into new EBs. During this maturation, morphologically intermediate forms (IB), measuring about 0.3-1.0 μm in diameter, can be observed inside the host cell. The IB has a central electron-dense core with radially arranged individual nucleoid fibers surrounding the core. Cytoplasmic granules are tightly packed at the periphery of the IB and are separated from the core by a translucent zone. At the end of the replication cycle, less and highly condensed EBs can be observed (Litwin et al., 1961; Eb et al., 1976; Costerton et al., 1976; Louis et al., 1980; Soloff et al., 1982; Matsumoto, 1982a; Matsumoto, 1988; Vanrompay et al., 1996; Rockey and Matsumoto, 2000). Immature, less condensed EBs have few fibrous elements transmigrating the granular cytoplasm towards the condensed electron-dense nucleoid. The latter lies adjacent to the inner bacterial membrane. Mature, more condensed EBs posses a homogeneous ovoid, elongated or irregular electron-dense nucleoid separated from cytoplasmic elements by a very distinct electron-transparent space.

Electron microscopy and freeze-fracture structures clearly indicate the existence of curious surface projections on both EBs and RBs (Fig 1.2) (Matsumoto et al., 1976; Matsumoto, 1982b). These projections extend from the chlamydial surface into the inclusion membrane. Matsumoto et al., (1999) also observed images of arrayed-rosettes in negatively stained EB preparations. It is tempting to speculate that these dome-like and

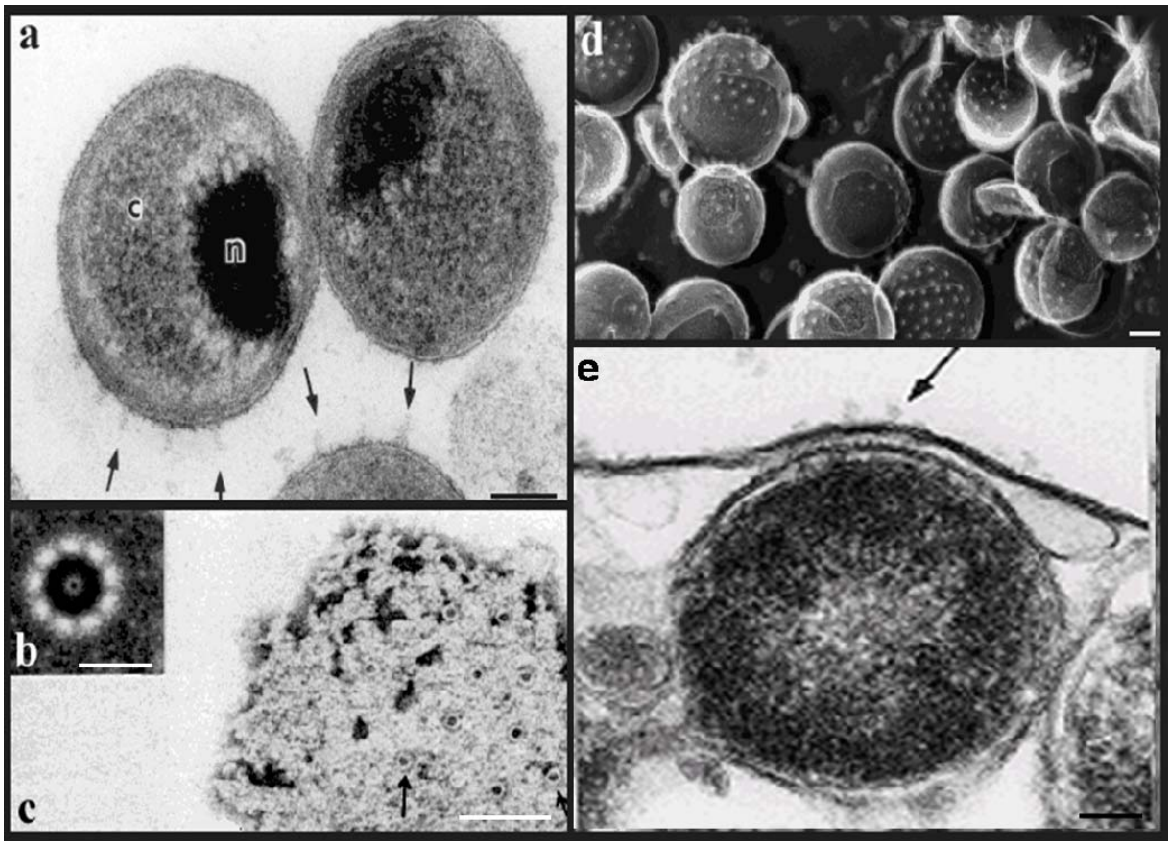

Fig. 1.2. Electron micrographs of *Cp. psittaci* and *C. trachomatis* EB and RB.

a) Transmission electron microscopic picture of an elementary body of *Cp. psittaci* Cal 10. The sample has been treated with ruthenium red to enhance the electron opacity of the surface projections (arrows). The most obvious feature is the eccentric, electron dense (black) DNA core (n), which is tightly compacted onto chlamydial histone protein. Histones are basic, DNA-binding proteins commonly found in higher plant or animal cells, but unusual in bacteria. Note that the EB cytoplasm (c) has a granular appearance due to the presence of 70S ribosomes. Surrounding the cytoplasm is a lipid cytoplasmic membrane and a rigid outer envelope (both ~8 nm) containing a regularly packed hexagonal structure of 16.7 nm. This structure, together with extensive -S-S- bridging of the sulphur amino-acid rich outer envelope proteins, probably accounts for much of the outer envelope strength. (bar 0.15 μm). Adapted from Matsumoto (1988).

b) Rotational symmetry of a *Cp. psittaci* Cal 10 EB. *Cp. psittaci* rosette made up of 9 subunits (bar 30 nm). Adapted from Matsumoto (1979).

c) Negatively stained envelope fragment of a *Cp. psittaci* Cal 10 EB. Note the dark centred rosettes (arrows). (bar 0.1 μm). Adapted from Matsumoto (1979).

d) Deep freeze-etching replica of *C. trachomatis* D-12N EBs within a McCoy cell inclusion at 44 hours PI. Note the projections radiating from the surface of each EB. (bar 0.1 μm). Adapted from Matsumoto (http://www.chlamydiae.com/docs/biology_biol_EB.asp).

e) Transmission electron microscopic picture of a reticulate body of *Cp. psittaci* Cal 10 showing the connection between reticulate bodies and the inclusion membrane of isolated inclusions (bar 0.1μm). Adapted from Matsumoto (1988).

needle-like surface projections correspond to Type III Secretion pores used to deliver virulence proteins into the host cell (Bavoil and Hsia, 1998).

3.2. Developmental cycle

Cp. psittaci is an obligate intracellular bacterium replicating within a non-acidified vacuole, termed an inclusion. Within the inclusion, *Cp. psittaci* undergoes a unique biphasic developmental cycle alternating between the elementary body or EB, which guarantees extracellular survival and infection of host cells and the reticulate body or RB, which is responsible for intracellular replication and generation of infectious progenitor bacteria.

Infection is initiated by the attachment and subsequent parasite-mediated endocytosis of the infectious EB. EBs are sometimes seen in association with clathrin-coated pits. *In vitro*, EBs rely on the actin cytoskeleton to facilitate their entry into host cells. Once internalized, EBs are redistributed from the periphery of the cell and aggregate at the Golgi region of the cell that corresponds to the Microtubule Organizing Centre (MTOC). Internalized EBs occupy small vesicles or inclusions, which avoid fusion with lysosomes and fusion with any other endocytic organelles. Thus, the nascent inclusion is derived from host plasma membrane proteins and lipids. Within the first several hours after internalization, EBs begin to differentiate into RBs, which is characterized by a loss in infectivity, nucleoid decondensation and enlargement of the organism. By 8 hours post infection, RBs start to replicate by binary fission and RBs are in close association with the inclusion membrane (Fig. 1.3). Numerous mitochondria surround the inclusion (Vanrompay et al., 1996). Host kinesin possibly plays a role in mitochondrial recruitment to the inclusion. *Cp. psittaci* may not be as capable as other chlamydial species at generating its own ATP, and therefore, a close association of the inclusion with mitochondria might provide an alternative mechanism to obtain ATP from the host.

Unlike bacteria that replicate in the host cell cytosol and have free access to cytosolic nutrients, *Cp. psittaci* must import host nutrients across the inclusion membrane. Chlamydial organisms indeed require amino acids and nucleotides from host pools, but the mechanisms that facilitate delivery of these molecules into the inclusion have not been clearly defined. Because the inclusion is disconnected from the endosomal/lysosomal pathway, delivery of nutrients by fusion of endocytic vesicles is unlikely. Furthermore, although exocytic vesicles containing sphingomyelin are known to fuse with the inclusion,

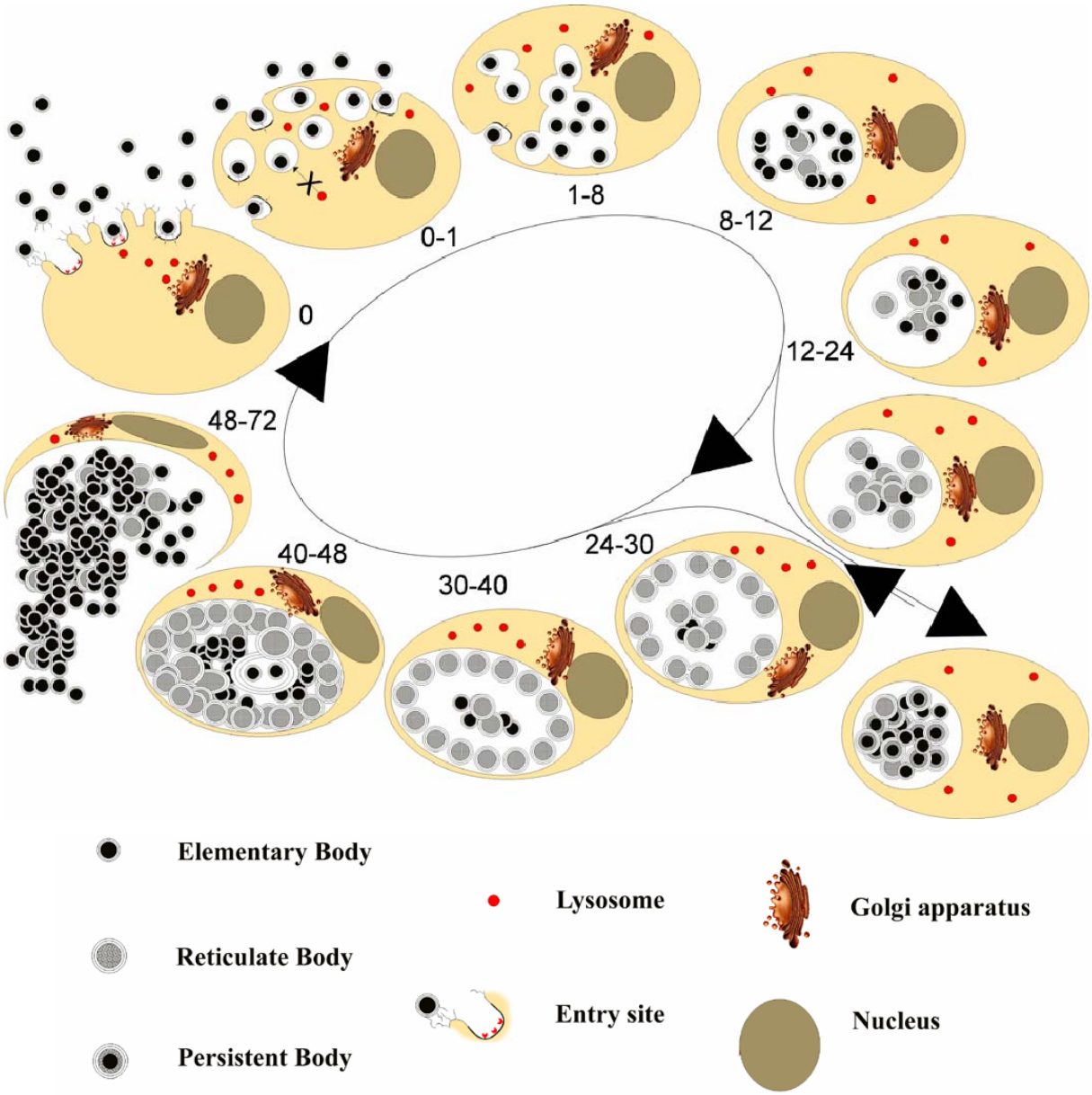

Fig. 1.3. Chlamydial development cycle. Numbers refer to hours post infection (Geens, 2005)

and may be a source of nutrients and lipids, the luminal contents of these vesicles have not been identified.Recent data demonstrate that the inclusion membrane is freely permeable to small molecules with a molecular weight of 100-520 Da (Hackstadt et al., 1999; Grieshaber et al., 2002). After delivery into the lumen by passive diffusion, specific membrane transporters located in the bacterial membrane would facilitate uptake into the

organisms themselves. In addition, specific membrane transporters would still be needed to transport molecules larger than 520 Da across the inclusion membrane. However, no such membrane transporters have been localized to the inclusion membrane as of yet.

As the developmental cycle progresses, the inclusion enlarges to accommodate the increasing numbers of bacteria. The growing inclusion intercepts biosynthetic membrane trafficking pathways and acquires the ability to fuse with a subset of Golgi-derived vesicles, thereby increasing its membrane surface (Hackstadt, 1999). When more and more RBs are actively replicating the next 20 hours (each RB can be responsible of a progeny of up to 1000 new RBs), the inner side of the inclusion membrane becomes overcrowded, leading to RB 'detachment' from the inclusion membrane. Detachment of RBs could be the signal to convert into intermediate bodies and new infectious EBs. At the end of the cycle, approximately 50 hours post infection, the host cell and its inclusion(s) either lyse or the EBs and some non-differentiated RBs are liberated via reverse endocytosis.

In some cases, the developmental cycle can be altered towards persistence. Persistent chlamydiae fail to complete development from RBs into infectious EBs but retain metabolic activity. Persistent RBs are oval shaped to strongly enlarged, morphologically aberrant and form small inclusions. They accumulate chromosomes because DNA replication continues, but do not divide. Persistent growth forms have been associated with chronic infections. These cryptic persistent forms can rapidly retransform to normal RBs and infectious EBs. How chlamydiae modulate between normal and persistent growth is not yet understood (Belland et al., 2003; Hogan et al., 2004; Gieffers et al., 2004).

4. Avian chlamydiosis

4.1. Strain Classification

The family *Chlamydiaceae* was recently reclassified into two genera and nine species. The new genera *Chlamydia* and *Chlamydophila* correlate with the former species *Chlamydia trachomatis* and *Chlamydia psittaci.* The genus *Chlamydia* includes *C. trachomatis* (human), *C. suis* (swine), and *C. muridarum* (mouse, hamster). The genus *Chlamydophila* includes *Cp. psittaci* (avian), *Cp. felis* (cats), *Cp. abortus* (sheep, goats, cattle), *Cp. caviae* (guinea pigs), and the former species *Cp. pneumoniae* (human) and *Cp. pecorum* (ruminants). All known avian strains belong to the species *Chlamydophila psittaci*, which includes six avian serovars (serovars A through F), and two mammalian

isolates (WC and M56) (Andersen, 1991b; Vanrompay et al., 1993; Vanrompay et al., 1997). WC and M56 are isolates from epizootics in cattle and muskrats, respectively. The avian serovars are relatively host-specific. Serovars A and B are usually associated with psittacine birds and pigeons, respectively. The natural hosts of the other serovars are more uncertain. Serovar C has primarily been isolated from ducks and geese, and serovar D mainly from turkeys. Serovar F was isolate from a psittacine bird and from turkeys. The host range of serovar E is the most diverse of the strains: it is isolated from about 20% of pigeons, from many cases of fatal chlamydiosis in ratites, from outbreaks in ducks and turkeys, and occasionally from humans. All serovars should be considered to be readily transmissible to humans.

Cp. psittaci 16S rRNA genes differ by < 0.8%. Several strains have an extrachromosomal plasmid and many strains are susceptible to bacteriophage Chp1 (Storey et al., 1989a; 1989b). At present, analysis of the MOMP encoding outer membrane protein A *(ompA)* gene is more often used to characterize avian *Cp. psittaci* strains in to genotypes A to F and E/B (Geens et al., 2005b). So far, genotype E/B has mainly been isolated from ducks.

4.2. *Epidemiology*

Psittacosis outbreaks of 1929-1930 and 1930-1938 were attributed to psittacine birds. However, in the next years it became clear that *Cp. psittaci* infections were not limited to psittacine birds, but could also affect other bird species. In 1939, the bacterium was isolated from two South African racing pigeons and soon, additional pigeon isolates were obtained, this time from Californian racing pigeons. At that time, Psittacosis in two New York citizens could be attributed to contact with infected feral pigeons. In 1942, serological evidence showed ducks and turkeys to be frequently infected and within the next 3 years, human infections due to contact with infected ducks were reported in California and New York. In the early 1950s, *Cp. psittaci* could be isolated from turkeys and from humans in contact with infected turkeys, during severe respiratory outbreaks in the US turkey industry (Meyer, 1967). The incidence of severe *Cp. psittaci* epidemics in US poultry declined during the 1960s. However, avian chlamydiosis remained a continuing threat to both birds and humans. During the 1980s, chlamydiosis was reported again in US turkeys (Grimes and Wyrick, 1991) and in the 1990s also in the European turkeys (Vanrompay et al., 1993a; Ryll et al., 1994; Hafez and Sting, 1997). Nowadays,

Cp. psittaci is nearly endemic in the Belgian turkey industry (Van Loock et al., 2005a; Verminnen et al., 2006), and there is evidence to believe that it is also endemic in other European countries (Van Loock et al., 2005a; Sting et al., 2006). However, devastating, explosive outbreaks like in 'ancient days' are rare and respiratory signs without or with low mortality characterize outbreaks nowadays. More recently, an increase in the number of chlamydiosis outbreaks in ducks and in people in contact with infected ducks has been reported (Laroucau, 2007, personal communication; Laroucau et al., 2008; Andersen and Vanrompay, 2008). In the past, human infections associated with outbreaks in ducks have already been described (Arzey and Arzey, 1990; Arzey et al., 1990; Martinov and Popov, 1992; Hinton et al., 1993; Goupil et al., 1998; Lederer and Muller, 1999; Bennedsen and Filskov, 2000).

The list of avian species in which *Cp. psittaci* infections occur increased rapidly. Today, *Cp. psittaci* has been demonstrated in about 465 bird species comprising 30 different bird orders (Kaleta and Taday, 2003). The highest infection rates are found in Psittacine birds (*Psittacidae*) and pigeons (*Columbiformes*). The prevalence in psittacine birds ranges between 16 and 81% and a mortality rate of 50% or even higher is not unusual (Raso et al., 2002; Dovc et al., 2005)l. *Psittacidae* are predominant chlamydial reservoirs, especially under captive conditions, and nowadays these birds are extremely popular pets (Chahota et al., 2006; Vanrompay et al., 2007).

Data on the seropositivity of racing pigeons range from 35.9 to 60%. Thirty-eight studies on the seroprevalence of *Cp. psittaci* in feral pigeons conducted from 1966 to 2005, revealed a seropositivity ranging from 12.5 to 95.6% (Laroucau et al., 2005; Tanaka et al., 2005; Haag-Wackernagel, 2005; Mitevski et al., 2005; Prukner-Radovcic et al., 2005). More recent studies, performed in feral pigeons in Italy, Bosnia and Herzegovina, and Macedonia revealed a seropositivity of 48.5%, 26.5% and 19.2%, respectively (Ilieski et al., 2006; Residbegovic et al., 2007; Ceglie et al., 2007). Free-living pigeons are distributed in urban and rural area over the world and are in close contact with humans in most outdoor public places.

Birds mostly living on sea shores and other waters, like gees, ducks, gulls and penguins are more frequently infected than hens, pheasants and quails (Kaleta and Taday, 2003). Common reservoirs of *Cp. psittaci* include wild and feral birds such as sea gulls, ducks, herons, egrets, pigeons, blackbirds, grackles, house sparrows, and killdeer, all of which freely intermingle with domestic birds. Highly virulent strains of *Cp. psittaci* can be

carried by and excreted in large numbers by sea gulls and egrets without any apparent effect on these hosts.

4.3. Transmission between birds

Cp. psittaci transmission primarily occurs from one infected bird to another susceptible bird in close proximity. *Cp. psittaci* is excreted in the faeces and nasal discharges. Faecal shedding occurs intermittently and can be activated by stress caused by nutritional deficiencies, prolonged transport, overcrowding, chilling, breeding, egglaying, treatment or handling. Bacterial excretion periods during natural infection vary according to strain virulence, infection dose and host immune status. However, shedding may occur for several months. Transmission of chlamydiae is mainly by inhalation of contaminated material and sometimes by ingestion. Large numbers of *Cp. psittaci* can be found in respiratory tract exudate and fecal material of infected birds. The importance of the respiratory exudate has become more apparent. In turkeys, the lateral nasal glands become infected early and remains infected for more than 60 days. Choanal/oropharyngeal swabs are more consistent for isolation of the agent than faecal swabs, especially during early stages of infection. Direct aerosol transmission through aerosolization of respiratory exudate must be considered as the primary method of transmission.

Avian species, including domestic poultry sharing aquatic or moist soil habitats with wild infected aquatic birds may become infection via contaminated water. Granivorous birds like pigeons, doves, pheasants and house sparrows may become infected by dust inhalation in faecal contaminated barnyards and grain storage sites. The consumption of infected carcasses may transmit *Cp. psittaci* to host species that are predators or scavengers of other birds.

Transmission of *Cp. psittaci* in the nest is possible. In many species, such as *Columbiformes*, cormorants, egrets, and herons, transmission from parent to young may occur through feeding, by regurgitation, while contamination of the nesting site with infective exudates or faeces may be important in other species such as snow geese, gulls and shorebirds. *Cp. psittaci* can be transmitted from bird to bird by blood-sucking ectoparasites such as lice, mites and flies or, less commonly, through bites or wounds (Longbottom and Coulter, 2003). Transmission of *Cp. psittaci* by arthropod vectors would be facilitated in the nest environment.

Vertical transmission has been demonstrated in turkeys, chickens, ducks, parakeets, seagulls and snow geese, although the occurrence appears to be fairly low (Wittenbrink et al., 1993). However, it could serve as a method of introducing chlamydia into a poultry flock.

Cp. psittaci can be introduced into susceptible pet birds and poultry through the wild bird population. Contaminated feed or equipment can also be a source of infection, and feed should therefore be protected from wild birds. Careful cleaning of equipment is extremely important as *Cp. psittaci* can survive in faeces and bedding for up to thirty days. Cleaning and disinfection with most detergents and disinfectants will inactivate *Cp. psittaci*, as this Gram-negative bacterium has a high lipid content.

4.4. Clinical disease

Depending on the chlamydial strain and the avian host, chlamydiae cause pericarditis, air sacculitis, pneumonia, lateral nasal adenitis, peritonitis, hepatitis, and splenitis. Generalized infections result in fever, anorexia, lethargy, diarrhea, and occasionally shock and death. Chlamydiosis is a very common chronic infection of psittacine birds. Infections cause conjunctivitis, enteritis, air sacculitis, pneumonitis, and hepatosplenomegaly. Droppings are often green to yellow-green. Many of the birds become chronically infected but show no clinical signs until stressed. These birds often shed chlamydiae intermittently and serve as a source of infection for humans and other birds.

Chlamydiosis is also a common chronic infection of pigeons. Clinical signs include conjunctivitis, blepharitis, and rhinitis (Andersen, 1996). Survivors can become asymptomatic carriers

Infected turkeys show vasculitis, pericarditis, pneumonitis, air sacculitis, and hepatosplenomegaly, and lateral nasal adenitis at necropsy. Mortality rates of 5–40% may occur unless early antibiotic treatment is instituted (Grimes et al., 1970; Andersen, 1996). Experimentally, virulent turkey strains cause little, if any, disease in chickens, pigeons, and sparrows; however, cockatiels and parakeets succumb rapidly to infection by these agents. Turkey outbreaks with low mortality and little or no human involvement usually are caused by the ”pigeon serovar”. The mortality rate is usually less than 5%.

Chlamydiosis in ducks is rare in the United States (Andersen 1996), but is a serious economic and occupational health problem in Europe (Andersen, 1996). Trembling,

conjunctivitis, rhinitis, and diarrhea are observed in infected ducks. Mortality ranges up to 30%.

4.5. Diagnosis

The methods used to diagnose *Cp. psittaci* infections are 1) direct visualization of the agent in clinical specimens by staining techniques, 2) isolation of the agent from clinical specimens followed by identification of the isolated agent, 3) detection of specific chlamydial antigens or genes in clinical specimens, and 4) serological tests in which antibodies are measured, preferably by demonstrating rising titers in paired acute and convalescent sera. Different diagnostic methods used for detection of a *Cp. psittaci* infection and their advantages and disadvantages are listed in Table 1.1.

4.5.1. Sample collection and storage of specimens

The laboratory diagnosis of avian chlamydiosis usually includes the isolation and identification of the organism from the host. Because *Cp. psittaci* requires living cells to multiply, isolation requires inoculation of either laboratory animals or cell cultures. Specimens should be collected aseptically, as contaminant bacteria may interfere with isolation of the chlamydiae.

Proper handling of clinical samples is necessary to prevent loss of infectivity. If specimens are used to inoculate animals or cell cultures immediately, most diluents will be adequate; however, if the specimen is to be shipped or stored, a special diluent should be used. A diluent consisting of sucrose–phosphate–glutamate (SPG) was developed for rickettsiae and has proven satisfactory for transport of chlamydial field samples (Bovarnick and Snyder, 1950; Spencer and Johnson, 1983). The recommended medium for chlamydiae consists of SPG buffer (sucrose, 74.6 g/liter; KH_2PO_4, 0.512 g/liter; K_2HPO_4, 1.237 g/liter; and L-glutamic acid, 0.721 g/liter), which can be sterilized by autoclaving. To this is added fetal calf serum (10%), vancomycin and streptomycin (100 mg/ml), and nystatin and gentamicin (50 mg/ml). The antibiotics reduce the effect of contamination even when samples are shipped at ambient temperatures. This medium also serves as a laboratory diluent and a medium for freezing of chlamydiae.

For isolation and identification of *Cp. psittaci* from acute and subclinical cases of chlamydiosis, the following samples should be collected: pharyngeal/choanal slit swabs (Andersen, 1996), lung tissue, thickened exudate-coated air sacs and free exudates,

Table 1.1. Different diagnostic methods used for detection of a *Cp. psittaci* infection and their advantages and disadvantages.

Diagnostic methods	advantages	disadvantages
Cytological staining	- easy - cheap - quick - no sophisticated equipment needed - dead and live bacteria can be demonstrated	- non-specific - less sensitive - non-automated - interpretation by experienced person
Immunocytochemie	- more sensitive and specific than cytology - easy - quick - dead and live bacteria can be demonstrated	- cross-reaction with other bacteria (when MAb is against LPS) - interpretation by experienced person - more expensive - fluorescence microscope required - non-automated
Immunohistochemistry	- automation possible - detection in the morphological context	- more labor intensive than immunocychemistry - histology laboratory required - MAb detecting *Cp.psittaci*-specific antigen in formalin-fixed samples needed - more time consuming than immunocytochemistry - more expensive
Antigen-ELISA	- quick - multiple samples can be tested at once - quantification - easy - dead and live bacteria can be demonstrated	- commercial kits often insensitive - non-specific if the target is LPS or Hsp60 - ELISA reader needed
Culture	- propagation for further investigations - more specific than direct antigen detection - direct evidence of live bacteria - quantification of live bacteria	- transport and storage of samples is critical - BSL3 laboratory - time consuming - expensive - labor intensive - trained personnel required - not all strains can be cultured

Table 1.1. Different diagnostic methods used for detection of a *Cp. psittaci* infection and their advantages and disadvantages (continued).

Diagnostic methods	advantages	disadvantages
Molecular diagnosis (PCR, Micro array)	- highly sensitive - highly specific - quick - automation possible - multiple samples can be tested at once - possibility of direct typing on clinical samples - can be quantitative - can detect live and dead bacteria	- expensive - specialized equipment needed - trained personnel needed
Serology: antibody ELISA	- easy - quick - multiple samples can be tested at once - quantification is possible - automation possible - valuable for epidemiological research	- convalescent sera (retrospective diagnosis) - not a proof that the organism is still present - tests detecting antibodies against LPS, hsp60 or whole organisms are non-specific - less sensitive than molecular diagnosis - ELISA reader needed

thickened pericardial tissues and exudates, sections of enlarged spleen and liver, intestinal mucosa at sites of hyperemia, colon contents, and conjunctival and nasal discharges. In the live bird, pharyngeal/choanal slit swabs, fecal samples, and cloacal swabs are the preferred specimens (Andersen, 1996). All three specimens should be collected, as no one sample will provide an isolation in all cases. Other specimens that can be collected, depending on the disease signs present, include whole blood, conjunctival scrapings or exudates, and peritoneal exudates. When companion birds are being tested for subclinical infection before placement in facilities, repeated sampling is necessary.

The same tissues can be collected for PCR and other molecular tests. If the DNA extraction will not be done immediately, it may be beneficial to collect the specimen in a DNA stabilization buffer (DeGraves et al., 2003).

The stability of chlamydiae during storage depends upon the material in which it is contained. Chlamydiae in tissue specimens or yolk-sac suspension can be preserved indefinitely by storage at -70° C (Andersen and Vanrompay, 2003; Andersen, 2004). Note that chlamydiae harvested from cell culture require special media during freezing. A satisfactory method for freezing them is to replace the cell culture medium with SPG buffer before freezing, as the organism is highly susceptible to the presence of sodium ions in the medium (Spencer and Johnson, 1983). The transport medium described earlier will provide adequate stability during freezing. At 4° C, the organism can survive with gradual loss of infectivity for 30 days or longer when in heavily infected tissues or in SPG transport medium (Spencer and Johnson, 1983).

4.5.2. Microscopic examination of specimens

4.5.2.1. Cytological Staining

Chlamydiae can be detected in smears of exudates and feces and in impression smears of liver and spleen by a variety of techniques. Giemsa, Stamp and Giménez stains are commonly used. The modified Giménez technique (Vanrompay et al., 1992), using carbol fuchsin and malachite green, is routinely used by several laboratories for detection of chlamydial inclusions in smears. The organisms appear as red to purple stained bacteria (Fig 1.4). These stainings can be helpful but are far less sensitive and specific than immunochemical stainings or molecular detection methods since other Gram-negative bacteria like for instance *E. coli* may also stain red due to their affinity for carbol fuchsin.

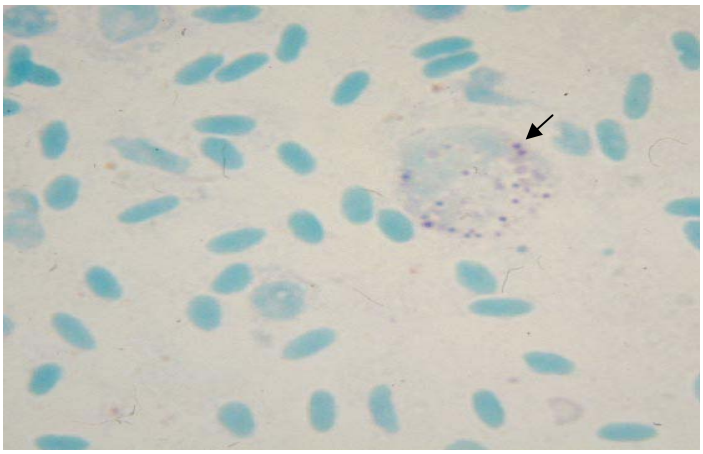

Fig 1.4. Giménez staining of an impression smear of the spleen from an infected and diseased pigeon. Note *Cp. psittaci* (red) inside a macrophage. (x 1000).

4.5.2.2. Immunohistochemical staining

Immunohistochemistry is another method for detection of chlamydiae in cytologic and histologic preparations (Moore and Petrak, 1985; Tappe et al., 1989). The use of immunohistochemical staining on formalin-fixed sections is gaining in popularity because a number of laboratories are using automatic staining equipment. The technique also allows laboratories to take routine samples for histopathology and then retrospectively examine them for chlamydiae, if necessary. The technique is more sensitive than histochemical staining, but experience is required to interpret findings. Cross-reactions with some bacteria, fungi, and epithelial intercellular material dictate that morphology must be considered in making a positive diagnosis.

Chlamydial antigen is most often found in macrophages in areas of inflammation. Antigen can be found in the lateral nasal gland up to 50 days post infection. Inflamed air sacs and pericardium also consistently contain antigen. However, care must be taken to avoid misinterpreting hemosiderin as antigen. Antigen-positive tissue and hematoxylin-and-eosin-stained sections are used as controls.

For immunohistochemical staining of paraffin sections, a family-specific monoclonal detecting all chlamydia and chlamydophila or a polyclonal antibody is used as the primary antibody. The antibodies should be produced to formalin-inactivated chlamydiae and selected for reaction with formalin-inactivated chlamydiae. They are mostly detecting the family-specific epitope on the lipopolysaccharide of *Chlamydiales*. The specific procedure

used for staining the paraffin sections will depend on the equipment available, as most techniques will give satisfactory results.

4.5.3. Enzyme-linked immunosorbent assays to detect chlamydial antigens

A number of manufacturers are producing enzyme-linked immunosorbent assay (ELISA) kits for detection of *Chlamydia trachomatis* in humans. These test kits will detect *Cp. psittaci* as they react to the lipopolysaccharide (LPS) or family (*Chlamydiales*) antigen. The advantages of ELISA are that it is rapid, does not require a high level of expertise, and is safe for the technician because the sample can be inactivated. A number of the test kits have been evaluated for use in avian samples (Vanrompay et al., 1994), although none of the kits are licensed for use in veterinary medicine. One of the problems of some of these tests is that the chlamydiae LPSs share some epitopes with the LPSs in other gram-negative bacteria, which can lead to a high number of false positives. This problem has been reduced or eliminated in the more recently developed kits by the use of monoclonal antibodies. However, these kits still lack sensitivity: a few hundred organisms are needed for a positive reaction. The general rule with these tests is that a diagnosis of chlamydiosis can be made when a strong positive reaction is seen along with clinical signs of chlamydiosis. Because of the number of false positives, a positive test without signs cannot be considered significant.

4.5.4. Isolation of the organism

4.5.4.1. Preparation of inoculum

Tissue specimens, fecal samples, and swabs are routinely used as samples for the isolation of chlamydiae. The processing of the samples is similar for inoculation of cell cultures, embryonated eggs, or laboratory animals. Diluents such as beef heart infusion broth, phosphate-buffered saline (PBS; pH 7.2), and cell culture media often are used to prepare a 20%–40% suspension of the homogenized sample. These diluents, with antibiotics, are satisfactory when the samples are to be inoculated within 24 h and will not be frozen. For routine use, a number of laboratories are using SPG buffers, such as the transport medium described earlier or Bovarnick's buffer (1950; Spencer and Johnson, 1983). These buffers have the added advantage of stabilizing the agent during refrigeration or freezing of the samples.

Before the inoculation of animals or cell cultures, contaminated samples must be treated by one of three basic methods: treatment with antibiotics (Bevan and Bracewell, 1986), treatment with antibiotics plus low-speed centrifugation (Andersen and Vanrompay, 2003), or treatment with antibiotics plus filtration (Bevan and Bracewell, 1986). A number of antibiotics that do not inhibit chlamydiae are available. A standard procedure is to homogenize the sample in diluent containing streptomycin, vancomycin, and kanamycin. Amphotericin B will control yeast and fungal growth. Other antibiotic solutions are often used, but penicillin, tetracycline, and chlooramphenicol should be avoided, as they will inhibit the growth of chlamydiae.

If the sample is lightly contaminated, adequate treatment for inoculation into chicken embryos, guinea pigs, mice, or cell culture consists of homogenization of the sample in an antibiotic solution. The sample is often left in the antibiotic solution for 24 h before the inoculation of cell cultures or animals. If the sample is heavily contaminated, such as a fecal sample, it should be homogenized in antibiotics and centrifuged at 1000–2000 × *g* for 30 min. The surface layer and the bottom layer should be discarded; the supernatant fluid is collected and recentrifuged, and the final supernatant fluid is inoculated into the experimental host or cell culture. If contamination persists, the sample may also be passed through a filter of 0.45–0.8 μm pore size.

4.5.4.2. Cell cultures and SPF embryonated chicken eggs

Cell cultures are the most common and convenient method for the isolation of *Cp. psittaci.* The most commonly used cell lines are BGM, McCoy, HeLa, Vero, and L-929, although a number of other cell cultures can be used. A study showed BGM to be the most sensitive, with Vero and L-929 listed as satisfactory (Vanrompay et al., 1992). Standard cell culture medium is used, containing 5%–10% fetal calf serum and antibiotics like vancomycin, streptomycin and amphotericin B as they do not inhibit the growth of chlamydiae.

The laboratory equipment and supplies available will determine the type of vessel used to grow the cell culture monolayer for incubation with the homogenated samples. The equipment, however, must be suitable for the following procedures: identification by direct fluorescent antibody (FA) or another appropriate technique of staining of the infected monolayer; centrifugation of the inoculum into the monolayer to enhance infectivity; possible blind passage at 3 or 6 days post inoculation to increase sensitivity of

isolation; examination of the sample two to three times during a passage; and protection of humans against possible infection. Small flat-bottomed vials (1-dram shell vials) or bottles, with 12-mm diameter glass coverslips, will meet these requirements and are often used (Bevan et al., 1978; Bevan and Bracewell, 1986) because the cell culture monolayer can be grown directly on the coverslip. Several vials are inoculated with each sample to permit fixing and staining at various times and to permit repassaging of negative samples after 6 days of inoculation.

Chlamydiae can be isolated from cells that are replicating normally. Most diagnosticians, however, prefer to use non-replicating cells for two reasons: to provide increased nutrients for the replication of chlamydiae, and because non-replicating cells can be maintained for longer periods for observation. Host-cell replication can be suppressed either by irradiation or by cytotoxic chemicals; the use of chemicals is becoming more common. Cytotoxic chemicals include 5-iodo-2-deoxyiodine, cytocholasin B, cycloheximide, and emetine hydrochloride (Paul, 1982). Cycloheximide (0.5-2.0 mg/ml) is most commonly used.

Chlamydiae are known to have a low infection rate in cell cultures, and methods to enhance the infection rate are often used. A common method to increase attachment of the chlamydiae to the cells is to centrifuge (500-1500 x g) the inoculum onto the monolayer for 30–90 min. Adsorption is enhanced by temperatures near 37 °C. Incubation is usually at 37–39 °C. Cultures must be examined at regular intervals by an appropriate staining method for the identification of chlamydiae. Staining is usually done at days 3 and days 6. Cultures that are negative at day 6 are harvested and repassed. Disruption of the monolayer by freeze-thawing should be avoided, as it can destroy the chlamydiae.

A number of laboratories still use chicken embryos for primary isolation of chlamydiae. The standard procedure is to inject up to 0.3 ml of inoculum into the yolk sacs of 6-day-old embryos (Andersen and Vanrompay, 2003). Replication of the chlamydiae usually will cause the death of the embryo within 5–12 days after inoculation. If no deaths occur, two additional blind passages are usually made before calling the sample negative. Chlamydial infection typically causes vascular congestion of the yolk-sac membranes, which are harvested and homogenized as a 20% yolk-sac suspension. This suspension can be frozen to preserve the strain or inoculated into other eggs or into cell culture monolayers. Identification of *Cp. psittaci* is usually performed by staining of yolk-sac impression smears by either cytological or immunohostochemical stainings.

4.5.4.3. Staining cell monolayers or yolk-sac impression smears

The preferred method for fixing of the monolayer is to remove the medium, wash once with PBS, and fix with acetone for 2–10 min. If the cell culture vessel is plastic, the monolayer can be fixed with a mixture of 50% acetone and 50% methyl alcohol or with 100% methyl alcohol. The preferred method for staining is the direct FA method (Bevan and Bracewell, 1986; Andersen and Vanrompay, 2003; Andersen, 2004) (Fig. 1.5.A), but a number of other methods can be used. With the FA method, the fluorescein-conjugated anti-chlamydia serum is applied to the glass slide and incubated for minimum 30 min at 37° C. The slides are then washed with PBS and aqua dest, air-dried, and mounted. The chlamydial inclusions will fluoresce a light green color under ultraviolet microscopy. Commercially prepared conjugates using monoclonal antibodies are available. Conjugates also can be prepared from polyclonal sera from rabbits, guinea pigs, sheep, or goats. The main problem is obtaining a specific, high-titre antiserum. The fluorescein conjugate is prepared using standard techniques (Bevan and Bracewell, 1986; Andersen and Vanrompay, 2003; Andersen, 2004).

Other techniques to demonstrate chlamydial inclusions are the indirect immunofluorescent technique, the immunoperoxidase technique (Moore and Petrak, 1985), and direct staining techniques, such as Giemsa, or Macchiavello's stains. The immunoperoxidase, Macchiavello, and Giminez (Fig. 1.5.B) staining techniques allow the use of standard light microscopes.

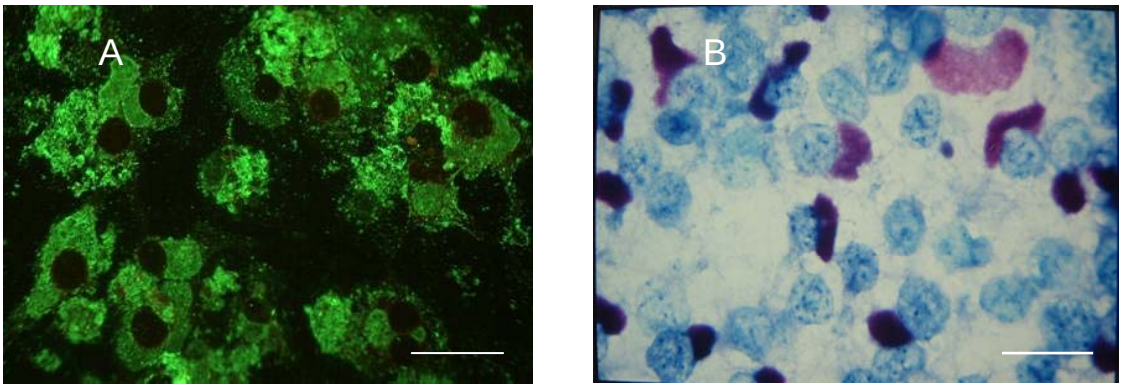

Fig. 1.5. **A**. Direct immunofluorescence staining of *Cp. psittaci* replicating in BGM cells. Note the green chlamydia inclusions inside the cells. (bar 20 µm). **B**. Modified Giménez staining of *Cp. psittaci* replicating in BGM cells. Note the red inclusions inside the cells (bar 20 µm).

4.5.5. Polymerase chain reaction

The possibilities of diagnostic detection of *Chlamydophila psittaci* are considerably improved with the introduction of molecular methods, particularly the polymerase chain reaction (PCR), which permits direct identification from clinical specimens and accelerates diagnosis from several days to a single working day. Tests published in the literature utilize two different genomic target regions for amplification, namely the ribosomal RNA gene region (Messmer et al., 1997; Everett et al., 1999c; Madico et al., 2000) and the gene encoding the MOMP antigen designated *omp1* or *ompA* (Hewinson et al., 1991; Kaltenboeck et al., 1997b; Yoshida et al., 1998).

The sensitivity of the PCR largely depends on the amount and quality of the extracted DNA. Optimal extraction of nucleic acids from a wide range of clinical samples is one of the most under appreciated, but nevertheless challenging and important steps. Proper extraction must efficiently release nucleic acids from samples, remove PCR inhibitors, avert the degradation of nucleic acids, and ensure adequate concentration of the nucleic acids after extraction. This is relatively easy achievable for viral and mammalian amplification targets, but much more difficult and inconsistent for nucleic acid targets of bacteria, as these pathogens frequently possess highly resistant outer membranes, and specific methods for disruption of these membranes are necessary for quantitative recovery of nucleic acids from these organisms. In some cases, concentration of target organisms by physical means increases the assays' sensitivity, but it is important to ascertain the enrichment effect (DeGraves et al., 2003). Such methods include immunomagnetic, centrifugal, or filter concentration (Lindqvist et al., 1997; Al-Soud et al., 1998; Radstrom et al., 2003). Several in-house-made and commercial DNA extraction methods have been described (Wilson et al., 1996; Sachse and Hotzel, 2003; Harkinezhad et al., 2007). Alternatively, commercial DNA extraction kits can be used for the extraction of chlamydial DNA. In our hands the QIAamp® DNA Mini Kit (Qiagen) performed the best for pharyngeal swabs while the High Pure PCR Template Preparation Kit (Roche) performed best for cloacal swabs and faeces. The use of commercial kits can be recommended especially when dealing with PCR inhibitors. They contain a special buffer reagent for lyses of the bacterial and eukaryotic host cells mostly based on a guanidine-detergent lysing solution first described by Chomczynski and Sacchi (1987). Simultaneous disruption of cells and inactivation of nucleases is achieved by lysis in strong denaturing reagents, for instance 4M guanidinium isothiocyanate (GIT) or 4M guanidinium

hydrochloride. Guanidinium isothiocyanate (GIT) is 2.5 times more effective on a molar basis than guanidine HCl. Both anion and cation act as denaturants in the GIT buffer but only the guanidine cation in GIT-HCl buffer. An optimal RNase digestion is intended to remove cellular RNA. The lysate is then centrifuged through a mini-column, where the released DNA is effectively bound to a solid phase: modified silica, hydroxyl apatite, or a special filter membrane. After washing, the DNA can be eluted with an elution buffer or water. DNA prepared in this manner is usually of high purity and free of PCR inhibitors.

Tissue sample homogenization in cell culture medium, PBS, or TBS and/or freeze-thaw cycles of samples lead to rapid loss of chlamydial target DNA. Specimens that contain low target numbers before freezing often become negative after cryostorage. The preservation of DNA from degradation is critically important. De Graves et al., (2003) advise to collect all specimens for PCR analysis in a DNA stabilization reagent. Such reagents are commercially available; for instance the RNA/DNA Stabilization Reagent for Blood/Bone Marrow® from Roche Applied Science. This stabilization reagent is based on the denaturation of proteins in a concentrated solution of guanidinium isothiocyanate and a reducing agent, as introduced by Chirgwin et al., (1979) for the inactivation of ribonuclease during RNA isolations. However, it also works perfectly well to stabilize DNA as demonstrated by De Graves et al., (2003) extracting chlamydial DNA from 10% (w/v) tissue suspensions of lungs from mice and by Harkinezhad et al., (2007), unpublished results) extracting chlamydial DNA from avian pharyngeal and cloacal swabs stored in this reagent as well as faeces.

The choice of a PCR test depends on the skill of the personnel in the laboratory and the equipment available. Nested procedures are more sensitive than traditional PCR and in most cases are also more sensitive than real-time PCR. Recently, two highly sensitive nested PCR assays have been developed to detect *Chlamydophila psittaci* in avian samples (Sachse and Hotzel, 2003; Van Loock et al., 2005b). The nested PCR of Sachse and Hotzel (2003) is based on a first amplification generating a genus-specific *ompA* product (576-597 bp) followed by a second amplification using one species-specific and one genus-specific primer generating a *Chlamydophila psittaci*-specific amplicon (389-404 bp). PCR results are visualized by agarose gel electrophoresis using ethidium bromide containing gels. The sensitivity of the nested PCR-EIA was established at 1 to 0.1 infection forming unit (IFU). Van Loock *et al*. (2005b) developed a nested PCR-enzyme immuno assay (PCR-EIA) (Fig. 1.6). The fluorescein–biotin labelled PCR products were

immobilized on streptavidin-coated microtiter plates and detected with anti-fluorescein peroxidase conjugate and a colorimetric substrate, although detection by use of a fluorimeter is also possible by using this method. The sensitivity of the nested PCR-EIA was established at 0.1 infection-forming units (IFU). Specificity was 100%. An internal inhibition control was included to rule out the presence of inhibitors of DNA amplification.

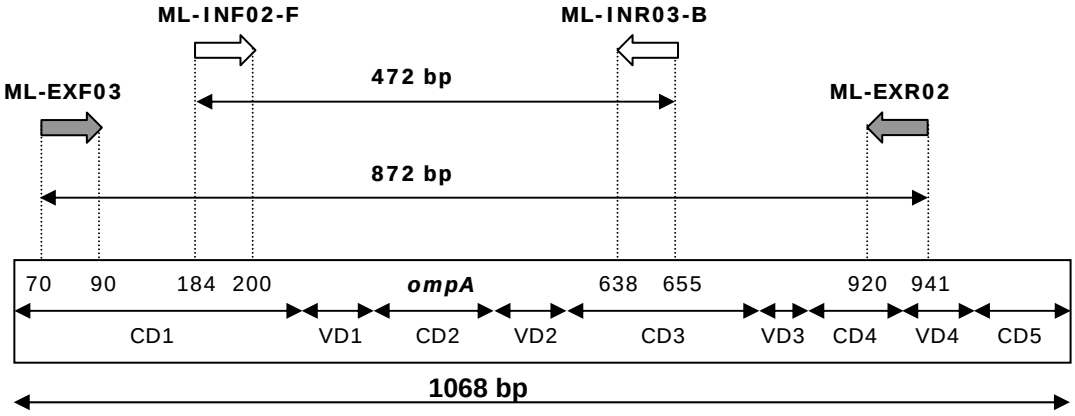

Fig. 1.6. Location of the outer and inner primers in the *ompA* gene. Numbering according to *ompA* sequences in Genbank.

A number of real-time PCR tests have been developed. The real-time PCR systems have the advantage that their sensitivity approaches that of the nested PCR systems, and they require no additional pipetting or handling of the PCR product following the initial set-up of the PCR mix. This reduces both the time needed for the test and the incidence of contamination. Recently a SYBR Green-based real-time PCR was developed targeting the rDNA ribosomal spacer of *Chlamydophila psittaci* (Geens et al., 2005b). The test could detect 10rDNA copies/µL DNA extract and could detect all known *ompA Chlamydophila psittaci* genotypes (Geens et al., 2005a).

4.5.6. DNA microarray-based detection

Micro-arrays can be coupled with PCR where they serve as a set of parallel dot-blots to enhance product detection and identification of bacterial isolates. Recently, Sachse *et al*. (2005) developed a DNA microarray-based detection and identification method for

Chlamydia and *Chlamydophila spp*. The test was developed using the ArrayTube platform (CLONDIAG®chip technologies). Unique species-specific hybridization patterns were obtained for all nine species of the family *Chlamydiaceae* on both microarray types. The present assay proved suitable for unambiguous species identification of chlamydial cell cultures and showed a potential for direct detection of these bacteria from clinical tissue. Recently, Clondiag has also developed an *ompA*-based *Cp. psittaci* genotyping microarray (D. Vanrompay, personal communication).

4.5.7. Serology

Despite the introduction of very sensitive and specific tests like PCR, the idea of serological diagnosis still lingers in the minds of several veterinary clinicians. However, serology alone is not particularly useful in diagnosing a current chlamydial infection in birds because of the high prevalence of this infection in birds and the long-term, up to several months, persistence of anti-chlamydial antibodies. In most bird species, there is a high background rate of anti-chlamydial antibodies, and until we have more information on the disease pattern in certain bird species in relation to direct identification of the bacteria and serology, we are unable to comment on the real significance of antibody titers obtained. Thus, to determine if a single bird is infected, serology should always be used in conjunction with antigen or gene detection or paired sera should be examined. However, obligatory examination of paired sera removes serology from immediate clinical relevance. A positive test is evidence that the bird was infected by the bacterium but does not necessarily indicate an active infection. False negative results can occur in birds with acute infections that are sampled before sero-conversion. Treatment with antibiotics may also delay and/or diminish the antibody response. The main serological methods that are being used for detecting chlamydial antibodies are: 1) various methods of elementary body agglutination (EBA), 2) the complement fixation test (CF), 3) an indirect (micro) immunofluorescence (MIF) test and 4) several commercial ELISAs. The EBA detects primarily IgM antibodies and thus can detect early infections (Grimes et al., 1994; Grimes and Arizmendi, 1996; Grimes, 1996). Titers > 1:10 in budgerigars, cockatiels and lovebirds; and titers > 1:20 in larger birds are frequently seen in cases of recent infection. A negative result does not guarantee that a bird is free of infection as the sensitivity of the test is rather low. The direct complement fixation (DCF) test detects avian IgG but not IgM. An advantage is that there is a readily available microprocedure. Disadvantages are:

1) test antigens commercially unavailable, 2) the test can not be used for testing sera from avian species whose immunoglobulins do not fix complement, such as small psittacine birds, 3) it is only relatively sensitive, 4) it cannot be used to differentiate between IgG and IgM antibodies, making it necessary to test paired sera, and 5) the technique is fairly laborious when there is a large number of samples to be tested. The modified DCF test is more sensitive but has the same disadvantages as the DCF test (Grimes et al., 1993; Grimes et al., 1994; Tully, Jr. et al., 1996; Andersen, 2004). The indirect IF test detects all immunoglobulin isotypes and is, as is the MIF test, widely used to detect *C. trachomatis*, *Cp. pneumoniae* and *Cp. psittaci* antibodies in human sera using selected strains of these bacteria (Salinas et al., 1993b; Wong et al., 1994; Fernandez et al., 2004). The MIF test appears to be more sensitive than the complement fixation tests. Some years ago a large number of commercially available ELISAs were evaluated for demonstrating *Cp.psittaci* antibodies in birds. All of these ELISAs were highly sensitive but showed low specificity, as they were mainly based on the use of whole chlamydial organisms, LPS, or chlamydial outer membrane fractions of LPS and lipoglycoprotein nature (Sting and Hafez, 1992; Benheim et al., 1993; Fudge, 1993; Ley et al., 1993; Tully, Jr. et al., 1996). When using these sources and antigen, false-positives due to the presence of antibodies cross-reactive to the chlamydial LPS or hsp60 cannot be ruled out. More recently, peptide-based ELISA systems, or ELISAs using recombinant LPS, have become commercially available for the specific detection of *C. trachomatis*, *Cp.pneumoniae* and *Cp.abortus* antibodies (Medac, Savyon, Labsystems), (Kaltenboeck et al., 1997a; Morre et al., 2002; Hoymans et al., 2003; Tiran et al., 2004). These tests performed as well as the MIF assay, but are less time-consuming, less expensive, and easier to perform. In the future this principle might also be useful in the serodiagnosis of *Cp. psittaci* infections. At present, an ELISA using recombinant MOMP of *Cp. psittaci* has already been described (Vanrompay et al., 2001a; Verminnen et al., 2005) for testing avian sera. The test is not species-specific as the recombinant MOMP, produced by transiently tranfected COS7 cells (Vanrompay et al., 1998), comprises all four variable domains as well as conserved domains and thus detects antibodies against all members of the genus *Chlamydophila* and *Chlamydia* (Vanrompay et al., 2004). However, this is not a problem when testing avian sera as birds can only become infected by *Cp.psittaci*. Unfortunately, *Cp. psittaci*-specific ELISAs are not yet available.

5. Public health significance

Cp. psittaci can infect humans and should be handled carefully under conditions of bio-containment. Humans most often become infected by inhaling the organism when the urine, respiratory secretions or dried faeces of infected birds is dispersed in the air as very fine droplets or dust particles. Other sources of exposure include mouth-to-beak contact, a bite from an infected bird or handling the plumage and tissues of infected birds. Therefore, post-mortem examinations of infected birds and handling of cultures should be done in laminar flow hoods or with proper protective equipment. Human infection can result from transient exposures. The disease is of public health significance because of the popularity of psittacine birds as pets and the increased placement of these birds in child-care facilities and in homes for the aged. Risk of psittacosis is not only limited to direct contact with birds but also associated with rural environment and activities like mowing or trimming lawns without a grass catcher and with gardening, which suggests that these outdoor activities may expose individuals to the infectious agent (Telfer et al., 2005; Fenga et al., 2007). Person-to-person transmission of psittacosis is possible but it is believed to be rare (Hughes et al., 1997; Ito et al., 2002).

The incubation period is usually 5–14 days; however, longer incubation periods are known. Human infections vary from inapparent to severe systemic disease with interstitial pneumonia and occasionally encephalitis. The disease is rarely fatal in properly treated patients; therefore, awareness of the danger and early diagnosis are important. Infected humans typically develop headache, chills, malaise and myalgia, with or without signs of respiratory involvement. Pulmonary involvement is common; auscultatory findings, however, may appear to be normal or to underestimate the extent of involvement. Several severe psittacosis (pneumonia) cases were recently documented (Chorazy et al., 2006; Strambu et al., 2006; Haas et al., 2006; Pandeli and Ernest, 2006) and recently *Cp. psittaci* has been associated with ocular lymphoma although this still is a matter of debate as conflicting results have been found (Ferreri et al., 2004; Chanudet et al., 2006; Rosado et al., 2006; Zhang et al., 2007). However, ‘severe’ psittacosis cases are probably only the tip of the iceberg. What lies underneath are less severe, clinically unnoticed infections, which are misdiagnosed due to symptoms similar to those of other respiratory pathogens, or even asymptomatic infections (Harkinezhad et al., 2007; Vanrompay et al., 2007). The impact of these types of *Cp. psittaci* infections on human health is difficult to determine. We can try to extrapolate our knowledge on avian infections to human psittacosis. As in birds,

carrier status might occur, as well as pathogenic interactions with other respiratory pathogens (Van Loock et al., 2006b).

Diagnosis can indeed be difficult and is usually established through testing paired sera or only one serum sample by use of the micro-immunofluorescence (MIF) test which is more sensitive and specific than the previously (and sometimes still) used complement fixation (CF) test. However, the MIF test shows cross-reactivity with other chlamydial species. Highly sensitive nucleic acid amplification assays can be used to specifically detect *Cp. psittaci*. Culture is also possible, but technically rather difficult requiring laboratory biosafety level 3. Like in birds, tetracyclines are the drugs of choice for treating human psittacosis. Doxycycline or tetracycline is usually administered unless contraindicated like for pregnant women and children < 9 years, where erythromycin can be used. The length of treatment will vary with the drug, but should be continued for at least 14 days for tetracycline.

Psittacosis is a notifiable disease in Australia and the U.S. and in most European countries like Belgium, France, Germany, Italy, Switserland, An overview of reported psittacosis cases in several countries is presented in Table 1.2.

Table 1.2. Human cases of psittacosis (available data on the internet)

Country/Territory	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	per million of habitants
Austria	1			1	2	3	0	3	6				0.36
Argentina					38	1							0.5
Australia	86	35	55	81	99	137	213	200	239	164	171	62	5.54
Belgium	0	0	8	12	13	10	23	39	12	7	2	3	0.95
Bosnia and Herzegovina	162						0	0	0				-
Chile					2								-
Croatia	7	4	0	5	8	3		5	4				1.10
Czech Republic	3	5	6			0	3	0	0				
Denmark	0				31	8	13	14	8	22	7	11	2.18
Finland	2	1	2	0	0	0			0				-
F.Y.R. of Macedonia	0			14	0	0	5	0	0				-
Germany	134	124	155	109	86	53	40	42	15	33	26	12	0.51
Hungary	0	0	0	1	5	1	6	85	7	140	29	28	0.60
Japan				23	18	35	54	44	40	34			0.27
Poland	2	2	0	2	0	5		2	2				0.05

Table 1.2. Human cases of psittacosis (available data on the internet) (continued)

Country/Territory	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	per million of habitants
Slovakia	2	0	1	3	10	0	0	1	0				0.18
Spain						5	4	0	1				0.06
Sweden	25	66	30	29	24	12	13	12	7	5	2	9	1.3
The Netherlands	0	28		25	36	24	17	27	33	49	59	27	1.7
U.K./Great Britain	353	322	293	207	204	106	68	100	62	59			1.75
U.K./N. Ireland	52	37	0	0		44	16	15	13				8.61
Ukraine	161	2	3	2	0	0	0	0	0				_
U.S.A.	45	38	54	15	13		19	13	11				0.05

7. Prevention, control recommendations and legislation

7.1. Desinfection

Chlamydiae are highly susceptible to chemicals that affect their lipid content or the integrity of their cell walls. Cleaning of equipment and cages of infected birds is important because *Cp. psittaci* can survive for up to 30 days in faeces and bed materials. Disinfection with most common detergents and disinfectants will inactivate *Cp. psittaci*. The following disinfectants can be used to inactivate the organism: 1 in 1000 dilution of quaternary ammonium compounds, 70% isopropyl alcohol, 1% Lysol, 1 in 100 dilution of household bleach or chlorophenols (Longbottom and Coulter, 2003; Smith et al., 2005).

7.2. Chlamydiaceae vaccine development over the years

Protective immunity to *Chlamydiaceae* is believed to be effected primarily through the action of CD4+ T helper type 1 (Th1) lymphocytes, CD8+ T lymphocytes, mononuclear phagocytes, and cytokines secreted by these cells. In addition, the role of local antibodies in mucosal secretions is not to be underestimated.

Initial attempts to develop an effective vaccine for controlling both animal and human chlamydial infections began with the use of inactivated or live whole organism preparations in the 1950s. In general, such preparations offered a reasonable level of protection, although they have been more successful in protecting animal infections than human infections.

Initial vaccine studies investigated the efficacy of formalin-inactivated whole organisms derived from *Cp. abortus* infected ovine placental material, but the large amounts of material required for this proved impractical. Subsequent studies with embryonated egg grown material resulted in the commercialization of a single *Cp. abortus* strain (A22), yolk sac derived, formaldehyde-inactivated, Falba-adjuvanted vaccine in 1958 by the Wellcome Foundation Ltd. This vaccine was effective at controlling ovine enzootic abortion (OEA) for approximately 20 years until disease started to reappear in vaccinated sheep flocks in the late 1970s. Initially, it was thought that field isolates differing from the vaccine strain or emergence of more virulent wild type strains was the cause of the vaccine breakdown. However, the subsequent inclusion of a second *Cp. abortus* strain (S26/3), isolated from a vaccinated ewe in 1979, into the vaccine did not improve the protective efficacy, and the vaccine was eventually withdrawn in 1992. The

failure of the vaccine was most likely due to production problems, since these strains continue to provide good levels of protection in experimental challenge model systems. During the 1960s unsuccessful attempts were made to develop inactivated and live vaccines against trachoma using both human and non-human primates (Grayston et al., 1963; Woolridge et al., 1967; Dhir et al., 1967; Collier and Blyth, 1967; Wang et al., 1967). These vaccines reduced disease in some individuals but they enhanced disease in others resulting from stimulation of an enhanced delayed-type hypersensitivity (DTH) response. Therefore, the use of whole organisms for developing human chlamydial vaccines was essentially abandoned.

In the early 1980s an attenuated strain of *Cp. abortus* was developed as a live vaccine at the Institut National de la Recherche Agronomique, Nouzilly, France (Rodolakis and Souriau, 1983; Rodolakis and Bernard, 1984). This live vaccine (Enzovax, Intervet UK Ltd.; Cevac Chlamydophila, Cevac Animal Health) is one of the 5 commercially available vaccines in Europe and the USA, the other four being inactivated whole organism based vaccines including: 1) Mydiavac (Novartis Animal Health UK Ltd.), 2) Ovax Clamidia (Fatro), 3) Enzabort EAE-Vibrio and 4) *Chlamydia psittaci* Bacterin (Colorado Serum). The live attenuated vaccine was produced by chemical mutagenesis, and consists of a temperature sensitive mutant strain that can grow at 35°C but not at 39.5 °C, the body temperature of sheep. These commercial live-attenuated and inactivated vaccines offer good protection against OEA and significantly reduce the shedding of infective organisms, a factor important in limiting the spread of infection to other animals. However, concerns remain over the safety of using live-attenuated vaccines, particularly since *Cp. abortus* can cause abortion and serious disease in pregnant women. There may also be a risk of the attenuated strain reverting to virulence, thus having the potential to cause disease and abortion in the vaccinated animal. Furthermore, the vaccine cannot be administered during pregnancy or to animals being treated with antibiotics limiting its use. In contrast, the inactivated vaccines can be administered to pregnant ewes, although care must be taken in handling and administering these vaccines as they are adjuvanted with mineral oils, which have the potential to cause tissue necrosis if accidentally self-injected. The only other animal chlamydial vaccines, which are commercially available are for *Cp. felis* infection in cats. There are 7 commercial vaccines available for cats. Five of them are live attenuated and two are inactivated vaccines. Felocell CVR-C Ch (Pfizer Animal Health) and Nobivac Forcat (Intervet) contain the attenuated *Cp.felis* strain Baker. The three others

are; Purevax RCCh (Merial), Purevax RCPCh (Merial) and Purevax RCPCh FeLV (Merial) and contain the attenuated *Cp.felis* strain 905. The two inactivated vaccines, Fevaxyn iCHPChlam and Fevaxyn Pentofel, (Fort Dodge Animal Health), are inactivated preparations comprising the *Cp. felis* Cello strain. Information on commercially available vaccines is shown in Table 1.3. Although vaccination is successful in reducing acute disease, it does not, however, prevent shedding of the organism and therefore chlamydial spreading in the population nor does it prevent re-infection (Wills et al., 1987). A small percentage of vaccinated cats may also develop post-vaccinal reactions such as fever, vomiting, anorexia and/or depression and lethargy. Vaccination of pregnant cats is also not advised.

Throughout the 1980s renewed efforts were made to develop a vaccine to protect from *C. trachomatis* infection. However, it was thought that chlamydial components, particularly heat shock protein 60 (hsp60), which was possibly responsible for the inflammatory damage caused by whole organism vaccines (Brunham and Peeling, 1994), should be avoided in a vaccine. Following the identification of the major outer membrane protein (MOMP) as the structurally and immunologically dominant protein (Caldwell et al., 1981), vaccine research largely focused on this protein. A certain level of protection has been achieved with COMC preparations, in which MOMP constitutes 90% or more of the protein content, using the guinea pig (Batteiger et al., 1993) and mouse (Pal et al., 1997) models for *C. trachomatis* genital tract infection, and in a mouse toxicity test for *Cp. felis* infection (Sandbulte et al., 1996).

More recently, mice have been shown to be protected from an upper genital tract challenge with *C. muridarum* following vaccination with a purified and refolded MOMP preparation (Pal et al., 2001). This study supports the view that conformational MOMP epitopes are important in protection and strengthen the argument for pursuing MOMP as a vaccine candidate.

Table 1.3. Commercially available chlamydial vaccines.

Vaccine name	Disease	Marketing authorization holder	Animal	Description
Felocell CVR-C Ch	Feline viral rhinotracheitis (FVR), feline calicivirus (FCV), feline panleukopenia (FPL), feline chlamydiosis	Pfizer Animal Health, USA	Cat	Attenuated: feline calicivirus (strain F9), feline herpes virus type 1 (strain G2620A), feline panleucopenia virus (strain MW-1) and *Cp. felis* (strain Baker)
Fevaxyn Pentofel	Feline panleucopenia, feline chlamydiosis, leukaemia and respiratory diseases caused by feline rhinotracheitis virus, feline calicivirus	Fort Dodge Animal Health, U.S.A	Cat	Inactivated: panleucopenia virus (CU4 strain), feline Calicivirus (255 strain), Feline Rhinotracheitis Virus (605 strain), *Cp. felis* (Cello strain),Feline Leukaemia Virus (61E strain)
Nobivac Forcat	Feline panleucopenia, feline chlamydiosis and respiratory diseases caused by feline herpesvirus, feline calicivirus	Intervet, the Nederlands	Cat	Attenuated : feline herpesvirus type 1(strain G2620A), feline calicivirus (strain F9), *Cp. felis* (strain Baker) and feline panleucopenia virus (strain MW-1)
Purevax RCCh	Feline viral rhinotracheitis, feline chlamydiosis and respiratory diseases caused by feline calicivirus,	Merial, France	Cat	Attenuated: feline rhinotracheitis herpesvirus (strain FVH F2), feline calicivirosis antigens (FCV 431 and FCV G1 strains) and *Cp. felis* (strain 905)
Purevax RCPCh	Feline viral rhinotracheitis, feline panleukopenia , feline chlamydiosis and respiratory diseases caused by feline calicivirus	Merial, France	Cat	Attenuated: feline rhinotracheitis herpesvirus (FVH F2 strain), feline calicivirosis antigens (FCV 431 and FCV G1 strains), *Cp. felis* (strain 905) and feline panleucopenia virus (PLI IV)
Fevaxyn i-CHP Clam	Feline panleukopenia, feline chlamydiosis	Fort Dodge Animal Health U.S.A.	Cat	Inactivated *Cp. felis* Cello strain (Cello, 1967)

Table 1.3. Commercially available chlamydial vaccines (continued).

Vaccine name	Disease	Marketing authorization holder	Animal	Description
Purevax RCPCh FeLV	Feline viral rhinotracheitis, feline panleukopenia (FPL), feline chlamydiosis, leukaemia and respiratory diseases caused by feline calicivirus	Merial, France	Cat	Attenuated: feline rhinotracheitis herpesvirus (FVH F2 strain), feline calicivirosis antigens (FCV 431 and FCV G1 strains), *Cp. felis* (strain 905), feline panleucopenia virus (PLI IV) and FeLV recombinant canarypox virus (vCP97)
Chlamydia psittaci Bacterin	Enzootic abortion of ewes	Colorado Serum, USA	Sheep	inactivated cultures of *Cp. psittaci* emulsified with a mineral oil adjuvant.
Enzabort EAE-Vibrio	Campylobacter and chlamydia	Colorado Serum, USA	Sheep	killed vaccine
Ovax Clamidia	Chlamydial abortion	Fatro, Italy	Sheep	egg-grown, formalin-inactivated, whole-organism
Ovilis®Enzovax	Chlamydial abortion	Intervet, the Nederlands	Sheep	an avirulent, temperature-sensitive, live chlamydia vaccine
Enzovax	Enzootic abortion of ewes	Intervet UK	Sheep	Attenuated vaccine
Mydiavac	Enzootic abortion of ewes	Novartis Animal health, UK Ltd.	Sheep	Inactivated whole-organism

Although vaccination with purified COMCs and refolded, purified MOMP preparations have been reasonably successful, the problems associated with the bulk growth of chlamydial organisms that is required for their preparation, make these options commercially non-viable. For these reasons, attention turned to the development of the cheaper and more cost-effective alternative of recombinant protein vaccines, particularly those based on MOMP. However, this approach proved difficult because the expression of full-length recombinant MOMP in *E. coli* was generally toxic. Sheep protection studies with *Cp. abortus* recombinant MOMP, expressed as insoluble inclusion bodies in a bacterial overexpression system, offered some protection against infection, although results were inconsistent (Herring et al., 1998). Another approach has been to express *Cp. abortus* MOMP, or fragments of MOMP, as overcoat protein on the surface of a plant virus (Herring et al., 1998). This method, although initially promising in mouse studies, ultimately proved unsuccessful and trials did not progress to sheep. Other studies with MOMP peptides (Allen et al., 1991; Knight et al., 1995) and oligopeptide vaccines (Su et al., 1995) containing antigenically common T-helper and neutralising B-cell epitopes have been variable. The disappointing results achieved with recombinant MOMP vaccination could be due to a lack of native protein structure and conformationally intact protective epitopes. Glycosylation of additional antigens may also be required to augment protection. Attempts to express or refold recombinant MOMP to a native confirmation have largely been unsuccessful, and are unlikely to yield large enough quantities of native recombinant MOMP to make them a viable option for use in commercial vaccines.

In recent years, attention has turned to DNA vaccination as a way of inducing a protective response against chlamydial infections (Zhang et al., 1999; Vanrompay et al., 1999b; Dong-Ji et al., 2000). One of the main advantages of this technology compared to the more conventional methods of vaccination is that the vaccine antigen is produced *de novo* by the host cell. The antigen is then processed in the same way as internalised viruses and intracellular bacterial pathogens, thus mimicking natural infection and resulting in humoral and cell mediated immune responses. However, in terms of human chlamydial infections, this method has generally been more successful at eliciting a protective response in the murine respiratory model (Zhang et al., 1997; Dong-Ji et al., 2000) than in the genital tract (Pal et al., 1999). Similarly, for animal infections, DNA vaccination has been more successful at inducing protective immune responses in turkeys infected with *Cp. psittaci* (Vanrompay et al., 1998; Vanrompay et al., 1999a; Vanrompay et al., 1999b; Vanrompay et al., 2001b), than in sheep (Longbottom, 2003) or mouse models (Hechard et

al., 2003a; Hechard et al., 2003b) infected with *Cp. abortus*. A *Cp. psittaci* DNA vaccine can be used in the presence of maternal antibodies (Van Loock et al., 2004). Thus, further research is needed to enhance the immunogenicity of DNA vaccines and to lower the production price of these vaccines.

There are currently no vaccines available against avian chlamydiosis although research on MOMP-based DNA vaccination in SPF turkeys was very promising (Vanrompay et al., 1999a; Vanrompay et al., 1999b). DNA vaccination significantly diminished clinical signs, lesions and chlamydial excretion though gave no full protection. But this is maybe impossible. So far, we have to rely on risk reduction strategies and treatment. Worldwide, psittacosis prevention and control recommendations and legislation are focusing on a reduction in human morbidity.

7.3. Legislation

Until recently, EU legislation for the importation of birds other than poultry from third countries was encompassed in Commission Decision 2000/666/EC (CEC, 2000) and 2005/760/EG, which were primarily concerned with restricting the introduction of Newcastle disease or avian influenza. Essentially these decisions required: 1) the bird must come from a flock in the country of origin where they have been kept for at least 21 days prior to export, 2) the birds are transported in cages or crates that contain only one species of bird or one species per compartment if compartimentalised and, 3) the birds are moved to designated licensed quarantine premises where they are held for 30 days and subjected to at least two veterinary inspections, usually beginning and end, before release. There was only one mention on *Cp. psittaci*: "if during quarantine it is suspected or confirmed that psittaciformes are infected with *Cp. psittaci*, all birds of the consignment must be treated by a method approved by the competent authority and the quarantine must be prolonged for at least two months following the last recorded case". Thus, these requirements did not apply to any other bird species nor were there any rules that applied to the management of psittacosis within the EU. Therefore, decisions 2000/666/EC and 2005/760/EC were repealed and a new commission regulation, EC no 318/2007 applies from 1 July 2007 after being published in the Official Journal of the EU. This regulation lays down the animal health conditions for imports of certain birds from third countries and parts thereof into the EU. Thus it is not applicable to: a) poultry, b) racing pigeons, c) birds imported from Andorra, Liechtenstein, Monaco, Norway, San Marino, Switzerland and the Vatican City,

and not to, d) third countries which can use an animal health certificate referred to in annex I of the regulation. The regulation also lays down the quarantine conditions. For instance: 1) approved quarantine facilities and centres, 2) direct transport of birds to quarantine stations, 3) attestation by the importers or their agents, 4) quarantine for at least 30 days, 5) examination, sampling and testing to be carried out by an official veterinarian, 6) actions in case of disease suspicion which are in case of chlamydiosis treatment of all birds and prolonged quarantine for at least two months following the date of the last recorded case. Importantly the regulation only allows imports of birds from approved breeding establishments, thus for birds other than poultry, only birds bred in captivity carrying an individual identification number and accompanied by an animal health certificate are allowed.

In the US, State regulatory agencies may impose quarantine on intrastate movement of diseased poultry flocks and may require antibiotic treatment of the flock prior to slaughter. Because regulations may vary from state to state, the appropriate public health and/or animal health agencies should be consulted as necessary. According to USDA regulations, movement of poultry, carcasses, or offal from any premise is prohibited where the existence of chlamydiosis has been proved by isolation of a chlamydial agent. The Animal and Plant Health Inspection Service of the USDA and the U.S. Department of Health and Human Services forbid interstate movement of birds from infected flocks, but there is no restriction on movement of eggs from such flocks.

In 2006, the US National Association of State Public Health Veterinarians (NASPHV; wwwnasphv.org) published a compendium of measures to control *Cp. psittaci* infections among humans and pet birds, including prevention and control recommendations such as: 1) protect persons at risk, 2) maintain accurate records of all bird-related transactions for at least one year to aid in identifying sources of infected birds and potentially exposed persons, 3) avoid purchasing or selling birds that have signs consistent with avian chlamydiosis, 4) isolate newly acquired, ill, or exposed birds, 5) test birds before they are to be boarded or sold on consignment, 6) screen groups of birds with frequent public contact routinely for anti-chlamydial antibodies, 7) practice preventive husbandry, 8) control the spread of the infection and finally 9) used disinfection measures.

Large-scale commercial import of psittacine birds ended in the US in 1993 with the implementation of the Wild Bird Conservation Act and the Veterinary Services of the Animal and Plant Health Inspection Service of the USDA regulates the legal importation of pet birds set forth in the Code of Federal Regulations, (Title 9, Chapter 1). The

Department of the Interior (DOI) monitors and regulates wildlife shipments at U.S. ports of entry and conducts investigations on smuggling and interstate trafficking of endangered and protected species including birds, which is extremely important as these birds are a potential source of new *Cp. psittaci* infections in domestic birds.

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Chapter II

Prevalence of *Chlamydophila psittaci* infections in a Flemish human population in contact with domestic and companion birds

This chapter is adapted from:

Taher Harkinezhad, Kristel Verminnen, Marc L. De Buyzere, Ernst R. Rietzschel, Sofie Bekaert and Daisy Vanrompay. 2008. Prevalence of *Chlamydophila psittaci* infections in a Flemish human population in contact with domestic and companion birds. Emerging Infectious Diseases. Submitted.

Abstract

Chlamydophila psittaci infections in man still present an epidemiological and diagnostic challenge. Psittacosis cases are underestimated. We therefore investigated the occurrence of *Cp. psittaci* infections in a representative Flemish population of 540 individuals. Participants, 264 males and 276 females, at the age of 36 to 57 years (median 46) were sampled pharyngeally and at the same moment a blood sample was obtained. Each individual completed a questionnaire allowing insight into professional and nonprofessional activities, smoking habits, medical history and contact frequency with different bird species and other animals. Pharyngeal swabs were analyzed using a *Cp. psittaci*-specific nested PCR/enzyme immunoassay (EIA), and a *Cp. pneumoniae*- specific nested PCR. Sera were tested for IgG/IgM by a recombinant *Cp. psittaci* MOMP-based ELISA, by MIF and by the Serion Chlamydia IgG/IgA ELISA. Results confirmed our suspicion on the underestimation of psittacosis in Belgium. *Psittaciformes* and racing pigeons were the main infection source. Non-exposition related risk factors for PCR or ELISA positivity were gender (women being more frequently infected) and excessive alcohol intake defined as an averaged intake of > 2 units daily for women and >3 units daily for men. We analyzed the effect of seropositivity and/or PCR positivity on inflammation (white blood cell count, high-sensitive CRP, fibrinogen). After adjustment for known confounders, we found no clear significant pattern suggesting an increased inflammatory burden.

1. Introduction

Members of the family *Chlamydiaceae* are obligate intracellular Gram-negative bacteria. The family comprises two genera, *Chlamydia* and *Chlamydophila*. *Chlamydia* has three species including *C. trachomatis* (humans), *C. suis* (swine) and *C. muridarum* (mice, hamsters).The genus *Chlamydophila* includes the species *Cp. psitttaci* (birds), *Cp.-abortus* (sheep, goats, cattle) *Cp. caviae* (guinea pigs), *Cp. felis* (cats), *Cp. pecorum* (sheep, cattle, swine) and *Cp. pneumoniae* (humans, koala's, horses) (Everett et al., 1999a). All animal infecting species are considered potentially infectious to humans although particularly *Chlamydophila psittaci* and *Cp. abortus*, are known to be transmissible from animals to humans, causing significant zoonotic infections.

The most important animal chlamydiosis of zoonotic character is psittacosis, a systemic disease in birds of acute, protracted, chronic or subclinical manifestation.

Although the causative agent *Cp. psittaci* is known to be very wide-spread in many avian species, not all carrier birds actually show symptoms of disease. However, they may transmit the infection to humans, the symptoms being mainly non-specific and influenza-like, but severe pneumonia, endocarditis and encephalitis are not uncommon. In Germany, the annual average of notified human cases of psittacosis is approximately 100 with several deaths (Sachse and Grossmann, 2002). A study in the United States revealed that, while the average prevalence may be at a few percent, up to 30% of households having purchased pet birds from infected flocks were affected by clinical psittacosis or were serologically positive (Moroney et al., 1998). The general situation in Europe, and thus also in Belgium is believed to be no different although human psittacosis cases are only sporadically reported, with 7, 2 and 3 cases in 2005, 2006 and 2007, respectively (www.iph.fgov.be). *Cp. psittaci* causes a symptomatic or asymptomatic course of infection depending on the virulence of the strain, the infective dose and the immune status of the host. An asymptomatic infection will be unnoticed, as people do not seek medical attention. On the other hand, in case of a symptomatic infection, flu-like symptoms occur which are often treated with antibiotics to prevent 'secondary bacterial' infections. Thus, in most symptomatic cases, diagnostic tests for *Cp. psittaci* are not performed. Therefore, the number of *Cp. psittaci* infections in humans is most likely underestimated.

Infections of workers in abattoirs in connection with the slaughtering of ducks, turkeys or geese, as well as cases among bird breeders are regularly reported in several European countries (Bracewell and Bevan, 1982; Newman et al., 1992; Bennedsen and Filskov, 2000; Harkinezhad et al., 2007; Vanrompay et al., 2007; Laroucau et al., 2008). Feral pigeons are quite commonly found infected in many urban habitats, which also raises serious questions as to the hazards to human health (Haag-Wackernagel, 2005; Laroucau et al., 2005; Mitevski et al., 2005). But, we better not forget the group of persons facing an elevated risk of infection because of frequent contacts with domestic and companion birds at work or in their spare time. As a consequence of the increasing habit of keeping parrots, parakeets, budgerigars, etc. as pet birds many more persons are at risk. However, the occurrence of *Cp. psittaci* infections in a generally healthy human population in contact with domestic and companion birds is not documented. The aim of the present study was to investigate the actual number of *Cp. psittaci* infections in such a human population, using recently developed molecular diagnostic techniques. We also tried to retain information on the relation between zoonotic risk, bird contact frequency and on the bird species most often involved in *Cp. psittaci* transmission to humans.

2. Material and methods

2.1. Study population and samples

Data are from a population survey (n=2524) of apparently healthy community-dwelling subjects, aged 35-55 years at study initiation, from the communities of Erpe-Mere and Nieuwerkerken. Subjects were recruited by random sampling of the population database of subjects within the age and geographic constraints. From the first 987 consecutive subjects entering the study (October 2002 until July 2003), pharyngeal specimens were taken using rayon-tipped, aluminium-shafted swabs (Copan, USA). Swabs were provided with DNA stabilization buffer (Roche) and stored at –80°C until tested by PCR. Additionally, blood was collected, and sera were stored at –80°C until serologically tested.

Each participant completed a questionnaire (reviewed by his/her primary care physician and a study nurse at study entry), designed to assess information on professional and non-professional activities, smoking habits, general health, personal medical history, use of medication, and contact with companion and/or domestic animals. In case of contact with birds, participants defined the bird species and contact frequency. The questionnaire was used to assign participants to groups having: 1) daily, 2) weekly, 3) sporadic or 4) no contact with birds. Swabs of 420 male (264) or female (276) participants having contact with companion/domesticated birds and of 120 of 567 randomly chosen participants having no contact with birds were examined. Persons were of 35 to 57 years. The ethical committee of Ghent University approved the study and a written informed consent was obtained from each participant prior to participating in the study.

2.2. Polymerase chain reactions

Pharyngeal DNA as well as positive control DNA (genomic DNA of *Cp. psittaci* strain 89/1051 and of *Cp. pneumoniae* strain TW183) was prepared as previosly decribed (Van loock et al., 2005b). Pharyngeal swabs were tested by a nested PCR enzyme immunoassay (PCR/EIA) which specifically targets the *Cp. psittaci ompA* gene (Van Loock et al., 2005b). The nested PCR/EIA was performed using external and internal primer pairs generating a biotin-fluorescein dual labeled *ompA* product of 451 bp, detected by spectrophotometry in streptavidin-coated ELISA plates. Subsequently, *Cp. psittaci* PCR/EIA positive specimens were also examined for the presence of *Cp. pneumoniae*, by

a *Cp. pneumoniae*- specific 16S-rRNA nested PCR (Messmer et al., 1997) using genus-specific external primers and species-specific internal primers generating PCR products of 436 and 127 bp, respectively. Results were visualized by agarose gel electrophoresis.

2.3. Serology

Sera of individuals positive by *Cp. psittaci* nested PCR/EIA and negative by *Cp. pneumoniae* nested PCR were examined for antibodies using: 1) a micro-immunofluorescence (MIF) test (Focus Diagnostics Inc.), detecting IgG and IgA against whole elementary bodies of *Cp. psittaci* genotype A strains 6BC and DD34 of which the lipopolysaccharide (LPS) had been removed, 2) the ELISA classic Chlamydia IgG/IgA (Virion/Serion), detecting antibodies against detergent extracted elementary bodies of the *Cp. psittaci* genotype D Borg strain, and 3) an ELISA detecting IgG and IgM against recombinant MOMP (rMOMP) of the genotype A *Cp. psittaci* strain 89/1051 (Verminnen et al., 2006). Sera, negative in both nested PCR's were also examined.

Tests were performed and interpreted as advised by the manufacturers diluting sera 1/20 and 1/100 in the Serion ELISA for IgG and IgA detection, respectively. In the MIF test, 1/10 diluted sera were used. The recombinant ELISA was performed on two-fold serum dilutions (starting at 1/100) of kaolin pre-treated sera (Novak et al., 1993). Recombinant MOMP was produced in pcDNA1::MOMP A transfected COS7 cells (Vanrompay et al., 1998). IgG and IgM titers were determined using rMOMP coated ELISA plates and 1:500 dilutions of horseradish peroxidase-labelled anti-human IgG (H+L) and anti-μ-chain specific antibodies (Nordic Immunological Laboratories, Tilburg, The Netherlands). Results were positive if the absorbance exceeded the cut off value of the mean of three negative control sera plus two times the standard deviation. Positive control sera originated from 3 humans infected while visiting a turkey farm experiencing a *Cp. psittaci* genotype A infection (Verminnen et al., 2008a).

2.4. Biochemical analyses

All subjects were fasting, refrained from smoking for at least 6 hours, and were screened for active infection/inflammation. Those with recent or active infection/inflammation were asked to return for blood sampling after their symptoms had subsided for at least 10 days. Serum parameters were measured using commercial reagents on a Modular P system (Roche Diagnostics, Mannheim, Germany) in an ISO 9002

certified reference laboratory.15 Hs-CRP concentrations were measured by a high-sensitive, particle-enhanced immunoturbidimetric method on an Integra 400 analyzer (Roche Diagnostics, Mannheim, Germany). Latex particles coated with mouse monoclonal anti-CRP antibodies were used. Precipitation was determined at 552 nm. The method was standardized with regard to the CRM 470 (RPPHS 91/0619) reference preparation of the IFCC.15 Coefficient of variation of all tests was <3.0%. Excessive alcohol intake was defined as an averaged intake of > 2 units daily for women and >3 units daily for men.

2.4. Statistics

Potential zoonotic risk factors were analyzed by SPSS 15.0.1.1.

3. Results

3.1. Contact frequency with different bird species

Based on the questionnaire, completed by all 540 participants, 254 (47%), 58 (10.7%), 108 (20%) and 120 (22.2%) had daily, weekly, sporadically or no contact with birds. In the daily-contact group (n = 254), most people had contact with canaries (65.3%), followed by *Psittaciformes* (46.4%) (Table 1). In the weekly-contact group (n=58) most people had contact with racing pigeons (63.7%) followed by canaries (60.3%). In the sporadic contact group (n = 108) most people had contact with *Psittaciformes* (29.63%) followed by canaries (28.7%).

Sixty one, 58.6 and 83.3% of the participants having daily, weekly or sporadically contact with birds, actually had contact with more than one bird species. Participants in contact with only one bird species are presented in Table 2.

3.2. Polymerase chain reactions

Sixty-nine of 540 (12.7%) pharyngeal swabs (44 and 25 of female and male participants, respectively) contained *C. psittaci* DNA. Positives included 57 of 254 (22.4%), 11 of 58 (19%), 0 of 108 (0%) and 1 of 120 (0.8%) persons having daily, weekly, sporadic, or no contact with domestic or companion birds, respectively. Thus, 471 of 540 (87.3%) examined individuals were negative. Of those, 197 (41.8%), 47 (10%), 108

(22.9%) and 119 (28.5%) had daily, weekly, sporadic, or no contact with birds, respectively. None of 69 *C. psittaci* positive swabs contained *C. pneumoniae* DNA.

Table 2.1 Contact frequency with different bird species

Birds	Daily n=254	Weekly n=58	Sporadic n=108	Total n=420
Canaries	166 (65.3%)	35 (60.3%)	31 (28.7%)	232 (55.2%)
Psittaciformes	118 (46.4%)	24 (41.3%)	32 (29.63%)	174 (41.4%)
Pigeons	58 (22.8%)	37 (63.7%)	24 (22.2%)	119 (28.3%)
Ducks	53 (20.8%)	14 (24%)	21 (19.4%)	90 (21.4%)
Geese	48 (19%)	21 (36.2%)	21 (19.4%)	88 (21%)
Turkeys	24 (9.4%)	7 (12%)	15 (13.8%)	46 (11%)

3.3. Serology

All 69 *Cp. psittaci* PCR positives and 96 *Cp. psittaci* and *Cp. pneumoniae* PCR negatives, were examined by the rMOMP ELISA, the MIF test and the Serion Chlamydia ELISA.

Twenty-eight on 69 (40.6%) sera (21 and 7 of female and male participants, respectively) reacted positive in the rMOMP ELISA. Positive sera originated from 24 and 4 people having daily or weekly contact with domestic or companion birds, respectively. Serum from the single PCR positive person out of the 'no-contact group' was negative in the rMOMP ELISA. IgG antibody titers ranged from 1/100 to 1/400, while IgM antibody titers were 1/100. Three of 28 (10.7%) sero-positives had only IgM, 11 individuals had IgM and IgG, and 14 had only IgG.

Only 3 of 69 (4.3%) sera of *Cp. psittaci* PCR positives reacted positive in the MIF test, revealing IgG titers of 16, 64 and 16, respectively and IgA titres of 16, <16 and 32, respectively. The rMOMP ELISA revealed IgG titers of 1/100, 1/100 and 1/200 in those 3 sera, respectively. Interestingly, positive control sera were negative in the MIF test. Surprisingly, all 69 *Cp. psittaci* PCR positives were negative in the Serion ELISA while

positive control sera contained IgG, but no IgA.

Ninety-six *Cp. psittaci* and *Cp. pneumoniae* PCR negatives were assigned to 4 groups having: 1) daily, 2) weekly, 3) sporadic or 4) no contact with birds. Interestingly, 11 of 43 (25.6%), 3 of 8 (37.5%), 1 of 4 (25%) and 3 of 41 (7.3%), thus 18 sera (18.7%) of individuals having daily, weekly, sporadic or no contact with birds, respectively, reacted positive in the rMOMP ELISA. Of those 18 positive sera, 9 and 9 were from female and male participants, respectively. The IgG titers ranged from 1/100 to 1/400 while the IgM titers were 1/100. Six of 18 (33.3%) had IgG and IgM, while 1 (5.5%) and 11 (61%) only had IgM or IgG antibodies, respectively. Sera of 96 PCR negatives were negative in the MIF test and in the Serion ELISA.

Zoonotic risk related to daily or weekly contact with one single bird species is presented in Table 2.3 and 2.4, respectively. Sporadic contact with one bird species gave no positives.

3.4. Statistics

Individuals in contact with *Psittaciformes*, especially parakeets and persons in contact with racing pigeons were significantly more frequently infected with *Cp. psittaci* (Table 2.2). Daily contact with especially racing pigeons and parakeets (Table 2.3) is significantly more dangerous than contact with canaries. Interestingly, weekly contact with *Psittaciformes* (parakeets + parrots) (Table 2.4) resulted in a significant higher infection rate than weekly contact with pigeons or canaries.

Thirteen (19%) of 69 PCR positives were smokers. However, smoking appeared to have no significant effect on acquiring psittacosis. The odd ratio for the PCR positives of 41-45 year old was higher (odds ratio [OR] 1.53, 95% confidence interval [CI] 0.88–2.66, $p < 0.16$) but was not significantly different regarding the studied population.

In a model containing age, body weight, presence of diabetes, excessive alcohol intake, educational achievement, fruit & vegetable intake, hypertension and smoking, non-exposition related risk factors for seropositivity were gender (for women compared to men: OR 2.620, 95% CI 1.296-5.293; $p< 0.007$) and excessive alcohol intake (for excessive users versus non-excessive: OR 2.215 95% CI 1.084-4.522; $p< 0.029$). Similarly, non-exposition related risk factors for PCR positivity were gender (for women compared to men: OR 1.942 95% CI 1.077-3.501; $p< 0.026$) and excessive alcohol intake (for excessive users versus non-excessive: OR 1.705 95% CI 0.923-3.148; $p< 0.088$).

We analyzed the effect of seropositivity and/or PCR positivity on inflammation (white blood cell count, high-sensitive CRP, fibrinogen). After adjustement for known confounders, we found no clear significant pattern suggesting an inceased inflammatory burden. In general, seropositivity trended to slightly higher levels of inflammatory variables (all non-significant), whilst PCR positivity trended to no-effect or even lower inflammatory levels. We tested wether the subgroup of subjects having at least weekly contact with psitacciformes had more inflammation (irrrespective of PCR or serostatus), but found no evidence of increased inflammatory burden.

Table 2.2. *Cp. psittaci* zooonotic risk related to contact of humans with one certain bird species.

Contact with:	Number (%) of persons on 540 examined individuals, in contact with only one bird species	PCR positives[a] (%)	PCR and/or ELISA[b] positives *(%)	OR (95% CI) [C]	P value
Psittacine birds	31 (5.7)	7 (22.5)	8 (25.8)	6.3 (2.6-15.2)	0.00001
Parakeet	23 (4.2)	6 (26.0)	6 (26)	5.9 (2.2-15.8)	0.00001
Parrot	8 (1.5)	1 (12.5)	2 (25)	4.9 (1-24.3)	0.050
Canary	69 (12.8)	9 (13.0)	12 (17.4)	4.8 (2.2-10.5)	0.00001
Pigeon	32 (5.9)	7 (22)	8 (25)	6.0 (2.5-14.7)	0.00001
Duck	2 (0.3)	0 (0)	0 (0)	-	-
Goose	5 (0.9)	0 (0)	0 (0)	-	-
Turkey	5 (0.9)	1 (20)	1 (20)	3.8 (0.43-33.8)	0.23
Total	144	24	29	-	-

[a]Each individual had only contact with one bird species; [b]rMOMP ELISA; [c]Odd ratio for PCR and/or ELISA positives with 95% confidence intervals

Table 2.3. *Cp. psittaci* zoonotic risk related to daily contact with one single bird species.

Total number of persons daily in contact with						
Parakeet	Parrot	Canary	Pigeon	Duck	Goose	Turkey
16	5	55	17	1	3	4
Number of persons (%) positive by PCR and/or rMOMP ELISA						
5 (31.2%)	1 (20%)	10 (18.2%)	7 (41.2%)	0 0	0 0	1 (20%)

Table 2.4. *Cp. psittaci* zoonotic risk related to weekly contact with one single bird species.

Total number of persons weekly in contact with						
Parakeet	Parrot	Canary	Pigeon	Duck	Goose	Turkey
3	2	8	9	0	1	1
Number of persons (%) positive by PCR and/or rMOMP ELISA						
1 (33.3%)	1 (50%)	2 (25%)	1 (11%)	0 0	0 0	0 0

4. Discussion

We strongly believe that the annually reported psittacosis cases do not reflect the real *Cp. psittaci* infection status in Belgium. Moreover, scientific reports of the COST855 on animal chlamydiosis and zoonotic implications indicate the underestimation of psittacosis in Europe (www.vetpathology.uzh.ch/forschung/CostAction855/). We therefore examined the prevalence of *Cp. psittaci* infections in a randomly selected apparently healthy human population sample of East-Flanders, in contact with domestic and/or companion birds.

Canaries and *Psittaciformes*, especially parakeets and parrots were the most popular pet birds, followed by racing pigeons, ducks, geese and turkeys. Individuals in contact with *Psittaciformes* and racing pigeons are more frequently infected. However, daily contact with *Psittaciformes* is as dangerous as weekly contact, while daily contact with racing pigeons is significantly more dangerous than weekly contact. This is in accordance with the literature, ascribing most psittacosis cases to contact with *Psittaciformes* (Smith et al., 2005; Kaibu et al., 2006; Heddema et al., 2006c). One single contact can be result in zoonotic transfer (Harkinezhad et al., 2007; Matsui et al., 2007). Psittacosis cases described in literature are on severe disease with pneumonia and in some cases even cardiac and neurological complications. However, in this study (which was primarily focused on cardiovascular disease and ageing, the main interest of the researchers was chronic inflammation. Those with recent or active infection/inflammation were asked to return for blood sampling after their symptoms had subsided for at least 10 days. Thus, no correlation between the presence of chlamydial DNA and clinical respiratory disease can be drawn.

Surprisingly, also canaries seemed to present a substantial zoonotic risk, as 45 of 69 (65.2%) PCR positives had contact with canaries. However, only 9 (20%) of them had exclusively contact with canaries. Thirty of them (66.6%) also had contact with *Psittaciformes*, while 21 (46.6%), 16 (35.5%), 16 (35.5%) and 8 (17.7%) also had contact with pigeons, ducks, geese or turkeys, respectively. Thus, *Psittaciformes*, rather then canaries, were more likely responsible for the high number of positives claiming to have contact with canaries. The disease occurs more often in *Psittaciformes* and the course of infection is also more severe, often leading to mortality as illustrated by Vanrompay et al., (1993a), isolating 45 *Cp. psittaci* strains from 264 dead birds, among them only four (8.8%) isolates of canaries and 25 (55.5%) isolates from *Psittaciformes*. Additionally, over

a period of 11 years (1991-2001), Dovc *et al*. (2005), found 14 of 1677 (0.8%) canaries to be sero-positive, compared to 238 of 3869 (6.2%) parrots.

Racing pigeons and especially daily contact with racing pigeons means a serious zoonotic risk. Although not examined, feral pigeons must also present a substantial zoonotic risk as several studies showed feral pigeons to be highly infected (Salinas et al., 1993a; Travnicek et al., 2002; Dovc et al., 2004; Tanaka et al., 2005; Heddema et al., 2006b). At present, there was one *Cp. psittaci* PCR positive person, claiming no contact with domestic or companion birds. Maybe, zoonotic transfer occurred through contact with infected feral pigeons or other wild birds such as waterfowl. On the other hand, the person could also became infected by human to human transmission although this is thought to be extremely rare (Hughes et al., 1997; Ito et al., 2002).

The rMOMP ELISA is routinely used in our department for epidemiology in birds as the test is highly sensitive and specific as compared to LPS- or whole organisms-based serological assays (Verminnen et al., 2006). However, birds only become infected with *Cp. psittaci*, while humans can become infected with other chlamydial species with *Cp. trachomatis* and *Cp. pneumoniae* being most prevalent. As MOMP carries family-specific epitopes, the presence of cross-reactive antibodies against *Cp. pneumoniae* and *Cp. trachomatis* must be ruled out. The presence of cross-reactive *Cp. trachomatis* antibodies can be examined by the *Cp. trachomatis* specific MOMP-peptide-based ELISA of Medac. However, there is no *Cp. pneumoniae* or *Cp. psittaci*-specific serological assay for humans. We used the Medac ELISA to check for cross-reactive antibodies (data not shown) and only 1 of 28 rMOMP ELISA positive sera was also positive for *Cp. trachomatis* IgA, none was positive for *Cp. trachomatis* IgG. However, we still cannot rule out cross-reactive antibodies to *Cp. pneumoniae* MOMP, but MOMP of *Cp. pneumoniae* is not immunodominant and all 28 rMOMP ELISA positives were negative by the *Cp. pneumoniae* nested PCR.

The MIF test, for long regarded as the serological ‘gold standard’, is poorly sensitive as only 3 out of 69 (4.3%) sera reacted positive, and moreover, positive control sera remained negative. The MIF test cannot be regarded as chlamydial specific, due to cross reaction of chlamydial LPS or heat shock protein with antibodies to other bacteria (Haralambieva et al., 2001) and like the rMOMP ELISA is not chlamydial species-specific (Bourke et al., 1989; Ozanne and Lefebvre, 1992). The latter was nicely demonstrated by Ceglie et al (2007), who found 24 of 34 (70.6%) pigeon sera positive for both *Cp. psittaci* and *Cp. pneumoniae*, with titers up to 1:256, although birds are not infected by *Cp.*

pneumoniae and molecular characterization of isolates clearly demonstrated the presence of *Cp. psittaci*. Additionally, the MIF test is subjective and tiring, only semi-quantitative and has poor reproducibility (Peeling et al., 2000).

The Serion ELISA is believed to be more sensitive than MIF. However, this was not true in our study as none of the 69 *Cp. psittaci* PCR positives reacted positive. Maybe, because the Serion ELISA uses genotype D antigen, while genotype A is being used in both MIF and rMOMP ELISA. The majority of PCR positives had contact with *Psittaciformes*, mostly being infected with genotype A. The Serion ELISA uses whole chlamydial organisms and is therefore not chlamydial species-specific.

In conclusion, *Cp. psittaci* infected racing pigeons and *Psittaciformes* present a main risk for psittacosis as compared to other domestic and companion birds. Zoonotic transfer can result in severe disease. However, as demonstrated in the present study, severe cases are probably only the tip of the iceberg. What lies underneath are asymptomatic infections or less severe, clinically unnoticed infections, which are misdiagnosed due to symptoms similar to those caused by other respiratory pathogens. The impact of these 'unnoticed' infections on human health is difficult to determine.

We demonstrated the usefulness of a highly sensitive and specific nested PCR/EIA for detecting *Cp. psittaci* DNA in human clinical specimens. In general, clinicians should be strongly recommended to use nucleic acid amplification tests for diagnosing psittacosis rather than serology as molecular tests are extremely sensitive and specific and present direct evidence of a *Cp. psittaci* infection.

Additionally, we also demonstrated the usefulness of serology for epidemiological research, as 18 of 96 PCR negatives were serologically positive. However, a *Cp. psittaci*-specific recombinant or peptide-based ELISA must be designed as present cross-reactive tests cannot be used for a human population, as *Cp. pneumoniae* is highly prevalent in humans.

Finally, psittacosis occurs more often than reported in Belgium. Psittacosis is most likely also underestimated in other European countries, stressing the need for more accurate diagnostic monitoring and reporting, a veterinary vaccine, and information campaigns with recommendations for psittacosis risk reduction strategies.

Acknowledgements

J.M. Ossewaarde is acknowledged for performing the Serion antibody ELISA. The Federal Department of Health, Food Chain Safety and Environment financed this study. The Asklepios research group, financed by the Fund for Scientific Research Flanders, is acknowledged for providing all samples. We gratefully acknowledge the funding by the Fonds voor Wetenschappelijk Onderzoek (research grant G042703, the Asklepios Study).

Chapter III

Chlamydophila psittaci infections in psittacine birds (*Psittaciformes)* and transmissions to humans

Part A:

First report on *Chlamydophila psittaci* genotype E/B transmission from parrots to men

Adapted from:

Harkinezhad T, **Verminnen K**, **Van Droogenbroeck C**, **Vanrompay D**. **2007.** *Chlamydophila psittaci* genotype E/B transmission from African grey parrots to humans**.** J Med. Microbiol. 2007; 56(8):1097-100.

Summary

Thirty-six birds from a parrot relief and breeding centre as well as the manager were examined for the presence of *Chlamydophila psittaci*. In the relief unit, 5 on 20 Grey amazon parrots showed depression, ruffled feathers, loss of weight and mild dyspnoea. The birds received no antibiotic treatment. Birds of the breeding unit, 14 Blue and gold macaws and 2 Green-winged macaws were healthy. They received doxycyclin at the start of each breeding season. The manager complained of shortness of breath but took no medication. Using a nested polymerase chain reaction enzyme immunoassay (nested PCR/EIA), *Cp. psittaci* was detected in the faeces of all five sick birds as well as in a nasal and pharyngeal swab of the manager. The veterinarian and her assistant became infected while sampling the parrots as pharyngeal and nasal swabs of both were positive in the nested PCR/EIA after visiting the parrot relief and breeding centre. Bacteria were isolated from 3 on 5 nested PCR/EIA positive birds, from the manager and the veterinarian, but not from the veterinary assistant. Using an *ompA* genotype-specific real-time PCR, *Cp. psittaci* genotype E/B was identified as the transmitted strain. All breeder birds tested *Cp. psittaci* negative. This is the first report on *C. psittaci* genotype E/B transmission from parrots to humans. In contradiction to genotype A strains which are thought to be highly virulent to both birds and men, the currently described genotype E/B strain apparently caused no severe clinical symptoms in both parrots and humans.

1. Introduction

Chlamydophila psittaci, an obligate intracellular bacterium has 7 known avian serovars, all considered transmissible to humans (Andersen, 1991b; Vanrompay et al., 1993a; Andersen, 1997). The serovars are identified by monoclonal antibodies recognizing serovar-specific epitopes on the major outer membrane protein (MOMP). These serovars correspond to 7 genotypes that are readily distinguished using outer membrane protein A (*ompA*) gene sequencing, restriction fragment length polymorphism (RFLP) analysis of the *ompA* gene (Vanrompay et al., 1997) or a recently developed genotype-specific real-time PCR (Geens et al., 2005b).

Genotype A is endemic among cockatoos, parrots, parakeets and lories (*Psittaciformes*) and is well known as a zoonotic agent. Genotype B is endemic among

pigeons. Genotypes C and D are mostly detected in non-*Psittaciformes*. They are highly pathogenic to domestic poultry and are admitted occupational hazards for slaughterhouse workers and generally for all people in contact with poultry, especially turkeys and ducks. Genotype E isolates were first isolated during an outbreak of pneumonitis in humans in the late 1920s and early 1930s. Subsequently, genotype E isolates have been obtained from a variety of avian hosts worldwide, including turkeys, ducks, pigeons, ostriches and rheas. Genotype F was first obtained from an American parakeet (Andersen, 1997) and eight years later also from a Belgian fattening turkey (Van Loock et al., 2005a).

Recently, a new genotype E/B was described (Geens et al., 2005a), being isolated from Italian urban pigeons, German commercial fattening Pekin ducks and Belgian fattening turkeys. Genotype E/B reacts with both the serovar E and B-specific monoclonal antibodies and generates the *ompA* RFLP pattern characteristic for genotype E. However, it differs from genotype E and B by a unique combination of a guanosine at positions 1,006 and 1,021 and a cytidine at position 1,022 resulting in an A instead of S at position 341 in the variable segment 4 of the MOMP. Genotype E/B can only be distinguished from genotypes E and B by *ompA* sequencing or by the recently developed genotype-specific real-time PCR (Geens et al., 2005b). Presently, the genotype-specific real-time PCR was used to examine the transmission of a *Cp. psittaci* genotype E/B strain from African grey parrots to humans.

2. Material and methods

2.1. Background

In November 2004, a parrot relief and breeding centre nearby Antwerp was visited in order to investigate the presence of *Cp. psittaci* infections in birds and their owners. The parrot relief unit housed twenty Grey Amazon parrots (*Psittacus erithacus)*, received from people moving houses or elderly people unable to nurse their birds any longer. The parrots were kept in separate cages. Additionally, 7 couples of Blue and gold macaws (*Ara ararauna*) and one couple of Green-winged macaws (*Ara chloroptera*) were kept for breeding. Relief and breeder birds were housed in clean, heated, separate, but adjacent rooms. Every day, the 56 year-old manager was handling the parrots of the relief unit, cleaning their cages, hand feeding and even nuzzling the birds. The man felt healthy although he complained of continued shortness of breath. He took no medication and was a non-smoker. The breeder birds were treated with doxycyclin (Soludox, Eurovet) in the

drinking water during 14 days before each breeding period and they were healthy. However, 5 out of 20 (25%) birds of the relief unit showed depression, ruffled feathers, loss of weight and mild dyspnoea. One of them was blind. They received no antibiotic treatment.

2.2. Samples

All birds as well as the manager were sampled using dacron-tipped aluminium shafted swabs (Fiers) filled with 2ml *Cp. psittaci* transport medium (Vanrompay *et al*., 1992) or DNA stabilization buffer (Roche) for chlamydial isolation or detection of the *ompA* gene, respectively. As bird catching was to be avoided due to stress, fresh droppings were collected from each cage floor sampling five different spots per cage. The manager was sampled by taking both pharyngeal and nasal swabs. The veterinarian and her assistant, were identically examined 'by chance' 7 weeks later, at the start of a psittacosis occupational risk study in which they voluntarily participated. Humans were sampled with informed consent. All swabs were kept on ice during transport and stored at –80°C until use.

2.3. Cp. psittaci identification and molecular characterization

The presence of the *Cp. psittaci ompA* gene was examined using a nested PCR/EIA as described by Van Loock *et al.,* (2005b). The nested PCR/EIA allows an ELISA-based detection of the amplified gene, which becomes biotin and fluorescein labelled during the amplification reaction. The presence of viable *Cp. psittaci* was examined by isolation in Buffalo Green Monkey (BGM) cells identifying the organisms by a direct immunofluorescence staining (IMAGEN™, Dakocytomation, Denmark), as previously described (Vanrompay et al., 1992). *Cp. psittaci* was characterized directly from the clinical specimens using a genotype-specific real-time PCR as described by Geens *et al*., (2005b). The latter, is a TaqMan probe-based real-time PCR able to distinguish the *ompA* gene of all 6 currently described avian *Cp. psittaci* genotypes A to F, plus the recently described genotype E/B.

3. Results and discussion

Notwithstanding possible air contact with infected birds of the relief unit, all breeders were *Cp. psittaci* negative in both nested PCR/EIA and culture. Regular doxycyclin treatment might have prevented spreading of the infection to the adjacent breeder unit. With the risk of developing tetracycline resistant strains, as described for the *Cp. psittaci* related species *Cp. suis* (Dugan et al., 2004; Dugan et al., 2007), this is surely not to be recommended as a preventive strategy. However, there is no avian *Cp. psittaci* vaccine and therefore owners of *Psittaciformes* frequently use tetracyclines in any case of respiratory disease or even prophylactically. One of the problems is that you can buy these drugs through the internet even without a prescription as a prescription is not needed in every country. Most people are unaware of the dangerous situation they might create by using these drugs frequently. They are ignorant on tetracylines being the drugs of choice when treating human psittacosis and the fact that mortality rate prior to the advent of antimicrobial treatment was approximately 15-20%. Vaccination as well as information campaigns on antibiotic use in birds with respect to zoonotic agents might prevent the development of tetracycline resistant *Cp. psittaci* strains.

For the relief unit, nested PCR/EIA revealed 5 out of 20 (25%) *Cp. psittaci* positive parrots and *Cp. psittaci* could be isolated from the faeces of three of them. The chlamydial *ompA* gene was detected in the nasal and pharyngeal swab of the manager. Nasal and pharyngeal swabs of the veterinarian and her assistant, examined 7 weeks after visiting the parrot and relief centre, also contained *Cp. psittaci* DNA. As in birds, the infection of the manager and the veterinarian were confirmed by isolating viable bacteria. *Cp. psittaci* isolates from the parrots, the manager and the veterinarian were all characterized as *ompA* genotype E/B. However, at that time, we could not isolate live chlamydial organisms from the veterinary assistant nor could we type the strain directly from the clinical specimens, as the genotype-specific real-time PCR remained negative. Of the three persons involved, the veterinary assistant was the one with least close contact to the parrots as she was only handing over the swabs to the veterinarian. In contradiction to the other two persons, she never went into the cages. This could explain why she was negative by culture and only positive by our nested PCR/EIA, which is able to detect 1 organism, while our genotype-specific real-time PCR has a detection limit of 10 chlamydial organisms.

During a subsequent medical consult, the manager was advised not to take antibiotics because he apparently showed no severe clinical symptoms. However, the manager

decided to close the parrot relief unit, not only due to our findings but also for economical reasons as heating costs became too high. All birds of the relief unit were treated with doxycycline and subsequently given to intimate Psittaciformes bird breeders. So far, there were no reports on sickness or abnormal mortality in these birds (personal communication, 2006). However, as already mentioned by Heddema *et al*. (2006c), the maintenance of accurate records of bird-related transactions for at least 1 year should be recommended and every new bird brought into a colony should be tested for *Cp. psittaci*. Track records should include the species of bird, bird identification, source of bird and any identified illness. Upon closure of the relief unit, the manager tested negative in the nested PCR/EIA and by culture.

As in the birds and the manager, the infection caused no severe clinical symptoms in the veterinarian and here assistant. In fact, the latter two persons were unaware of being infected. Therefore, they received no antibiotic treatment. Both persons tested negative in the nested PCR/EIA and in culture 10 weeks after visiting the parrot relief and breeding centre.

Worldwide, *Cp. psittaci* has been proven to infect at least 465 different avian species from 30 avian orders that include 153 *Psittaciformes* species (Kaleta and Taday, 2003). From 1988-2003, 935 human cases of psittacosis were reported to the US centers for disease control and prevention (CDC) (Smith et al., 2005). At present, approximately 100 cases of human psittacosis are reported annually in the United States, and one person may die from it each year. Many more cases may occur that are not correctly diagnosed or reported. However, the incidence of psittacosis in men seems to be increasing in developed countries, which is correlated to the import of exotic birds.

Birds of the order *Psittaciformes* seem to be highly susceptible as most veterinary case reports are on *Cp. psittaci* infections in *Cacatuidae* (Cockatoos) and *Psittacidae* (parrots, parakeets, lories), dealing with severe clinical signs such as eye discharge or swelling, laboured breathing, diarrhoea, poor appetite, lethargy, "fluffed up" appearance, or weakness and even mortality (Bracewell and Bevan, 1986; Kaleta and Taday, 2003; Chahota et al., 2006). Many of the birds become chronically infected but show no clinical signs until stressed. These birds often shed *Cp. psittaci* intermittently and serve as a source of infection for humans and other birds. Especially, Amazon parrots (88%), macaws (87%), budgerigars (81%), Cockatoos (80%), Conures and Senegal parrots (78%), African greys and Eclectus (75%), Grey cheek parrots (70%), lovebirds (68%) and Cockatiels (65%) are highly infected as serologically demonstrated (Fudge, 1997).

However, we do not really know if *Psittaciformes* are more susceptible to *Cp. psittaci* than other bird species. It might be that the disease is simply noticed and therefore diagnosed more often due to the severe clinical signs in these birds. For instance, when comparing epidemiological data of chlamydiosis in *Psittaciformes* and pigeons, the prevalence seems to be comparable ranging from 16 to 81% and 23 to 85%, repectively (Fudge, 1997; Travnicek et al., 2002). However, chlamydiosis in pigeons is less severe. Signs of uncomplicated chlamydiosis in pigeons are variable and often the disease is unnoticed. Those that develop acute disease are anorexic, unthrifty, and diarrhetic. Some develop conjunctivitis, swollen eyelids, and rhinitis. Respiratory difficulty is accompanied by rattling sounds. As disease progresses, birds become weak and emaciated. Recovered birds become carriers without signs of disease. Some birds progress through an infection showing no signs or, at the most, transient diarrhea before becoming carriers. Mortality is more due to secondary infections such as Salmonellosis and Trichomoniasis.

In fact, the overall clinical picture in a given species is the result of strain virulence and host immunogenetics. We do know less about bird immunogenetics but we do know that *Cp. psittaci* genotypes A, B, and F have been isolated from *Psittaciformes*. However, *Psittaciformes* are most frequently infected with genotype A strains which are highly virulent, are intensively excreted and are often causing mortality (Andersen, 1991b; Vanrompay et al., 1997). Pigeons on the other hand, mostly become infected by genotype B and sometimes genotype E strains. However, the term genotype only refers to the sequence of the outer membrane (*ompA*) gene encoding for the *Cp. psittaci* immunodominant major outer membrane protein (MOMP). We do know that the MOMP is a porine and also a possible bacterial adhesine, but the direct contributions of this protein to the virulence of the bacterium are not clear. Actually, we do not know why *Psittaciformes* are most frequently infected with genoytype A strains which apparently cause severe disease and mortaltity in these birds and why pigeons are most frequently infected by other genotypes which apparently are less virulent. The question could probably be solved if we would know more about host immunogenetics and bacterial virulence factors such as the Type Three Secretion System (TTSS), which was recently discovered in *Cp. psittaci* (Beeckman et al., 2006).

However, given the fact that *Psittaciformes* are almost always infected with genotype A strains and the proven zoonotic transmission of genotype A strains (Heddema et al., 2006c), we were surprised to find genotype E/B in both parrots and men. So far, genotype E/B has only been discovered in pigeons, ducks and turkeys. All birds appeared clinically

healthy except for the turkeys, which showed mild respiratory symptoms. Apparently, an asymptomatic psittacosis infection occurred in all three persons involved. Maybe, the E/B strain was indeed less virulent, although zoonotic transmission occurred. On the other hand, frequent exposure to *Cp. psittaci* could also explain the asymptomatic course of infection in these persons. However, we don't know anything about natural protection following a *Cp. psittaci* infection in humans. We do know that fattening turkeys can become infected with two different genotypes during one production period of 15 weeks. We do know that *Cp. psittaci* can go into a carrier status, characterized by dormant aberrant bodies, i.e., enlarged pleomorphic RBs, and reduced inclusion size inside the host cell with the absence of elementary bodies capable of infecting new cells (Goellner et al., 2006). In turkeys, a *Cp. psittaci* infection aggravated the outcome of a viral avian pneumovirus infection (Van Loock et al., 2006a) and an *Escherichia coli* infection in *Cp. psittaci* latently infected turkeys, aggravated the *E. coli* infection and reactivated chlamydial replication and excretion (Van Loock et al., 2006b). However, we can only speculate on similar pathogenic interactions between *Cp. psittaci* and human respiratory pathogens.

The number of psittacosis cases is surely underestimated in humans. For accurate diagnosis in both birds and humans we recommend the nested PCR/EIA, which detects *Cp. psittaci* specifically at high sensitivity. The genotype-specific real-time PCR is very convenient for tracking down the avian source and allows the identification of new *ompA* genotypes. Above all, a *Cp. psittaci* vaccine for *Psittaciformes* is urgently required, as it will definitely diminish the number of symptomatic and asymptomatic zoonotic infections in humans.

Chapter III

Chlamydophila psittaci infections in psittacine birds (*Psittaciformes)* and transmissions to humans

Part B:

Chlamydophila psittaci transmission from pet birds to humans

Adapted from:

Daisy Vanrompay, Taher Harkinezhad, Marijke Van de Walle, Delphine Beeckman, Caroline Van Droogenbroeck, Kristel Verminnen, Ruud Leten, An Martel, and **Katty Cauwerts**. **2007.** *Chlamydophila psittaci* transmission from pet birds to humans. Emerg. Infect. Dis. 13(7):1108-10

Abstract

We studied *Chlamydophila psittaci* zoonotic transmissions in 39 breeding facilities for *Psittaciformes* (cockatoos, parrots, parakeets, lories). Antibiotics were frequently used. Nevertheless, genotype A or E/B transmissions occurred in 14.9% of the humans involved. Information campaigns on sensible antibiotic use in *Psittaciformes* and a *Cp. psittaci* vaccine are urgently required.

1. Introduction

Chlamydophila psittaci, an obligate intracellular gram-negative bacterium has 7 known genotypes A to F and E/B (Geens et al., 2005a), all considered transmissible to humans causing psittacosis or parrot fever (Vanrompay et al., 1997; Andersen and Vanrompay, 2003). Genotypes are distinguished using outer membrane protein A (*ompA*) gene sequencing or by a recently developed *ompA* genotype-specific real-time PCR (Geens et al., 2005b). *C. psittaci* has been proven to infect 465 different avian species from 30 avian orders with at least 153 species from the order *Psittaciformes* (Kaleta and Taday, 2003). From 1988-2003, 935 human cases of psittacosis were reported to the US centers for disease control and prevention (Smith et al., 2005), most of them related to contact with *Psittaciformes*. Presently, approximately 100 psittacosis cases are reported annually in the United States, and one person may die from it each year. The incidence of psittacosis in men seems to be increasing in developed countries, which is related to the import of exotic birds. Many more cases occur that are not correctly diagnosed or reported.

2. The study

We investigated the occurrence of *C. psittaci* zoonotic transmissions in Belgian breeding facilities for *Psittaciforme*s (cockatoos, parrots, parakeets, lories). Participants were recruited through the Belgian Society for Parakeet and parrot breeders. Fifty breeding facilities received a sampling package, delivered by regular mail. The package contained a questionnaire designed to asses information on: a) the pet bird owners professional and non-professional activities, smoking habits, general health status, use of medication, allergy, clinical signs specifically related to psittacosis and on b) the birds

origin, housing, feeding, breeding, health status, medication and presence of other bird species. We also included dacron-tipped swabs and instructions for faecal (cage floor) or pharyngeal sampling in birds or humans, respectively (Fig 3.1). Additionally, we provided transport medium (Vanrompay et al., 1992) for isolation and DNA stabilization buffer (Roche) for PCR, to be added to the swabs after sampling. Packages returned by express mail one day after sampling were stored at –80°C until use. Sampling packages are convenient and above all safe for the investigator. Forty-one on 50 (82%) breeders returned the packages. Two packages were incomplete and therefore excluded, resulting in 308 birds and 46 humans from 39 *Psittaciformes* breeding facilities to be tested. We also got the opportunity to take pharyngeal samples from a veterinary student, involved in another study in those breeding facilities. The student was examined before starting his study and thereafter monthly during 4 months. All humans were examined with informed consent.

The presence of the *ompA* gene in birds and men was examined using a *C. psittaci*-specific nested PCR/EIA (Van Loock et al., 2005b). The presence of viable *C. psittaci* was examined by isolation in Buffalo Green Monkey cells identifying the organisms by a direct immunofluorescence staining (IMAGEN™, Dakocytomation), (Fig. 3.2), (Vanrompay et al., 1992). In case of zoonotic transmissions, the infection source was traced using a genotype-specific real-time PCR (Geens et al., 2005b) on specimens of both the birds and their owner.

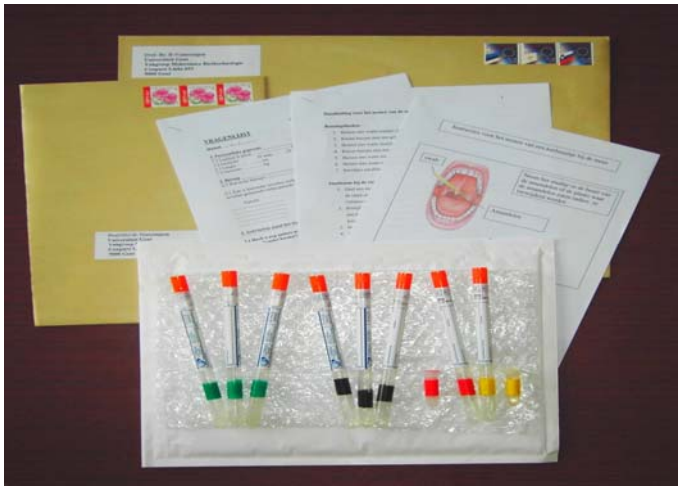

Fig. 3.1. Package sent to the breeders to sample their birds and themselves.

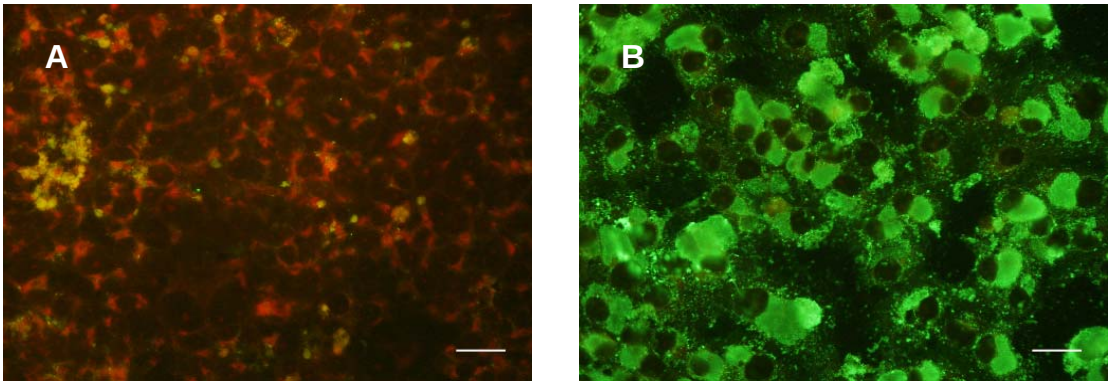

Fig. 3.2. Direct immunofluorescence (FITC) staining (IMAGEN®) of BGM cells 6 days after inoculation (bar 20 µm). Negative control (**A**), a positive parrot sample (**B**) with characteristic chlamydial inclusions inside the cytoplasm of the host cell.

Fifty-nine out of 308 (19.2%) *Psittaciformes* were positive in the nested PCR/EIA and bacteria were isolated from 25 (42.3%) PCR-positive birds. Thus, on a total of 39 tested breeding facilities, 8 (20.5%) were positive in both nested PCR/EIA and culture, and respiratory disease was present in all. Five additional breeding facilities were only nested PCR/EIA positive. Interestingly, one of them was currently treating the birds with doxycycline and the remaining 4 had recently used doxycycline, oxytetracycline or enrofloxacine. Treatment was successful as live *C. psittaci* were no longer present in these 5 breeding facilities and all birds appeared healthy. Thus, in total we had 13 on 39 (33.3%) positive breeding facilities with a significant correlation between faecal excretion of live chlamydial organisms and respiratory disease (OR 14.5, 95% CI 1.6-130.5, $p < 0.05$). The remaining 26 breeding facilities with healthy birds were negative in both PCR and culture. Table 3.1 shows the bird species that have been tested in this study. We are aware that we might have missed early infections as faecal excretion occurs following primary bacterial replication in the respiratory tract and septicaemia. Moreover, faecal shedding occurs intermittently and healthy carrier birds might not excrete for more than one year. Pharyngeal swabs would have been a better choice but psittacine owners are reluctant to catch their birds, as stress induces respiratory disease. Reactivation of a *C. psittaci* carrier status might be involved in this phenomenon.

Nested PCR/EIA revealed *Cp. psittaci* DNA in 6 out of 46 (13%) pet bird owners, all from different breeding facilities and all with birds diagnosed positive in both PCR and culture. Most of the infected persons (age 22, 24, 31, 38, 49 and 56) were relatively young as the mean age of the pet bird owners was 46. Live organisms were present in 4 out of 6

PCR positive humans and all 4 (non-smokers, non-allergic) showed mild respiratory illness (shortness of breath or rhinitis and coughing). However, although the study was performed in the summer period, 8 (20%) of the remaining 40 negative breeders complained of rhinitis and/or coughing during the last two weeks, sometimes (7.5%) together with a sore throat. Thus, it was impossible to draw statistical conclusions on the relationship between the presence of viable chlamydial organisms in humans and respiratory disease. Remarkably, the veterinary student (age 23) tested negative before starting his study but his first pharyngeal specimen after start visiting the breeders was positive in both PCR and culture. He remained infected until one month after ending his study but showed only mild respiratory illness. Thus, protective clothing, including air filter face masks are recommended for preventing occupational disease, as the infection risk may not be underestimated. The transmitted *C. psittaci* strains in 5 pet bird owners and in the veterinarian were all genotype A and those persons showed rhinitis and coughing while genotype E/B was present in one pet bird owner complaining of continued shortness of breath. Thus, in contradiction to the recently published reports on human psittacosis related to pet birds (Telfer et al., 2005; Kaibu et al., 2006; Heddema et al., 2006c), severe clinical signs with pneumonia did not occur in these humans and therefore none of them was treated. Interestingly, 10 on 39 (25.6%) humans had pneumonia since keeping *Psittaciformes*, while normally 8/1000 pneumonia cases occur each year in Belgium. Most likely, pet bird owners and veterinarians are regularly infected and maybe they become protected against severe disease. But, we don't know if they become carriers and what might be the consequences of this.

It was alarming that 18 on 39 (46.2%) breeders treated their birds with tetracycline, doxycycline or enrofloxacine over the past year. Four on 39 (10.2%) were even using tetracyclines prophylactically. Nevertheless, of those 18 breeding facilities, 8 (44%) still tested positive in the nested PCR/EIA while culture was positive in 3 (16.6%). With the risk of developing antibiotic resistant strains, as being described for *C. suis* (Dugan et al., 2004; Dugan et al., 2007), the regular use of antibiotics must be avoided. However, there is no vaccine and therefore pet bird owners frequently use tetracyclines in case of respiratory disease and sometimes even prophylactically. These drugs are sold on the internet even without a prescription as a prescription is not needed in every country. People are ignorant on tetracylines being the drugs of choice when treating human psittacosis and the fact that mortality rate prior to the advent of antimicrobial treatment was 15-20%.

In conclusion, vaccination and information campaigns on sensible antibiotic use in *Psittaciformes* are urgently needed to prevent psittacosis in men as well as the development of antibiotic resistant strains.

Table 3.1. Bird species tested from 39 breeding facilities.

No	Family	Genus and species	Common names	Number tested	Number Positives	Percentage positives/species
1	***Psittacidae***	*Agapornis personata*	Masked Lovebird	5	0	0
2		*Agapornis roseicollis*	Rosy-faced Lovebird	3	0	0
3		*Agapornis taranta*	Black-winged Lovebird	4	1	25
4		*Amazona aestiva aestiva*	Blue-fronted Amazon	2	1	50
5		*Amazona aestiva xanthopteryx*	Blue-fronted Yellow-shouldered Amazon	2	1	50
6		*Amazona amazonica amazonica*	Orange-winged Amazon	1	0	0
7		*Amazona auropalliata*	Yellow-naped Amazon	3	0	0
8		*Amazona autumnalis autumnalis*	Red-lored Amazon	6	3	50
9		*Amazona festiva bodini*	Bodinus' Amazon	1	0	0
10		*Amazona oratrix*	Yellow-headed Amazon	2	0	0
11		*Amazona viridigenalis*	Green-cheeked Amazon	1	0	0
12		*Aprosmictus erythropterus erythropterus*	Red-winged Parrot	2	0	0
13		*Ara ararauna*	Blue and Yellow Macaw	1	0	0
14		*Ara auricollis*	Yellow-collared Macaw	6	1	16
15		*Ara chloropterus*	Green-winged Macaw	1	0	0
16		*Aratinga jandaya*	Jandaya Conure	2	1	50
17		*Aratinga solstitialis*	Sun Conure	5	1	20
18		*Bolborhynchus aurifrons aurifrons*	Mountain Parakeet	6	1	16
19		*Bolborhynchus aymara*	Aymara Parakeet	5	2	40
20		*Bolborhynchus lineola lineola*	Barred Parakeet	6	2	33

Table 3.1. Bird species tested from 39 breeding facilities (continued).

No	Family	Genus and species	Common names	Number tested	Number Positives	Percentage positives/species
21		*Eclectus roratus roratus*	Grand Eclectus Parrot	11	1	9
22		*Forpus coelestis*	Pacific Parrotlet	13	3	23
23		*Loriculus philippensis regulus*	Central Island Hanging Parrot	6	0	0
24		*Melopsittacus undulatus*	Budgerigar	5	0	0
25		*Nannopsittaca dachilleae*	Amazonian Parrotlet	3	0	0
26		*Neophema pulchella*	Turquoise parrot	6	2	33.3
27		*Neophema splendida*	Scarlet-chested Parrot	6	2	33.3
28		*Neopsephotus bourkii*	Bourke's Parrot	5	3	60
29		*Northiella haematogaster haemetogaster*	Blue-bonnet	1	0	0
30		*Pionites melanocephala melanocephala*	Black-headed Caique	6	1	16.7
31		*Pionopsitta barrabandi barrabandi*	Barraband's Parrot	2	0	0
32		*Pionus menstruus*	Blue-headed Parrot	1	0	0
33		*Pionus menstruus menstruus*	Blue-headed Parrot	2	0	0
34		*Pionus senilis*	White-capped Parrot	1	0	0
35		*Platycercus*	Crimson rosella	10	0	0
36		*Platycercus adelaidae adelaidae*	Adelaide Parakeet(Rosella)	1	0	0
37		*Platycercus adscitus palliceps*	Pale-headed Rosella	1	0	0
38		*Platycercus barnardi barnardi*	Barnard's Parakeet	2	0	0
39		*Platycercus caledonicus*	Green Rosella	2	0	0
40		*Platycercus elegans elegans*	Crimson Rosella	4	0	0

Table 3.1. Bird species tested from 39 breeding facilities (continued).

No	Family	Genus and species	Common names	Number tested	Number Positives	Percentage positives/species
41		*Platycercus icterotis icterotis*	Western Rosella	1	0	0
42		*Platycercus zonarius semitorquatus*	Twenty-eight Parrot	1	0	0
43		*Platycercus zonarius semitorquatus*	Twenty-eight Parrot	1	0	0
44		*Platycercus zonarius zonarius*	Port Lincoln Parrot	2	0	0
45		*Poicephalus senegalus senegalus*	Senegal Parrot	6	0	0
46		*Poicephalus gulielmi gulielmi*	Jardine's Parrot	2	0	0
47		*Poicephalus meyeri meyeri*	Meyer's Parrot	1	0	0
48		*Polytelis alexandrae*	Princess Parrot	1	0	0
49		*Psephotus chrysopterygius*	Golden-shouldered Parrot	2	0	0
50		*Psephotus haematonotus haematonotus*	Red-rumped Parrot	6	3	50
51		*Psittacula cyanocephala*	Plum-headed Parakeet	1	0	0
52		*Psittacula derbyana*	Derbyan Parakeet	2	0	0
53		*Psittacula eupatria eupatria*	Alexandrine Parakeet	3	0	0
54		*Psittacula krameri*	Rose-ringed Parakeet	20	3	15
55		*Psittacus erithacus*	African Grey Parrot	22	2	9
56		*Psitteuteles goldiei*	Goldie's Lorikeet	2	1	50
57		*Psitteuteles iris iris*	Iris Lorikeet	2	1	50
58		*Psitteuteles versicolor*	Varied Lorikeet	2	0	0
59		*Pyrrhura rhodocephala*	Rose-crowned Conure	9	2	22.2
	Cacatuidae					
60		*Cacatua alba*	White Cockatoo	1	0	0

Table 3.1. Bird species tested from 39 breeding facilities (continued).

No	Family	Genus and species	Common names	Number tested	Number Positives	Percentage positives/species
61		*Cacatua leadbeateri leadbeateri*	Major Mitchell's Cockatoo	2	0	0
62		*Cacatua sulphurea sulphurea*	Sulpher-crested Cockatoo	1	0	0
63		*Eolophus roseicapillus roseicapillus*	Roseate Cockatoo	12	2	16.6
64		*Nymphicus hollandicus*	Cockatiel	21	8	38
	Loriidae					
65		*Charmosyna placentis subplacens*	Sclater's Red-flanked Lorikeet	2	1	50
66		*Lathamus discolor*	Swift Parrot	4	2	50
67		*Trichoglossus haematodus moluccanus*	Blue Mountain Lorikeet	6	3	50
68		*Trichoglossus rubritorquis*	Red-collared Lorikeet	2	1	50
	Platyceridae					
69		*Eunymphicus cornutus cornutus*	Hooded parrot	1	0	0

Chapter IV

Protection of budgerigars (*Melopsittacus undulatus*) against *Chlamydophila psittaci* challenge by DNA vaccination

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This chapter is adapted from:

Taher Harkinezhad, Katelijn Schautteet and Daisy Vanrompay. 2008. Protection of budgerigars (*Melopsittacus undulautus*) against *Chlamydophila psittaci* challenge by DNA vaccination.

Abstract

Plasmid DNA (pcDNA1::MOMP A) expressing the major outer membrane protein (MOMP) of *Chlamydophila psittaci* genotype A strain 89/1051 has been tested for its ability to induce protective immunity against *Cp. psittaci* challenge in budgerigars. Eight pairs of male and female budgerigars were housed in eight separate bird cages placed in two negative pressure isolators, four cages per group. All budgerigars were immunized twice intramuscularly with 100 µg plasmid DNA. Both groups received a primary DNA inoculation at day 0 followed by a booster inoculation 3 weeks later. Group 1 received pcDNA::MOMP A while group 2 received the placebo vaccine pcDNA1. Budgerigars were challenged by aerosol two weeks following the booster vaccination. The challenge consisted of 10^8 TCID50 of the homologous *Cp. psittaci* genotype A strain. Cloacal and pharyngeal swabs of all budgerigars, taken prior to the experimental infection were negative in both PCR and culture. However, all budgerigars showed low pre-existing serum antibody titers, indicative of a previous infection. Nevertheless, DNA immunization could significantly reduce clinical symptoms, macroscopic lesions, pharyngeal and cloacal excretion as well as chlamydial replication, even in the presence of pre-existing serum antibodies, as compared to the placebo-vaccinated controls.

1. Introduction

Chlamydophila psittaci, a Gram-negative obligate intracellular pathogen, causes chlamydiosis in *Psittaciformes* (cockatoos, parrots, parakeets, lories) and psittacosis or parrot fever in humans. The infection is highly prevalent in *Psittaciformes* as studies in wild and captive *Psittaciformes* in North and South America, Australia, Europe and Japan showed 16 to 81% of examined birds to be positive for *Cp. psittaci* (Yamashita and Hirai, 1981; Schwartz and Fraser, 1982; Hirai et al., 1984; Bracewell and Bevan, 1986; Dorrestein and Wiegman, 1989; Raso et al., 2002; Dovc et al., 2005). The outer membrane protein A (*ompA)* genotype A is found most often in these birds. The infection is also highly prevalent among captive Belgian *Psittaciformes* as we recently demonstrated by examining 39 Belgian *Psittaciformes* breeding facilities. Fifty-nine (19.2%) of 308 *Psittaciformes* were positive for *Cp. psittaci* by nested PCR/EIA, and bacteria were isolated from 25 (42.3%) birds with PCR-positive results. Eight on 39 (20.5%) breeding facilities were positive in both nested PCR/EIA and culture, and respiratory disease was

present at all these facilities (Vanrompay et al., 2007). Interestingly, we not only discovered 'the expected' genotype A, but for the first time also detected genotype E/B strains in *Psittaciformes* (Harkinezhad et al., 2007). Moreover, we also demonstrated the transmission of this genotype E/B from African grey parrots to humans. Thus, as in other countries, infected Belgian *Psittaciformes* present a zoonotic risk, which is clearly underestimated as shown in Chapters II and III.

In birds, the course of infection can be rather mild but on the other hand a mortality rate of 50% or even higher is not unusual (Dovc et al., 2007). Faecal and nasal excretions of diseased birds are the primary source of human infections (Smith et al., 2005; Heddema et al., 2006c; Harkinezhad et al., 2007; Vanrompay et al., 2007). However, apparent clinically healthy *Psittaciformes* also present a threat to human health, as many cockatoos, parrots, parakeets and lories never get rid of the bacterium once being infected and most of them actually become *Cp. psittaci* carriers, shedding the bacteria again after being stressed (Schachter et al., 1978; Andersen and Vanrompay, 2003).

Thus, a considerable number of people are at risk of becoming infected with this bacterium such as people working in pet shops, garden centres, quarantine stations and zoos. But, also visitors of these facilities and people keeping *Psittaciformes* as pets can become infected. Vaccinating *Psittaciformes* could significantly reduce the zoonotic risk. However, there is no commercial vaccine available against avian chlamydiosis. Maybe, DNA vaccination might be used for future vaccination of *Psittaciformes,* as the significance of DNA immunization as a means of preventing severe clinical signs, lesions and bacterial excretion, has been demonstrated in a specific pathogen free turkey and chicken experimental model of *Cp. psittaci* infection (Vanrompay et al., 1999a; Vanrompay et al., 1999b; Vanrompay et al., 2001a; Van Loock et al., 2004; Verminnen et al., 2005; Zhou et al., 2007). DNA vaccination induces humoral (B cell) and cellular (CD4/CD8) immune responses, only low doses are needed (µg), DNA vaccination is effective in the presence of maternal antibodies, reversion to virulence as compared to live bacteria is not possible, as compared to killed vaccines no toxic treatment is needed, DNA vaccines are easy to design and lyophilization is possible, transport and storage does not require a cold chain and DNA vaccines are more stable at room temperature.Therefore, we evaluated the ability of plasmid DNA expressing the major outer membrane protein (MOMP) of a *Cp. psittaci* genotype A strain to raise protective immunity in budgerigars (*Melopsittacus undulatus*) experimentally infected with the homologous *Cp. psittaci* strain. The *ompA* gene encoding the MOMP was used because it is the only chlamydial antigen

which has been unambiguously identified as protective antigen. MOMP also caries serovar- neutralising epitopes. Taking into account our previous DNA vaccination results in SPF turkeys, we tried to increase the immunogenicity of the vaccine further by optimizing the codon-usage.

2. Material and methods

2.1. Cp. psittaci strain

Cp. psittaci strain 89/1051, isolated from the liver of blue-fronted Amazon parrot (*Amazona aestiva aestiva)* (Vanrompay et al., 1993a), was used. The strain was previously characterised using both serovar-specific monoclonal antibodies, outer membrane protein A (*ompA*) restriction fragment length polymorphism (RFLP) analysis and sequencing of the *ompA* gene and was classified as an avian serovar/genotype A strain (Vanrompay et al., 1993a; Vanrompay et al., 1997; Geens et al., 2005a). The bacteria were grown in Buffalo Green Monkey (BGM) cells as previously described (Vanrompay et al., 1992) and the 50% tissue culture infective dose ($TCID_{50}$) was determined on BGM cells by the method of Spearman and Kaerber (Mayer et al., 1974).

2.2. DNA vaccine

Plasmid pcDNA1::MOMP A was constructed by sticky-end ligation of the outer membrane protein A (*omp A*) gene of *Cp. psittaci* genotype A strain 89/1051 into the *Eco*RI and *Bam*HI site of pcDNA1 (Vanrompay et al., 1998). The *ompA* gene was codon optimized for expression in avian cells (GenScript Corporation, USA). *E. coli* MC1061/P3 cells were transfected by electroporation (Gene Pulser; Bio-Rad, Nazareth, Belgium), and clones were selected on medium containing ampicillin plus tetracycline and grown in microtiter plates. The presence of inserts was confirmed by *Eco*RI and *Bam*HI restriction enzyme analysis of plasmid mini preparations (QIAGEN) and by PCR clone analysis using Sp6 and T7 primers, which flanked the cloning site. PCR clone analysis was performed in microtiter plates with the BioMek Thermal Cycler (Perkin-Elmer Cetus Zaventem, Belgium). First, 5 µl of each clone was subjected to PCR in a 50 µl final reaction mixture containing 50 mM KCl, 20 mM Tris-HCl (pH 8.3), 2 mM MgCl2, 0.1% Tween 20, 200 mM each deoxynucleoside triphosphate, 20 mM each primer, and 0.1 U of SuperTaq (15 U/ml) polymerase. Samples were subjected to 25 cycles of amplification.

Cycling conditions were as follows: denaturation for 30 s at 95°C, primer annealing for 1 min at 55°C, and primer extension for 2 min at 72°C. The sequence of the codon optimized *ompA* insert was confirmed by the VIB Genetic Service Facility (University of Antwerp, Antwerp, Belgium) using vector associated T7 and SP6 priming sites.

Finally, pcDNA1 control (placebo) and pcDNA1::MOMP A were grown in MC1061/P3 and purified using the Endo Free Giga plasmid preparation method (QIAGEN). DNA concentration was determined by measuring the optical density at 260 nm and was confirmed by comparing intensities of ethidium bromide-stained *Eco*RI/*Bam*HI restriction endonuclease fragments with standards of known concentrations. DNA was stored at -20°C in 1mM Tris (pH 7.8)-0.1 mM EDTA.

Expression of the *ompA* gene was confirmed by indirect imunofluorescence staining of DEAE dextran transfected COS-7 cells, transfected either with pcDNA1::MOMP A or pcDNA1 control plasmid, as described previously (Vanrompay et al., 1998). For each immunization 100 µg pcDNA1::MOMP A diluted in sterile saline (0.9% NaCl) was used.

2.3. Vaccination trial

The experimental design was evaluated and approved by the Ethical Commission for Animal Experiments of Ghent University. Sixteen one-year-old budgerigars were purchased from a private breeding facility in East-Flanders. We used conventional budgerigars, as SPF *Psittaciformes* are unavailable. Pharyngeal swabs of all birds of this breeding facility were negative in a *Cp. psittaci* species-specific nested PCR/EIA and in culture on BGM cells (Vanrompay et al., 2007). Eight pairs of male and female budgerigars were housed in eight separate bird cages placed in two negative pressure isolator units (IM 1500, Montair, Sevenum, The Netherlands) (Fig. 4.1), four cages per unit. In this way, two groups of four bird pairs each were created. Food (seed mixture for budgerigars, food sticks, sepia shells) and water were provided ad libitum.

All budgerigars were immunized intramuscularly (*m. quadriceps*) with 100 µg plasmid DNA in 100 µl sterile saline (0.9% NaCl). Both groups received a primary DNA inoculation at day one and one booster inoculation 3 weeks later. Group 1 received pcDNA::MOMP A while group 2 received the placebo vaccine pcDNA1. Budgerigars were challenged by aerosol (Cirrus Nebulizer, Intersurgical, UK) two weeks following the booster vaccination. The challenge consisted of 10^8 $TCID_{50}$ of the *Cp. psittaci* genotype A strain 89/1051. See Table 4.1 for experimental set-up of the vaccination trial.

Fig. 4.1. Negative pressure isolators were used for housing the birds.

2.4. Samples

All budgerigars were observed daily for clinical signs. Pharyngeal swabs were collected at the beginning of the experiment, at day 1 and at euthanasia, 21 days post challenge infection. Faecal excretion was evaluated at the day of challenge and subsequently every day until 20 days post challenge by taking swabs from fresh faecal droppings on the cage floor. All swabs were stored at -80°C in *Cp. psittaci* transport medium until tested. Blood samples for the quantification of MOMP- specific serum antibody titres were collected immediately prior to each DNA inoculation, immediately prior to the experimental infection and at the day of euthanasia. Blood samples were stored overnight at room temperature, centrifuged (325 x g, 10 minutes, 4°C) and afterwards serum was collected and frozen at -20°C until tested. At the time of euthanasia, 21 days post challenge, proliferative responses in spleen cells were determined. All budgerigars were examined for macroscopic lesions. Macroscopic lesions were scored (Table 4.2) as previously described (Vanrompay et al., 1994). Cryostat tissue sections of the lungs, conchae, abdominal and thoracic airsacs, pericardium, kidney, liver and gut (caecum) were examined for the presence of chlamydial antigen.

Table 4.1. Experimental set-up of the vaccination experiment

Time	Manipulation	Samples		
Day 0	Primary vaccination	Pharyngeal swabs	Cloacal swabs	Blood
Day 14	Booster vaccination	Pharyngeal swabs	Cage floor swabs	Blood
Day 35	Challenge	-	Cage floor swabs	Blood
Cage floor swabs were taken every day until euthanasia				
Day 56	Euthanasia	Pharyngeal swabs	Cloacal swabs	Blood

2.5. Cp. psittaci excretion

Pharyngeal and cloacal swabs were examined for the presence of viable *Cp. psittaci* by culture in BGM cells, as previously described (Vanrompay et al., 1994). The number of BGM cells with chlamydial inclusions was counted in five ad random selected microscopic fields (Radiance 2000MP, Bio-Rad; 600 x). For each cage or bird pair, the excretion was scored from 0 to 5. Score 0 indicated no chlamydophilae present; score 1 was given when a mean of 1-5 elementary bodies was present in the absence of replicating reticulate bodies; score 2, 3, 4 and 5 were given when a mean of 1-5, 6-10,11-20 and more than 20 inclusion positive cells was present. Pharyngeal and cloacal shedding is presented as a mean score per bird pair ± standard deviation (S.D.).

2.6. Cp. psittaci detection in the tissue organs

Cryostat tissue sections of different organs were examined by the IMAGEN^{TM} direct immunofluorescence staining (Novo Nordisk Diagnostics, Cambridge, UK), as previously described (Vanrompay et al., 1994). The presence of chlamydophila antigen in tissues of the placebo-vaccinated control group and of the pcDNA1::MOMP A vaccinated group was scored as mentioned above for swab cultures.

Table 4.2. Score system for macroscopic lesions

Tissue	Score		
	+	++	+++
Conchae	congestion	necrosis	necrosis and granulomata
Lungs	congestion bilateral	grey foci unilateral	grey foci bilateral
Airsacs	opacity	focal fibrinous airsacculitis	diffuse fibrinous airsacculitis
Pericard	serous pericarditis	fibrinous pericarditis	fibrinous adhesive pericarditis
Liver	congestion	pale	pale and soft
Spleen	slightly enlarged	moderately enlarged	severely enlarged
Kidney	slightly enlarged	moderately enlarged	severely enlarged
Gut	slightly congested	severely congested	enteritis

2.7. Antibody responses

Enzyme-linked immunosorbent assays (ELISAs) were performed on sera being pre-treated with kaolin to remove background activity (Novak et al., 1993). MOMP-specific antibody titres were determined using standard protocols with rMOMP as antigen directly coated on the plates. Recombinant MOMP was produced in COS-7 cells, transiently transfected with pcDNA1::MOMP A, as described previously (Vanrompay et al., 1998).

For the determination of antibody titres in serum, 1/1000 and 1/2000 dilutions of cross-reactive biotinylated anti-chicken/turkey IgG (H+L) antibody (Invitrogen) and peroxidase-conjugated streptavidin (Invitrogen) were used, respectively. Anti-MOMP immunoglobulin titres were presented as the reciprocal of the highest serum dilution that gave an optical density (OD_{405}) above the cut-off value. The cut-off value was the mean OD of a negative African grey parrot serum ± twice the standard deviation (S.D.).

2.8. Lymphocyte proliferative responses

Leukocytes were isolated from the spleen of each budgerigar, at 21 days post challenge. Lymphocyte proliferative tests were performed as previously described (Vanrompay et al., 1999b). Briefly, non-adherent cells were grown in duplicate in 96-well tissue culture plates at 6 x 10^5 cells in 150 µl of Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% heat-inactivated foetal calf serum (FCS; Integro,

Zaandam, The Netherlands), 1% non-essential amino acids (Life Technologies), 1% sodium pyruvate (Life Technologies), 1% L-glutamine (Life Technologies), 1% gentamycine (Life Technologies) and 5 x 10^{-5} M ß-mercaptoethanol (ß-ME; Life Technologies). For antigen proliferation, 20 µg of rMOMP was added to individual wells. Negative and positive controls included cells stimulated with either plain medium or with 10 µg concanavalin A (Con A), respectively. Cells were incubated at 39.5°C in a humidified incubator with 5% CO_2. Con A or antigen-induced proliferation was measured by incorporation of ^{3}H-thymidine (1 µCi/well) during at last 16 hours of culture, at days 2 (ConA) and 6 (DNA primed groups), respectively. Cultures were harvested onto glass fibre filter strips with a cell harvester (Skatron, Liers, Norway). The radioactivity incorporated into the DNA was measured with a β-scintillation counter (Perkin-Elmer, Life Science, Brussels, Belgium) and was expressed as a stimulation index (SI: mean counts per minute in the presence of antigen divided by the mean counts per minute in the absence of antigen).

2.9. Statistics

The two-tailed Student *t* test, Mann-Whitney test and one tailed Spearman's rank correlation was employed for statistical analyses. Results were considered significantly different at the level of $P < 0.05$.

3. Results

3. 1. Clinical symptoms

Clinical signs were first observed at five days post challenge. At that time, two non-vaccinated birds showed mild dyspnoea. Another non-vaccinated bird had diarrhoea (green watery droppings) and was shaking his head as a sign of rhinitis. Vaccinated birds appeared healthy. From day 6 onwards till the end of the experiment, all non-vaccinated birds showed severe dyspnoea and diarrhoea, all but one (87.5%) had fluffy feathers. Three out of eight birds (37.5%) showed lethargy and two out of eight birds (25%) were shaking their heads regularly (Figure 4.2). Symptoms in the non-vaccinated group were most severe from post inoculation day 6 till day 15. In the vaccinated group, two out of eight birds (25%) had diarrhoea at post inoculation day 11, four out of eight birds (50%)

showed dyspnoea from post inoculation day 10 till day 13 and at the same time five out of eight (62.5%) birds had fluffy feathers and one out of eight birds (12.5%) were lethargic. At euthanasia, at 21 days post challenge, only one out eight (12.5%) vaccinated birds showed fluffy feathers and was less active. All other vaccinated birds were more active as compared to the non-vaccinated control group as three out of four male birds were flaunting and some birds even played with the toys (mirror, bell) inside the cage. During the experiment, two out of eight (25%) vaccinated birds lost 7.0 and 7.7% of their body weight, respectively. In the non-vaccinated group, six out of eight (75%) birds lost 2.5, 2.6, 3.0, 5, 8.1 and 15.0% of their body weight, respectively.

3. 2. Cp. psittaci excretion

Faecal excretion was examined by bacterial culture in BGM cells. Faecal *Cp. psittaci* excretion was not observed before challenge and during the first four days post challenge. At five days post challenge, fresh faecal droppings present on the cage floor of three out of four (75%) non-vaccinated bird pairs were culture positive. Mean culture scores per bird

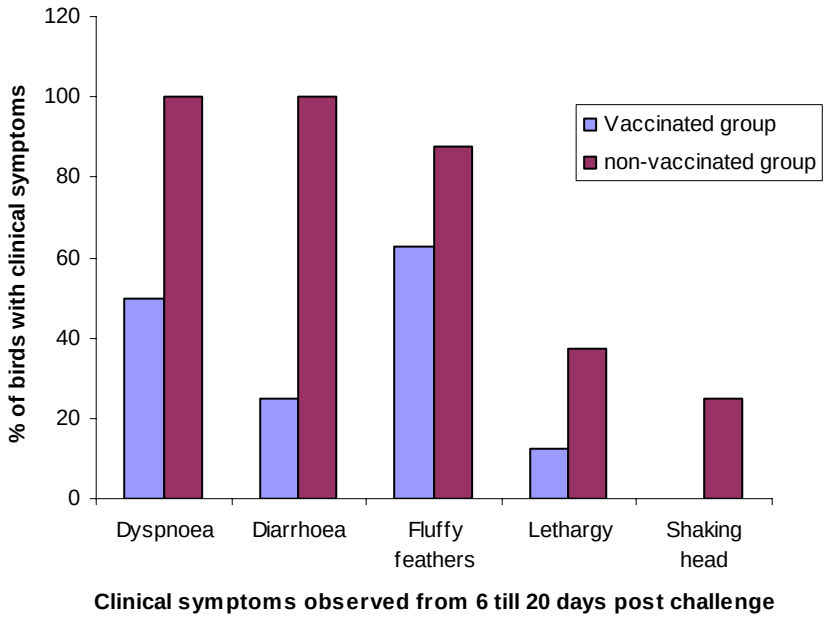

Fig. 4.2. Percentage of budgerigars with clinical signs observed in vaccinated and non-vaccinated groups from 6 till 20 days post challenge.

pair are presented in Figure 4.2. One day later, all four (100%) non-vaccinated bird pairs excreted live bacteria and the faeces of all pairs remained positive until 20 days post challenge. In the four pcDNA1::MOMP A vaccinated bird pairs, bacterial excretion was first observed at 6 days post challenge. At that time, two out of four (50%) vaccinated bird pairs were shedding *Cp. psittaci* and one day later, the faeces of all vaccinated bird pairs became culture positive. All four vaccinated bird pairs remained culture positive until 14 days post challenge. On day 15, two out of 4 (50%) vaccinated bird pairs were culture positive and from day 17 to 20, one out of four (25%) bird pairs was positive. Overall, non-vaccinated bird pairs showed significantly ($p<0.01$) higher mean culture scores as compared to the non-vaccinated control group (Figure 4.3). At 21 days post challenge, only one out of eight (12.5%) cloacal swabs of the vaccinated birds was positive with a mean immunofluorescence score of 0.37. On the other hand, all (100%) cloacal swabs of the non-vaccinated budgerigars were culture positive with a mean immunofluorescence score of 3.25.

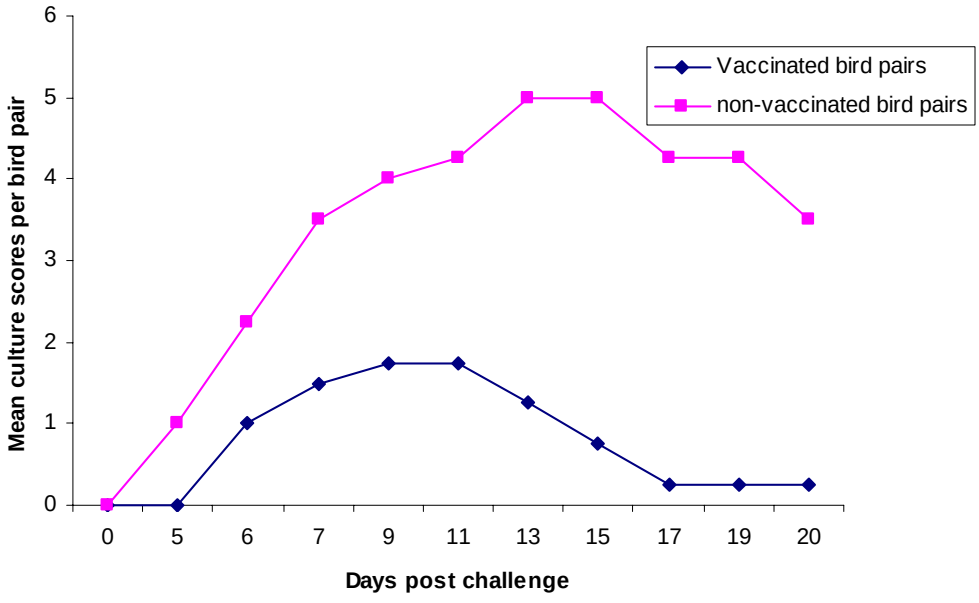

Fig. 4.3. Presence of *Cp. psittaci* in fresh faecal droppings of vaccinated and non-vaccinated bird pairs following challenge. The results of vaccinated and non-vaccinated birds were singnificantly different from day 5 until day 20.

3.3. Macroscopic lesions

At euthanasia, budgerigars were examined for the presence of macroscopic lesions. Lesion scores in individual birds of vaccinated and non-vaccinated groups are presented in Table 4.3. Macroscopic lesions were clearly more severe in the non-vaccinated group, with prominent lesions in especially the respiratory tract and the spleen as all budgerigars showed focal to diffuse fibrinous airsacculitis, bilateral congestion to bilateral pneumonia and a slightly to severely enlarged spleen. Additionally, all birds showed congestion of the gut, all but one (87.5%) had a congested liver, 5 out of 8 (62.5%) showed serous to fibrinous adhesive pericarditis, 3 out of 8 (37.5%) showed slightly to moderately enlarged kidneys and 2 out of 8 (25%) showed necrosis of the conchae. Macroscopic lesions in the vaccinated group were less severe and as in the non-vaccinated group mainly located in the respiratory tract and the spleen. Actually, all vaccinated budgerigars showed mild lesions in the lower respiratory tract, 6 out of 8 (75%) had a slightly enlarged spleen and 5 out of 8 (62.5%) showed mild lesions in the upper respiratory tract. No further lesions were observed in this group.

3. 4. Cp. psittaci presence in different tissues

Immunofluorescence staining of frozen tissue sections revealed strong chlamydial replication in the upper (conchae and trachea) and lower (lungs and airsacs) respiratory tract of all non-vaccinated budgerigars (Table 4.4). With the exception of the pericard of one bird, replication was less prominent in the pericard, liver, kidney and gut of non-vaccinated budgerigars. However, all non-vaccinated birds experienced a systemic infection as bacterial replication was clearly observed outside the respiratory tract resulting in faecal shedding in all birds until the end of the experiment at 21 days post challenge. The infection probably also spreads throughout the body of immunized birds as faecal shedding occurred in all four vaccinated bird pairs from 7 until 14 days post challenge. However, the infection seems to be eliminated more efficiently as demonstrated by diminished faecal shedding during subsequent days and by the absence of bacteria in 6 out of 8 (75%) cloacal swabs, at 21 days post challenge. Notwithstanding this, replicating *Cp. psittaci* organisms were still present in the upper and especially lower respiratory tract of all but one (87.5%) vaccinated budgerigars at 21 days post challenge.

Table 4.3. Macroscopic lesion scores in tissues of budgerigars, 3 weeks post *Cp. psittaci* challenge.

Tissue	Macroscopic lesion scores in tissues of budgerigars															
	Vaccinated bird number								Non-vaccinated bird number							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Conchae	+	-	+	+	-	+	+	-	-	-	-	-	++	-	-	++
Lungs	+	-	+	+	+	+	+	+	+	++	++	++	+++	+	+++	+
Airsacs	+	+	+	+	+	+	+	+	+++	++	++	++	+++	++	++	+++
Pericard	-	-	-	-	-	-	-	-	+++	-	-	-	+++	+	+	+++
Spleen	+	+	+	+	+	+	-	-	+++	+	+	+	++	+	++	+
Liver	-	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+
Kidney	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	++
Gut	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+

Table 4.4. Immunofluorescence scores for the presence of *Cp. psittaci* antigen in tissues of vaccinated and non-vaccinated budgerigars.

	Immunofluorescence scores for the presence of *Cp. psittaci* antigen															
	Vaccinated bird number								Non-vaccinated bird number							
Tissue	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Conchae	++	-	+	+	++	+	++	-	+	+	++	+	++	++	++	++
Trachea	+	-	+	+	-	-	+		++	+++	++	++	++	++	+	+
Lungs	+	-	-	-	+	-	+	-	++	++	+++	++	++	++	+	+++
Th. airsacs[a]	++	-	++	++	+	++	+++	-	+++	++++	++++	++	++++	+++	++	++++
Abd. airsacs[b]	++	-	++	++	+++	+++	+	+	+++	++++	++++	+++++	+++	+++++	++	+++
Pericard	-	-	-	-	-	-	-	-	+	-	+	+	++	+++	+	+
Liver	-	-	-	-	-	-	-	-	+	++	+	+	+	-	+	+
Kidney	-	-	-	-	-	-	-	-	++	+	++	++	+	+	+	++
Gut	-	-	-	-	-	-	-	-	+	++	+	-	+	+	+	+

[a]Th. airsacs: thoracal airsacs; [b]Abd. airsacs: abdominal airsacs.

3. 5. MOMP antibody responses

All budgerigars had anti-MOMP antibody titers (titer of 60 to 120) before being vaccinated with the *Cp. psittaci* genotype A vaccine pcDNA1::MOMP A or the placebo vaccine pcDNA1 (Fig. 4.4). MOMP antibody titers raised at least four-fold in all pcDNA1::MOMP A vaccinated birds, except for bird number 8, in which the MOMP antibody titer raised only two-fold. The effect of the booster vaccination was apparently less spectacular as antibody titers following pcDNA1::MOMP A booster vaccination raised only further in 3 out of 8 (37.5%) immunized budgerigars. Following challenge, antibody titers stayed identical in 4 out of 8 (50%), declined in 3 out of 8 (37.5%) and raised in 1 of 8 (12.5%) immunized budgerigars. Strangely, antibody titers raised four-fold in 2 out of 8 (25%) pcDNA1-vaccinated controls at 3 weeks post primary-vaccination. However, at the day of the experimental infection, MOMP-antibodies were absent in 2 out of 8 (25%) non-vaccinated control birds, and MOMP-antibody titers were 60 and 120 in 2 out of 8 (25%) and 4 out of 8 (50%) pcDNA1 immunized birds, respectively. Antibody titers in non-vaccinated controls did not augment following challenge, except for bird numbers 10, 12 and 16. There was a significant ($P<0.05$) difference in mean antibody titers between the vaccinated and non-vaccinated groups at 3 weeks post primary-vaccination and at all subsequent time points during the experiment.

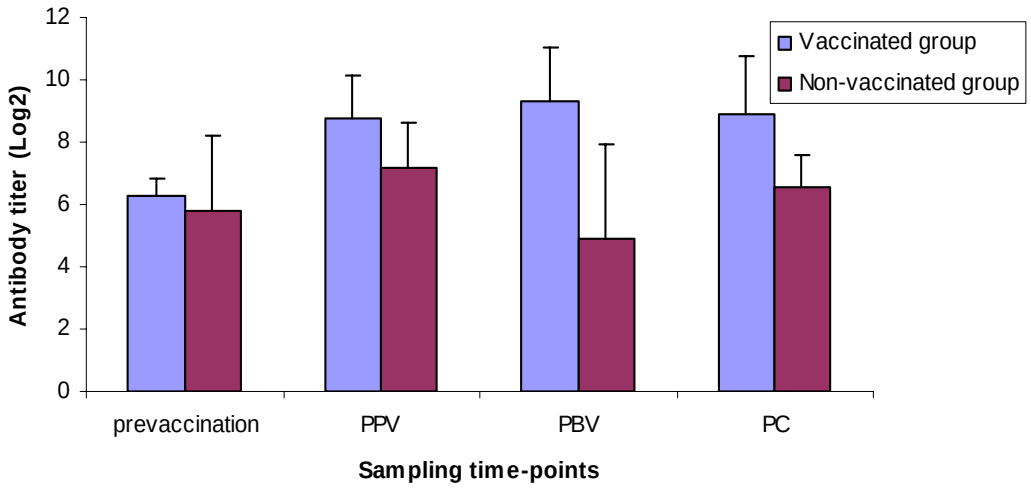

Fig. 4.4. MOMP specific antibody titers. The vaccinated group was intramuscular immunized with pcDNA1::MOMP. Budgerigars in the non-vaccinated group received an empty pcDNA1 plasmid (placebo). Sampling time-points: day of the primary vaccination (Prevaccination); post primary vaccination (PPV); post booster vaccination (PBV); post challenge (PC).

3.6. MOMP-specific lymphocyte proliferation test

Proliferative responses to rMOMP (genotype A strain 89/1051) of spleen lymphocytes of pcDNA1::MOMP A or pcDNA1 immunized control budgerigars were determined 21 days following challenge with the *Cp. psittaci* genotype A strain 89/1051. The spleen lymphocytes of pcDNA1::MOMP A immunized birds displayed significantly higher proliferative responses than the spleen lymphocytes of the placebo-vaccinated budgerigars (Table 4.5).

Table 4.5. Proliferative responses of spleen lymphocytes of immunized and non-immunized budgerigars to recombinant major outer membrane protein (rMOMP) at day 21 post challenge.

Group (n)*	Mean stimulation index ± S.D.
Non-vaccinated (8)	11.0 ± 2.25
Vaccinated (8)	25.45 ± 4.25

*Number of budgerigars per group.

4. Discussion

Reported human psittacosis cases are most often related to contact with *Cp. psittaci* infected poultry (ducks and turkeys) or *Psittaciformes*. *Psittaciformes* are kept in specialized breeding facilities, zoos and quarantine stations, but with respect to public health the increasing habit of keeping cockatoos, parrots, parakeets and lories as pets in many households, needs even more attention. In 2001, 750.000 budgerigars were kept as pet in the U.K. and households owning budgerigars comprised 2.8% of U.K. pet owners (http://www.defra.gov.uk/animalh/ and http://www.pfma.org.uk/). We have no recent data, nor do we have information from other countries. However, the current situation in Europe is most likely more or less the same as the one described for the U.K. in 2001, as *Psittaciformes* are still extremely popular pets.

Vaccination of *Psittaciformes* against *Cp. psittaci* infections might diminish the zoonotic risk, but successful vaccination could also improve the breeding performances of captive *Psittaciformes* and their general health status. Commercial *Cp. psittaci* vaccines are not existent in birds. Previously, we demonstrated the use of major outer membrane

protein (MOMP)-based DNA vaccination as a mean of preventing severe clinical signs and lesions in a specific pathogen free (SPF) turkey model of *Cp. psittaci* infection. The major outer membrane protein is an immunodominant protein representing the majority of the surface exposed proteins of *Cp. psittaci* and the MOMP contains serovar-specific neutralizing epitopes (Zhang et al., 1987; Kikuta et al., 1991; Sandbulte et al., 1996).

In the present study, pcDNA1::MOMP A immunized budgerigars were significantly protected against severe clinical signs and lesions following challenge with a very high dose (10^8 $TCID_{50}$) of the highly virulent homologous *Cp. psittaci* genotype A strain, 89/1051. Non-vaccinated budgerigars showed severe clinical signs and lesions and in contradiction to the vaccinated birds, excreted high amounts of *Cp. psittaci* during 3 weeks post challenge. However, notwithstanding the severity of the disease, none of the placebo-vaccinated control budgerigars died, as observed in specific pathogen free (SPF) turkeys infected with this highly virulent strain (Vanrompay et al., 1995b). This might be due to the pre-existing anti-MOMP serum antibody titers detected in all budgerigars prior to the experimental infection. Indeed, pharyngeal and cloacal swabs taken prior to the experimental infection were negative in both nested PCR and culture, but nevertheless, all budgerigars must at least once have been infected, during their stay in the breeding facility as they all had pre-existing MOMP serum antibody titers. Indeed, the prevalence of *Cp. psittaci* infections is high among *Psittaciformes* (Vanrompay et al., 2007) and therefore previous exposure to the organism is hardly surprising. Moreover, *Cp. psittaci* serum antibody titers in *Psittaciformes* can remain for months.

Interestingly, DNA vaccination could significantly reduce clinical symptoms, macroscopic lesions, bacterial excretion as well as chlamydial replication, even in the presence of serum antibodies originating from a previous infection. Maybe not surprisingly, as MOMP-antibody titers in pre-sera were rather low (maximum 120) and experimental DNA immunization is known to be effective in turkeys having high maternal antibody titers (Van Loock et al., 2004). Nevertheless, this could be an essential observation regarding future vaccination, as *Cp. psittaci* genotype A strains are endemic in *Psittaciformes* and most birds, if not all, will have antibodies against the organism.

As demonstrated before, serum antibodies did not protect against a respiratory *Cp. psittaci* infection, as all non-vaccinated controls got sick, even when having (low) pre-existing MOMP-serum antibody titers. We have no information on protective mucosal antibody titres in these birds prior to immunization, but the experimental infective dose was extremely high, thus clinical disease in non-vaccinated budgerigars was actually

inevitable, even in the presence of higher serum antibody levels and/or protective mucosal antibodies The experimental infective dose most likely did not reflect the natural infective dose. Actually, the latter is unknown and might vary depending on the virulence of the strain. Thus, it might be interesting to determine the protective capacity of the DNA vaccine using lower infective doses.

Budgerigars 2 and 8, responding best to both primary and booster vaccinations and showing no secondary antibody responses upon challenge were best protected. Similar observations have been made following MOMP-based DNA immunization in SPF turkeys. In turkeys, best protection occurred in immunized turkeys that upon challenge did not demonstrate secondary antibody responses, as the bacteria were probably unable to replicate to boosting levels (Vanrompay et al., 1999a; Vanrompay et al., 1999b; Vanrompay et al., 2001a; Van Loock et al., 2004).

During former DNA vaccination studies in turkeys, non-vaccinated birds showed a primary antibody response upon challenge. At present, bird number 13 and 16 showed a MOMP-specific immune response following immunization with the placebo vaccine, which could only mean that they experienced a *Cp. psittaci* infection during the first 3 weeks of the experiment. However, pharyngeal and faecal swabs of these two birds were PCR and culture negative at the day of the experimental infection, and at that time, 2 weeks post booster (placebo) vaccination, antibody titers were lower than after the primary-vaccination. Placebo-vaccinated birds 13 and 16 were most severely infected regarding clinical signs, macroscopic lesions and presence of bacteria in cloacal swabs at the end of the experiment. Maybe, these 2 birds were *Cp. psittaci* 'carriers' and handling them reactivated the persistent infection leading to a four-fold raise in antibody titer during the first 3 weeks of the experiment. However, bacterial shedding by these two birds prior to the experimental infection must have been low, as their cage mates (birds number 12 and 15) did not became infected. However, we could not proof the 'carrier hypothesis' as swabs prior to challenge were negative and the ones collected following the experimental infection only contained genotype A.

Currently, we have no real explanation for the absence of a significant antibody response in all but one (bird number 16) non-vaccinated controls. Maybe, circulating antibodies remained longer in vaccinated birds and serum antibodies in non-vaccinated birds were more difficult to detect as most antibodies were 'captured' by the extreme high amount of bacteria presenting in these heavily infected birds.

Evidence for the mobilization of T cell memory in response to challenge was shown by the significantly increased spleen lymphocyte proliferative response upon challenge when compared to the placebo-vaccinated control group. However, 10-fold increases in SI between vaccinated and non-vaccinated budgerigars as observed in previously performed DNA vaccination experiments in SPF turkeys, could not be demonstrated.

The essential observation of this report is that a codon-optimized, MOMP-based DNA vaccine is capable of circumventing the influence of circulating antibody inhibition. Budgerigars in the present study showed relatively moderate levels of pre-existing circulating antibodies and a totally inhibited antibody response to the vaccine construct in the presence of higher circulating antibody titers cannot be excluded. However, circulating antibody titers affected neither the induction of vaccine-specific T cell response nor significant protection in budgerigars. Therefore, prime-boost DNA vaccination may be useful for preventing *Cp. psittaci* infections in *Psittaciformes*, a bird population in which *Cp. psittaci* infections are nearly endemic. In addition, research on DNA vaccination in persistently infected birds would be useful in order to study possible clearance of the infection by DNA immunization.

Acknowledgments

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Chapter V

General conclusion and perspectives

General discussion and perspectives

In humans, *Cp. psittaci* causes psittacosis or parrot fever usually characterized by fever chills, headache, dyspnoea and cough. The chest X-ray often shows an infiltrate (Yung and Grayson, 1988; Schlossberg et al., 1993; Smith et al., 2005). However, the disease seems to vary considerably in severity as the clinical features of the infection can range from none to sepsis with multi-organ failure requiring intensive-care-unit admission (Heddema et al., 2006c). People usually contract the infection by inhaling an aerosol of droppings of infected birds.

Psittacosis is found worldwide and the incidence seems to be increasing in developed countries, which is correlated to the import of exotic birds. However, the actual number of human infections is probably still underestimated because psittacosis is difficult to diagnose, is covered by antimicrobials, which may be employed empirically for therapy of community-acquired pneumonia, and often is not reported. Annually reported psittacosis cases in Belgium were 7 in 2005, 2 in 2006 and 3 in 2007 (www.iph.fgov.be). Thus, specific data need to be collected to assess the current epidemiological situation concerning zoonotic transmission of *Cp. psittaci* in Belgium. Additionally, molecular analysis of the strains isolated from humans and birds would improve our knowledge of risk factors for bird-to-human transmission. Recent advances in molecular diagnosis of *Cp. psittaci* infections such as nested PCR/EIA (Van Loock et al., 2005b) and Real-time PCR (Geens et al., 2005b; Heddema et al., 2006a) have been recognized as powerful techniques for rapid, specific and sensitive diagnosis of chlamydial infections and were therefore used in the present study to obtain current epidemiological data.

Thus, it was our first purpose to examine the prevalence of *Cp. psittaci* infections in a generally healthy human population in contact with domestic and/or companion birds to gain insight on the real occurrence of psittacosis, as we strongly believe that the annually reported psittacosis cases do not reflect the real *Cp. psittaci* infection status of our general Belgian population. The prevalence study would also allow us to define the general human population being most at risk of getting psittacosis. Results obtained would allow subsequent examination of the prevalence and the clinical consequences of *Cp. psittaci* infections in a human population being highly at risk of getting psittacosis. Finally, it was our purpose to use the obtained knowledge on bird species and *Cp. psittaci* genotypes, most often involved in psittacosis, to design a recombinant vaccine and to evaluate the protective capacity of the vaccine in a well-defined avian experimental infection model.

In the first part of our research, we indeed confirmed the underestimation of human *Cp. psittaci* infections in Belgium as 69 (12.7%) pharyngeal swabs of 540 examined generally healthy Flemish persons contained *Cp. psittaci* DNA as demonstrated by the *Cp. psittaci*-specific nested PCR/EIA. *Cp. psittaci* PCR positives included 57 of 254 (22.4%), 11 of 58 (19%), 0 of 108 (0%) and 1 on 120 (0.8%) persons who, according the questionnaire, claimed to have daily, weekly, sporadic, or no contact with domestic or companion birds, respectively. Thus, contact frequency with birds seems to be an important risk factor. Interestingly, individuals in contact with parakeets, parrots (*Psittaciformes*) and racing pigeons are more at risk of becoming infected with *Cp. psittaci* than those in contact with other bird species. Additionally, daily contact with racing pigeons is significantly more dangerous than weekly contact. However, this is not the case for *Psittaciformes,* as the infection risk for humans is the same whether they are daily or only weekly in contact with parakeets or parrots. Thus, infected *Psittaciformes* are strictly seen more dangerous to public health. This could be due to the presence of predominantly *Cp. psittaci* genotype A strains in *Psittaciformes* against genotype B and sometimes E strains in pigeons. The latter genotypes are regarded less virulent for birds as they replicate slower and produce smaller inclusions with lower percentages of infectious elementary bodies (Vanrompay et al., 1993b). Thus, maybe bacterial excretion levels are not that high in pigeons, presenting a lower risk for bird to human transmission of *Cp. psittaci*. But how can we than explain the high percentage of positive individuals (44.6%) having daily contact with racing pigeons. Perhaps, because racing pigeons were kept as a group, all together creating high infection pressure, whereas parakeets and parrots were mostly housed individually and in case of parakeets sometimes as pairs.

Cp. psittaci zoonotic transmission from *Psittaciformes* can cause severe disease in humans (Samra et al., 1991; Newton et al., 1996; Kay, 1997; Soni et al., 1999; Henrion et al., 2002; Bourne et al., 2003; Saito et al., 2005; Kaibu et al., 2006; Heddema et al., 2006c). However, as demonstrated in our prevalence study, these severe cases are probably only the tip of the iceberg. What lies underneath are less severe, clinically unnoticed infections, which are misdiagnosed due to symptoms similar to those caused by other respiratory pathogens, or even asymptomatic infections. The impact of these types of *Cp. psittaci* infections on human health still needs to be determined. Nothing is known of natural protection, the consequences of recurrent infections, and the possible pathogenic interactions between *Cp. psittaci* and other human respiratory pathogens. We can try to extrapolate our knowledge on avian infections to human psittacosis. *Cp. psittaci* can go

into a carrier status in birds and although not proven it is also thought to be possible in men. Dormant aberrant bodies, i.e., enlarged pleomorphic reticulate bodies, and reduced inclusion size inside the host cell with the absence of elementary bodies capable of infecting new cells, characterize a carrier status (Goellner et al., 2006). In turkeys, an *Escherichia coli* superinfection in *Cp. psittaci* carriers aggravated the *E. coli* infection and reactivated chlamydial replication and excretion (Van Loock et al., 2006b). Additionally, a *Cp. psittaci* infection aggravated the outcome of a viral avian pneumovirus infection in turkeys (Van Loock et al., 2006a). However, we can only speculate on similar pathogenic interactions between *Cp. psittaci* and other human respiratory pathogens, but we do know that co-occurrence of *Cp. psittaci* with other human respiratory pathogens has been reported (Yano et al., 1990; Nakajima et al., 1996).

Interestingly, the prevalence study also demonstrated the usefulness of a nested PCR/EIA for detecting psittacosis in humans. The PCR/EIA was originally designed for demonstrating chlamydiosis in birds and is highly specific as primers were specifically designed for amplifying *Cp. psittaci* DNA and no other chlamydial DNA. The latter is extremely important when examining human specimens as humans can become infected with several chlamydial species. Clinicians should be strongly recommended to use nucleic acid amplification tests for diagnosing psittacosis in humans rather than serology as these molecular tests are extremely sensitive and specific, and present direct evidence of a *Cp. psittaci* infection. However, the U.S. Centers for Disease Control and Prevention (CDC), the U.S. National Association of State Public Health Veterinarians (NASPHV) and the U.S. Council of State and Territorial Epidemiologists (CSTE), world-wide all recognized as established 'advisory organizations', still promote the use of culture and serology for establishing clinical diagnosis of psittacosis (www.nasph.org; www.avma.org). This is incomprehensible as, the main serological tests commonly used for screening, the complement fixation test (CFT) and the so-called 'gold standard' MIF test (more sensitive than CFT), both do not distinguish *Cp. psittaci* from *Cp. pneumoniae* and *C. trachomatis*. Moreover, several researchers (Wong et al., 1994; Smith et al., 1997; Telfer et al., 2005; Fenga et al., 2007; Matsui et al., 2007) and also our present study showed that the MIF test is actually not a good test because of its lack of specificity, sensitivity, reproducibility, objectivity and agreed protocol. In addition, obligatory examination of paired sera removes serology from immediate clinical relevance. Treatment with antibiotics may also delay and/or diminish the antibody response, so a third serum sample might even be needed. Thus, to our opinion, these serological tests

might be helpful for comparative epidemiology, but it is quite a different matter to be confident about the meaning of their result in an individual human patient. Culture is also recommended by the CDC, the NASPHV and CSTE to confirm human psittacosis. However, culture can only be performed in specialized biosafety level 3 (BSL3) laboratories, it takes at least 3 days, sampling, transport and storage of specimens requires special attention, culture is technically difficult and some strains simply don't grow in cell cultures or embryonated eggs. Thus, nucleic acid amplification tests should be used to confirm psittacosis in humans and this should be made clear to the authorities, a currently task undertaken by the European COST action 855 on Animal chlamydiosis and its zoonotic implications. Nucleic acid amplification tests present important benefits as they allow rapid outbreak management and subsequent genotyping of the strains involved using for instance, a genotype-specific real-time PCR (Geens et al., 2005b), as demonstrated in Chapter III or a genotyping micro array (Sachse et al., 2005), allowing to trace the avian infection source.

In the second part of the research, we studied zoonotic transmissions of *Chlamydophila psittaci* in 39 Belgian breeding facilities for *Psittaciformes* (cockatoos, parrots, parakeets, lories) that frequently used antimicrobial drugs. Genotypes A (6 persons) and E/B (one person) were detected in 14.9% of humans at these facilities. Additionally, we illustrated the occupational risk for veterinarians and their assistants in a case report on *Cp.psittaci* genotype E/B transmission from African grey parrots to humans. We showed the need for information on sensible antimicrobial drug use in *Psittaciformes* as tetracycline resistant zoonotic *Cp. psittaci* strains could develop by frequent prophylactic use of these drugs. These drugs are sold on the internet, even without a prescription as a prescription is not needed in every country. Legal authorities should urgently deal with this problem, which presents a serious threat to public health.

Our findings contributed to raising the awareness of chlamydial infections, as well as their zoonotic dimensions. Ultimately, this should allow the introduction of improved prevention and control measures such as: 1) protection of persons at risk, advising them to wear protective clothing, gloves and a fitted respirator with appropriate P3 filter when cleaning cages or handling infected birds. Necropsies should be performed in a biological safety cabinet, preferably wetting the carcass with detergent and water to prevent aerosolization of the infectious particles, 2) Maintain accurate records of all bird-related transactions for at least one year to aid in identifying sources of infected birds and potentially exposed persons as currently trace-backs of infected birds to distributors

and breeders is often not possible because of limited regulation of the pet bird industry, 3) avoid to purchase or sell birds that have signs consistent with avian chlamydiosis, 4) isolate newly acquired birds, ill, or exposed birds, 5) test birds before they are to be boarded or sold on consignment, 6) screen groups of birds with frequent public contact routinely by nucleic acid amplification tests, 7) practice preventive husbandry (avoid contact with wild birds), 8) control the spread of an infection, 9) quarantine of all infected and exposed birds, 10) use disinfection measures, 11) bird importation regulations.

Zoonotic risk reduction strategies are urgently needed, but an avian vaccine would significantly reduce the risk of *Cp. psittaci* bird to human transfer. At present, only vaccines for chlamydioses in cats and sheep are commercially available, and some of those in use are known to have considerable limitations (Longbottom and Livingstone, 2006). Vaccination studies based on DNA vaccines and recombinant MOMP have recently been performed in turkeys. *Cp. psittaci* DNA vaccination significantly protected SPF turkeys against severe clinical disease and significantly reduced chlamydial excretion, as well as the lesions caused by this pathogen, whereas recombinant MOMP vaccination induced less protective immune responses (Van Loock et al., 2004; Verminnen et al., 2005). A DNA vaccination protein-boost study in SPF turkeys has been completed and shown to generate both cellular and humoral immune responses (Verminnen et al., 2005), although these responses were less protective than DNA prime-boost strategies. Therefore, we evaluated the ability of plasmid DNA (pcDNA1::MOMP A) expressing the major outer membrane protein (MOMP) of the *Cp. psittaci* genotype A strain 89/1051 to induce protective immunity against *Cp. psittaci* challenge in budgerigars. DNA immunization could significantly reduce clinical symptoms, macroscopic lesions, pharyngeal and cloacal excretion as well as chlamydial replication, even in the presence of pre-existing serum antibodies, as compared to the placebo-vaccinated controls. However, full protection was not achieved, if albeit possible.

We are convinced that the protection ability of the candidate vaccine can be further improved. There are several approaches to enhance the efficacy of DNA vaccines such as; improving uptake of plasmid into the cell and nucleus including gene gun delivery (Vanrompay et al., 1999a; Babiuk et al., 2003), phage delivery (Clark and March, 2004; March et al., 2004), liposome mediated transfection (Gregoriadis et al., 2002) and generating complexes of plasmid DNA with cationic polymers to improve transfection efficiencies (Verminnen et al., 2008b). *In vivo* electroporation has been shown to improve

the efficacy of DNA vaccination in large animals (Babiuk et al., 2002; Scheerlinck et al., 2004), but is rather difficult to use in smaller, stress-sensitive animals such as pet birds. Enhancing the immune response to the antigen is another strategy that can improve the efficacy of DNA vaccines. This can be achieved by incorporating genes encoding cytokines (IFN-γ, IL-1β, IL-2, IL-8, IL-15, IL18, GM-CSF, TNF-4β and lymphotactine) or cytidine phosphate guanosine (CpG) motifs into the plasmid (Gurunathan et al., 2000; Krieg and Davis, 2001; Babiuk et al., 2003; Garmory et al., 2003; Loots et al., 2006). Moreover, identification of suitable adjuvants is as critical as the choice of antigen for improvement of the protective efficacy of the vaccine.

In the future, we would like to enhance the protective capacity of our *Cp. psittaci* genotype A DNA vaccine further by using a complex of a cationic polymer with codon-optimized pcDNA1::MOMP A, as constructed by Verminnen *et al*.,(2008b) for *Cp. psittaci* genotype D vaccination of turkeys. Additionally, we would like to repeat the vaccination experiment in budgerigars using lower infective doses, as 10^8 $TCID_{50}$ of the highly virulent *Cp. psittaci* strain 89/1051 most certainly does not reflect the natural infective dose.

An important issue that needs to be addressed in order to prevent zoonotic transmission to humans is legislation. A new EU commission regulation, EC no 318/2007 applies from 1 July 2007 after being published in the Official Journal of the EU. This regulation lays down the animal health conditions for imports of certain birds from third countries and parts thereof into the EU. It is not applicable to: a) fowl, turkeys, guinea fowl, ducks, geese, quails, pigeons, pheasants, partridges, ratites reared or kept in captivity for breeding, the production of meat or eggs for consumption, or for restocking supplies fo games (poultry), b) racing pigeons which are introduced to the territory of the Community from a neighbouring third country where they are normally resident and then immediately released with the expectation that they will fly back to that third country, c) birds imported from Andorra, Liechtenstein, Monaco, Norway, San Marino, Switzerland and the Vatican City State, d)birds intended for zoos, circuses , amusement parks or experiments, e) pet animals referred to in the third paragraph of Article 1 of Directive 92/65/EEC, accompanying their owner, f) birds imported for conservation programmes approved by the competent authority of the Member State of destination. The regulation also lays down the quarantine conditions. For instance: 1) approved quarantine facilities and centres, 2) direct transport of birds to quarantine stations, 3) attestation by the importers or their

agents, 4) quarantine for at least 30 days, 5) examination, sampling and testing to be carried out by an official veterinarian, 6) actions in case of disease suspicion which are in case of chlamydiosis treatment of all birds and prolonged quarantine for at least two months following the date of the last recorded case. Importantly the regulation only allows imports of birds from approved breeding establishments, thus for birds other than poultry, only birds bred in captivity carrying an individual identification number and accompanied by an animal health certificate are allowed.

Thus, current EU psittacosis legislation is strictly on import of birds. There is no legislation/regulation on risk reduction strategies in member states, or on outbreak management. Some years ago there was the Dutch foundation, "Bestrijdingsplan papegaaienziekte – B.P.P.", but it no longer exists because the proposed risk reduction strategies were too expensive. The foundation tried to: a) improve the birds' health, b) reduce contact of humans with psittacosis, c) improve the awareness of bird sellers and their customers. Bird breeders could voluntarily participate. Ten percent of their birds were examined twice a year and when *Cp. psittaci* was present, all birds had to be treated with antibiotics. If negative, breeders received a "psittacosis-negative" certificate. Maybe, we should reconsider the implementation of such a psittacosis risk reduction strategy, but this time on EU level.

Summary

Summary

Psittacosis, also known as parrot fever is a bacterial infection in humans that can cause asymptomatic infections but also severe pneumonia with serious health problems. It is caused by *Chlamydophila psittaci*, an obligate intracellular Gram-negative bacterium, formerly known as *Chlamydia psittaci*. *Cp. psittaci* includes 9 known outer membrane gene A (*ompA*) genotypes designated A to F, E/B, M56, and WC (Everett *et al*., 1999; Geens *et al*., 2005a). The 7 first genotypes are known to naturally infect birds. All *Cp. psittaci* genotypes should be considered to be readily transmissible to humans. Infected birds shed the bacteria through faeces and nasal discharges, and humans become infected by inhaling an aerosol of these materials. Psittacosis is a notifiable disease in most countries. Psittacosis is found worldwide and the incidence is increasing in developing countries, which is correlated to the import of exotic birds. However, the actual number of psittacosis cases is probably still underestimated because psittacosis is difficult to diagnose, is covered by antimicrobials which may be employed empirically for therapy of respiratory disease and/or community-acquired pneumonia, and often is not reported.

The aim of the present thesis was to collect specific data to assess the current epidemiological situation concerning zoonotic transmission of *Cp. psittaci* in a Flemish population having contact with domestic and/or companion birds. We tried to define the human population being most at risk of getting psittacosis and subsequently tried to gather information on the prevalence and the clinical consequences of psittacosis in this human population. Finally, the obtained information on bird species and *Cp. psittaci* genotypes being most often involved in bird to human transmission, was going to be used for designing a recombinant *Cp. psittaci* vaccine and evaluating its protective capacity in a well-defined avian experimental infection model.

Chapter I gives and overview on the history, taxonomy, and infection biology of chlamydial infections in general. Moreover, different aspects of avian chlamydiosis such as epidemiology, transmission between birds, clinical disease, diagnosis, treatment, public health significance, prevention, control measurements and current legislation, are reviewed.

In **Chapter II** we investigated the occurrence of *Cp. psittaci* infections in a representative Flemish population of 540 individuals claiming to have daily, weekly, sporadically or no contact with domesticated and/or companion birds. Participants, 264 males and 276 females, at the age of 36 to 57 years (median 46) were sampled

pharyngeally and at the same moment a blood sample was obtained. Each individual completed a questionnaire allowing insight into professional and nonprofessional activities, smoking habits, medical history and contact frequency with different bird species and other animals. Pharyngeal swabs were analyzed using a *Cp. psittaci*-specific nested PCR/enzyme immunoassay (EIA), and a *Cp. pneumoniae*- specific nested PCR. Sera were tested for IgG/IgM by a recombinant *Cp. psittaci* MOMP-based ELISA, by MIF and by the Serion Chlamydia IgG/IgA ELISA. Canaries and *Psittaciformes*, (especially parakeets and parrots) were the most popular pet birds, followed by racing pigeons, ducks, geese and finally turkeys. Individuals in contact with *Psittaciformes* and racing pigeons are more at risk of becoming infected with *Cp. psittaci*. Daily contact with racing pigeons is significantly more dangerous than weekly contact. However, this is not the case for *Psittaciformes,* as the infection risk for humans is the same whether they are daily or only weekly in contact with parakeets or parrots. Racing pigeons were kept as a group, all together creating a high infection pressure, whereas parakeets and parrots were mostly housed individually and in case of parakeets sometimes as pairs. Thus, infected *Psittaciformes* are strictly seen more dangerous to public health.

Additionally, we showed the usefulness of a highly sensitive and specific nested PCR/EIA for detecting *Cp. psittaci* DNA in human clinical specimens and the urgent need for the development of a sensitive but especially *Cp. psittaci* specific recombinant or peptide-based antibody detection test for testing human sera. Results stress the need for more accurate diagnostic monitoring and reporting, a veterinary vaccine, and information campaigns with recommendations for psittacosis risk reduction strategies.

In chapter III, we describe the transmission of a *Cp. psittaci* genotype E/B strain from African green parrots to humans hereby demonstrating for the first time the zoonotic potential of *Cp. psittaci* genotype E/B strains. Results of this study also underline the occupational risk for veterinarians. In Chapter III, we additionally describe the transmission of *Cp. psittaci* from *Psittaciformes* (cockatiels, parakeets, parrots, lories) to humans in a zoonotic risk study in a human population proven (chapter II) to be highly at risk of getting psittacosis. A population of 308 *Psittaciformes* from 39 breeding facilities as well as 46 of the pet bird owners were tested for *Cp. psittaci* infections. Fifty-nine (19.2%) birds were positive for *Cp. psittaci* by nested PCR/EIA detecting the chlamydial outer membrane protein A (*ompA*) gene in faecal samples. *Cp. psittaci* were cultured from 25 (42.3%) PCR- positive samples inoculating them onto BGM cells and subsequently identifying the bacteria by immunofluorescence staining. Eight of 39 (20.5%) tested

breeding facilities were positive in both nested PCR/EIA and culture and respiratory disease was present at all eight. Five breeding facilities gave only positive results in the nested PCR/EIA and birds showed no clinical signs. Birds in one of these facilities were currently being treated with doxycycline, and the remaining four breeding facilities recently used doxycycline, oxytetracycline, or enrofloxacin. In 13 of 39 (33.3%) *Cp. psittaci* – positive breeding facilities a significant correlation between faecal excretion of viable chlamydophila and respiratory disease was shown (odds ratio 14.5, 95% confidence interval 1.6-130.5, $p<0.05$). The remaining 26 breeding facilities with healthy birds had negative results for PCR and culture. Nested PCR/EIA showed the presence of *Cp. psttaci* DNA in pharyngeal samples of 6 on 46 (13%) pet bird owners. Interestingly, birds of those pet bird owners were positive in both PCR and culture. Viable organisms could be isolated from 4 out of 6 PCR positive human samples. Subsequent *ompA* genotyping by use of a genotype-specific real-time PCR revealed the presence of genotypes A (5 of 6) and E/B (1 of 6). Only mild clinical signs appeared in these persons and none were treated. In our study, 18 (46.2%) of 39 breeding facilities had treated their birds with tetracycline, doxyclycline, or enrofloxacin in the past year. Four (10.2%) of 39 also used tetracyclines prophylactically. Thus, a *Cp. psittaci* vaccine for *Psittaciformes* and information on sensible use of antibiotics are needed to prevent psittacosis in humans and birds as well as possible development of drug-resistant zoonotic *Cp. psittaci* strains.

In chapter IV, we describe the development of a *Cp. psittaci* genotype A DNA vaccine for budgerigars (*Melopsittacus undulatus*). Codon-optimized plasmid DNA (pcDNA1::MOMP A) expressing the major outer membrane protein (MOMP) of *Chlamydophila psittaci* genotype A strain 89/1051 has been tested for its ability to induce protective immunity against *Cp. psittaci* challenge in budgerigars. Eight pairs of male and female budgerigars were housed in eight separate bird cages placed in two negative pressure isolators, four cages per group. All budgerigars were immunized twice intramuscularly with 100 µg plasmid DNA. Both groups received a primary DNA inoculation at day 1, followed by a booster inoculation 3 weeks later. Group 1 received pcDNA::MOMP A while group 2 received the placebo vaccine pcDNA1. Budgerigars were challenged by aerosol two weeks following the booster vaccination. The challenge consisted of 10^8 $TCID_{50}$ of the homologous *Cp. psittaci* genotype A strain. Cloacal and pharyngeal swabs of all budgerigars, taken at the beginning of the experiment and prior to the experimental infection were negative in both PCR and culture. However, all

budgerigars showed low pre-existing serum antibody titers, indicative for a previous infection. Nevertheless, DNA immunization could significantly reduce clinical symptoms, macroscopic lesions, pharyngeal and cloacal excretion as well as chlamydial replication, even in the presence of pre-existing serum antibodies, as compared to the placebo-vaccinated controls.

In conclusion, we clearly demonstrated the underestimation of human *Cp. psittaci* infections in Belgium and identified racing pigeons, but especially *Psittaciformes* as the main infection source for humans having contact with domestic and/or companion birds. We also demonstrated the occupational risk for veterinarians and their assistants of getting psittacosis from *Psittaciformes.* We stressed the need for 1) zoonotic risk reduction strategies, 2) information campaigns on sensible antibiotic use in *Psittaciformes*, 3) control on antibiotic availability through the internet, 4) a *Cp. psittaci*-specific antibody detection assay for testing human sera, as well as the need for 5) a *Cp. psittaci* vaccine, as it will definitely diminish the number of symptomatic and asymptomatic *Cp. psittaci* infections in humans and the risk of creating antibiotic resistant zoonotic *C. psittaci* strains. Future research will be directed towards improvement of protective immunity obtained by our DNA vaccine by generating complexes of plasmids with cationic polymers in order to increase plasmid delivery and towards the development of a recombinant or peptide-based *Cp. psittaci*-specific antibody enzyme-linked immunosorbent assay for testing human sera.

Samenvatting

Samenvatting

Psittacosis, ook gekend als de papegaaienziekte, is een humane bacteriële infectie veroorzaakt door *Chlamydophila psittaci*, een Gram-negatieve obligaat intracellulaire bacterie. De infectie kan asymptomatisch verlopen doch kan eveneens een erge longontsteking veroorzaken met eventueel ernstige complicaties en in een enkel geval kan de infectie zelfs dodelijk zijn. *Cp. psittaci* wordt onderverdeeld in 9 gekende 'outer membrane protein' A (*ompA*) genotypen, namelijk de genotypen A tot F, E/B, M56 en WC. De zeven eerste genotypen veroorzaken een infectie bij vogels. Al deze genotypen zijn potentieel gevaarlijk voor de mens daar zij kunnen overgedragen worden van vogels naar mensen. Geïnfecteerde vogels scheiden de bacterie uit via hun faeces maar ook via neussecreet en de mens wordt geïnfecteerd door het inademen van aërosolen van deze excreties. Psittacosis is een aangifteplichtige ziekte in de meeste Europese landen, De Verenigde Staten van Amerika, Australië en Japan.

Psittacosis komt bijgevolg wereldwijd voor en de incidentie neemt de laatste jaren toe. De reden voor deze toename is nog onbekend. Mogelijk zijn artsen veel alerter geworden, maar het kan ook zijn dat de ziekte besmettelijker aan het worden is. Ook is de stijging mogelijk te wijten aan onzorgvuldig gedrag van vogelhandelaren die de vogels onnodig vaak behandelen met antibiotica waardoor de infectie gemaskeerd wordt, waardoor de vogels zelf niet ziek lijken, maar de infectie wel verder kunnen verspreiden. Er is de laatste jaren een toename van het aantal psittacosis gevallen in de ons omringende landen (Nederland, Duitsland en Frankrijk). Doch wij zijn, samen met andere onderzoekers, van mening dat het werkelijke aantal psittacosis gevallen in deze landen en ook in België, nog steeds sterk onderschat wordt. De milde vorm van de ziekte doet immers denken aan griep, waardoor de infectie niet herkend wordt. Vaak wordt een antibioticumtherapie gestart bij een respiratoire infectie, zonder dat het oorzakelijke agens daadwerkelijk geïdentificeerd werd. Tevens wordt de infectie niet altijd gerapporteerd. Het werkelijke aantal psittacosis gevallen zal vermoedelijk veel hoger zijn dan de officiële cijfers laten zien. In België werden respectievelijk 7, 2 en 3 psittacosis gevallen gemeld in 2005, 2006 en 2007.

Aan de hand van deze thesis wilden wij de actuele epidemiologische situatie aangaande zoönotische *Cp. psittaci* transmissies in Vlaanderen verduidelijken en dit in eerste instantie bij mensen met vogels (gedomesticeerd/gezelschap) als huisdier. Het was onze bedoeling om een humane risicopopulatie binnen deze groep van mensen te

identificeren. Vervolgens werd de prevalentie van psittacosis en de klinische gevolgen hiervan onderzocht binnen deze geïdentificeerde humane risicopopulatie. Op deze wijze wilden we wetenschappelijke informatie bekomen over het vogelspecies en de *Cp. psittaci* stammen die het meest betrokken waren bij de overdracht van *Cp. psittaci* van vogels naar de mens. Deze informatie zou tenslotte gebruikt worden voor de doelgerichte aanmaak van een recombinant *Cp. psittaci* vaccin voor vogels. De beschermende capaciteiten van het vaccin zouden onderzocht worden in een experimenteel infectiemodel bij vogels.

In hoofdstuk I geven we eerst een algemeen literatuuroverzicht over de geschiedenis, de taxonomie en de biologie van chlamydiale infecties bij mens en dier. Vervolgens wordt een overzicht gegeven over *Cp. psittaci* infecties bij vogels waarbij de epidemiologie, de klinische aspecten van de infectie, de diagnose, de behandeling van de infectie, het belang voor de volksgezondheid alsmede preventieve maatregelen en de huidige wetgeving hieromtrent beschreven worden.

In hoofdstuk II onderzoeken we het voorkomen van *Cp. psittaci* infecties in een Vlaamse populatie van 540 mensen (264 mannen en 276 vrouwen), 36 tot 57 jaar oud (mediaan 46), die verklaarden dagelijks, wekelijks, sporadisch of nooit in contact te komen met gedomesticeerde en/of gezelschapsvogels. Bij al de deelnemers werd een faryngeale swab genomen alsmede een bloedstaal. Elke persoon vulde tevens een anamnese in met vragen over professionele en niet-professionele bezigheden, rookgewoonte, medische achtergrond en contactfrequentie met verschillende vogelspecies en andere dieren. De faryngeale swabs werden onderzocht met een *Cp. psittaci*-specifieke 'nested PCR enzyme immunoassay' (nested PCR/EIA) en *Cp. psittaci*-positive swabs werden tevens onderzocht met een *Cp.pneumoniae*-specifieke nested PCR. De sera werden onderzocht aan de hand van een recombinante enzyme-linked immunosorbend assay (ELISA) gebaseerd op de detectie van antistoffen tegenover het recombinant 'major outer membrane protein' (MOMP) van *Cp. psittaci*. De humane sera werden tevens onderzocht met de IgG/IgA micro-immunofluorescentie test en de Serion Chlamydia ELISA. De resultaten toonden aan dat kanaries en *Psittaciformes* (vnl. parkieten en papegaaien) de meest populaire aviaire huisdieren waren gevolgd door sportduiven, eenden, ganzen en kalkoenen. Personen die in contact kwamen met *Psittaciformes* en sportduiven liepen een significant hoger risico om geïnfecteerd te worden met *Cp. psittaci* dan mensen die in contact kwamen met andere vogelspecies. Dagelijks contact met sportduiven resulteerde in een significant hoger risico dan wekelijks contact. Dagelijks of wekelijks contact met *Psittaciformes* resulteerde in een even hoog aanzienlijk risico op

een *Cp. psittaci* infectie. Sportduiven werden steeds in groep gehouden, waardoor de contactpersonen waarschijnlijk werden blootgesteld aan een vrij hoge infectiedruk. Papegaaien en parkieten daarentegen werden door de onderzochte populatie particulieren meestal individueel gehouden, of in het geval van parkieten soms als koppel, maar zelden als groep. Geïnfecteerde *Psittaciformes* zijn bijgevolg, strikt gezien meer risicovol voor de volksgezondheid.

In hoofdstuk II tonen we ook het nut aan van een zeer gevoelige en specifieke nested PCR/EIA voor de detectie van *Cp. psittaci* DNA in humane klinische stalen. Tevens wordt duidelijke aangetoond dat de MIF test, een serologische test die momenteel nog steeds aangewend wordt voor de diagnose van psittacosis onvoldoende gevoelig en specifiek is. Het gebruikt van deze test wordt nog steeds geadviseerd door wereldwijd erkende instanties zoals de U.S. 'Centers for Disease Control and Prevention' (CDC), de U.S. National Association of State Public Health Veterinarians (NASPHV) en de U.S. Council of State and Territorial Epidemiologists (CSTE). Er is bijgevolg een dringende nood aan de ontwikkeling van een recombinante of peptide-gebaseerde ELISA voor de detectie van *Cp. psittaci*- specifieke antistoffen. Deze test zou dan naar onze mening gebruikt moeten worden voor epidemiologisch onderzoek, eerder dan voor de klinische diagnose van psittacosis bij de individuele patiënt. Hiervoor adviseren wij het gebruik van recent ontwikkelde gevoelige en specifieke moleculaire diagnostische testen.

In hoofdstuk III, richten we ons onderzoek op de kwekers van *Psittaciformes* (kaketoes, papegaaien, parkieten en lori's). Aan de hand van de resultaten van hoofdstuk II hebben we immers kunnen vaststellen dat er een hoog risico is voor deze personen om geïnfecteerd te worden met *Cp. psittaci*. We beschrijven de transmissie van een *Cp. psittaci* genotype E/B stam van Afrikaanse grijze roodstaart papegaaien naar de mens en tonen hierbij voor het eerst aan dat genotype E/B stammen kunnen overgedragen worden van vogels naar de mens. De resultaten van deze studie demonstreren ook het beroepsrisico voor dierenartsen om besmet te worden met *Cp. psittaci* door contact met geïnfecteerde vogels. In hoofdstuk III onderzochten we tevens een populatie van 308 *Psittaciformes* van 39 verschillende kweekfaciliteiten, alsmede 46 van de kwekers. Negenenvijftig (19.2%) vogels excreteerden *Cp. psittaci* in hun faeces zoals vastgesteld kon worden aan de hand van de nested PCR/EIA. *Cp. psittaci* kon bovendien geïsoleerd worden uit 25 (42.3%) PCR positieve stalen. Acht van de 39 (20.5%) kweekfaciliteiten werden positief bevonden a.d.h.v. de nested PCR/EIA en kiemisolatie in Buffalo Green Monkey (BGM) cellen. In alle 8 kweekfacilteiten waren er klachten van respiratoire

symptomen bij de vogels. Vijf kweekfaciliteiten waren enkel positief a.d.h.v. PCR en de vogels aldaar vertoonden geen klinische symptomen. In één van deze 5 kweekfaciliteiten werden de vogels op het moment van staalnamen behandeld met doxycycline en in de andere 4 kweekfaciliteiten waren de vogels recent behandeld geweest met doxycycline, oxytetracycline of enrofloxacine. In 13 van de 39 (33.3%) van de kweekfaciliteiten werd een significante correlatie waargenomen tussen fecale excretie van levende *Cp. psittaci* kiemen en de aanwezigheid van respiratoir symptomen bij de vogels. De overige 26 kweekfaciliteiten met gezonde vogels waren negatief a.d.h.v. PCR en kiemisolatie. Met behulp van de nested PCR/EIA konden we *Cp. psittaci* DNA aantonen in de faryngeale swabs van 6 van de 46 (13%) onderzochte kwekers. De vogels van deze geïnfecteerde kwekers waren positief a.d.h.v. PCR en kiemisolatie. We konden levende *Cp. psittaci* kiemen isoleren bij 4 van de 6 positieve kwekers. Met behulp van een genotype-specifieke real-time PCR werden 'outer membrane protein A' (*ompA*) genotype A (n=5) of E/B (n=1) stammen teruggevonden bij deze 6 kwekers. Opmerkelijk was dat 18 van de 39 (46.2%) kwekers verklaarden dat ze hun vogels het afgelopen jaar behandeld hadden met tetracycline, doxycycline of enrofloxacine. Vier van de 39 (10.2%) kwekers verklaarden dat ze tetracyclines profylactisch gebruikten. Uit deze studie blijkt de nood aan een *Cp. psittaci* vaccin voor *Psittaciformes*. Een dergelijk vaccin zou het aantal zoönotische transmissies naar de mens aanzienlijk kunnen reduceren. Bovendien blijkt uit deze studie dat we dringend informatie moeten verschaffen aangaande een verantwoord gebruik van antibiotica bij vogelkwekers om het ontstaan van antibioticum resistente zoönotische *Cp. psittaci* te voorkomen.

In hoofdstuk IV beschrijven we de ontwikkeling van een *Cp. psittaci* genotype A DNA vaccin voor grasparkieten (*Melopsittacus undulatus*). Er werd een plasmide (pcDNA1::MOMP A) aangemaakt dat het 'major outer membrane protein' (MOMP) van de *Cp. psittaci* genotype A stam 89/1051 tot expressie bracht. Hierbij werd een codon-geoptimaliseerd construct aangemaakt. Dit DNA vaccin werd vervolgens uitgetest op zijn vermogen om een beschermende immuunrespons te induceren bij grasparkieten, experimenteel geïnfecteerd met de homologe *Cp. psittaci* stam. Acht koppels (man + vrouw) grasparkieten werden gehuisvest in 8 afzonderlijke kooien en geplaatst in 2 negatieve druk isolatoren, 4 kooien per isolator. Al de grasparkieten werden twee maal intramusculair geïmmuniseerd met 100 µg plasmide DNA. Beide groepen kregen een primary-vaccinatie op dag één, 3 weken later gevolgd door een boostervaccinatie. Groep 1 werd gevaccineerd met pcDNA1::MOMP A terwijl groep 2 geïmmuniseerd werd met het

placebo vaccin pcDNA1. Twee weken na de boostervaccinatie werden de beide groepen aërogeen geïnfecteerd met 10^8 Tissue Culture Infective Dose 50 ($TCID_{50}$) van de homologe *Cp. psittaci* genotype A stam. Cloacale en faryngeale swabs genomen bij het begin van het experiment en vóór de experimentele infectie waren negatief a.d.h.v. PCR en kiemisolatie. Niet tegenstaande deze bevinding, hadden al de grasparkieten lage antistoftiters tegenover het MOMP van *Cp. psittaci*, zoals bleek uit het onderzoek van presera a.d.h.v. de MOMP-gebaseerde recombinante antistof ELISA. Dit wees uiteraard op een voorgaande infectie bij deze één jaar oude dieren. Ondanks de aanwezigheid van deze antistoffen in de beide groepen, werd in de pcDNA1::MOMP A gevaccineerde groep, in vergelijking met de placebo gevaccineerde groep, een significante reductie waargenomen van: 1) de klinische symptomen, 2) de pathologie vastgesteld bij lijkschouwing, 3) de faryngeale en cloacale kiemexcretie en 4) de vermeerdering van *Cp. psittaci* in het lichaam van deze vogels.

Als besluit kunnen we stellen dat we duidelijk aangetoond hebben dat het aantal psittacosis gevallen in België sterk onderschat wordt. Sportduiven, maar vooral *Psittaciformes* vormen een reëel *Cp. psittaci* infectie-risico voor mensen die in contact komen met gedomesticeerde en/of gezelschapsvogels. We hebben in hoofdstuk III ook duidelijk kunnen aantonen dat er een reëel beroepsgebonden risico is voor dierenartsen om geïnfecteerd te worden met *Cp. psittaci* wanneer zij in contact komen met *Psittaciformes.* Een éénmalig contact met geïnfecteerde Afrikaanse grijze roodstaartpapegaaien was voldoende om besmet te worden. We hebben aangetoond dat er een dringende nood is aan: 1) voorlichtingscampagnes om het risico op *Cp. psittaci* overdracht van vogels naar de mens te reduceren, 2) informatiecampagnes aangaande een doordacht en verantwoord gebruik van antibiotica door kwekers van *Psittaciformes*, maar misschien ook meer in het algemeen, door alle vogelkwekers en ook door vogelhandelaren, 3) maatregelen om de aankoop van antibiotica via het internet in te dijken en/of beter te controleren, 4) een *Cp. psittaci* -specifieke antistofdetectie test voor onderzoek van humane sera, 5) een *Cp. psittaci* vaccin voor vogels, in het bijzonder *Psittaciforme*s, om het risico op zoönotische transmissies naar de mens de reduceren maar ook om het risico op het ontstaan van antibiotica resistente *Cp. psittaci* stammen te voorkomen. Verder onderzoek zal bijgevolg gericht zijn op de ontwikkeling van een recombinante of peptide-gebaseerde *Cp. psittaci* antistofdetectie test voor onderzoek van humane sera en op de verdere optimalisatie van het DNA vaccin door o.a. koppeling van het plasmide aan kationische polymeren om de transfectie efficiëntie te verhogen.

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Curriculum vitae

Personal aspects

Name :	Harkinezhad Nelivan
Given Name:	Mohammad Taher
Date of birth:	23 August 1971
Family status:	Married ; one child
Native language:	Kurdish
Nationality:	Iranian

Contact options

Current work address:
Coupure Links 653
9000 Ghent
Belgium
Tel: +32 9 264 99 23
Fax: +32 9 264 62 19

E-mail address: t_harki@yahoo.com

Current home address:
Erpelsteeg 52
9000 Ghent
Belgium
Cell phone: +32 485 76 35 30

Education

PhD student, in Applied Biological Sciences: option, biotechnology of Cell and Gene, 2004-2008, Ghent University, Ghent, Belgium.
M.Sc. in Animal Science (Genetics and Animal Breeding), 1999 ,Tabriz University, Tabriz ,Iran.
B.Sc. in Animal Production ,1996 ,Tehran University ,Tehran , Iran
A.Sc. in Technology of Animal Production, 1994, Gilan University, Rasht, Iran.

Special training

Doctoral training in applied biological sciences, 2005-2008, Ghent University, Ghent, Belgium.
Awareness course concerning the Transport of Infectious and Related Substances by Air and Road, 2007, Ghent University, Ghent, Belgium.
Course of Permanent training "Low Countries studies", 2004, Ghent University, Ghent, Belgium.

Publications

Vanrompay, D., Hoang, T. Q., De Vos, L., Verminnen, K., **Harkinezhad, T.**, Chiers, K., Morre, S. A. and Cox, E., (2005). Specific-pathogen-free pigs as an animal model for studying Chlamydia trachomatis genital infection. Infect. Immun. 73[12], 8317-8321 .

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Van Droogenbroeck, C., Beeckman, D. S.A., **Harkinezhad, T**., Cox, E. and Vanrompay, D., (2008). Evaluation of the prophylactic use of ovotransferrin against chlamydiosis in SPF turkeys. Vetrinary Microbiology. Accepted.

Meetings

With oral presentation

Harkinezhad, T., Geens, T., Verminnen, K. and Vanrompay, D., (2005). Transfer of *Chlamydophila psittaci* from turkeys and parrots to man apparently without causing severe health problems. Meeting of COST Action 855 on Validation of new diagnostic tests for animal Chlamydioses. 10th June 2005. Ghent, Belgium

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With poster presentation

Harkinezhad, T., Beeckman, D., Van Droogenbroeck, C., Verminnen, K. and Vanrompay, D., (2007). Molecular epidemiology of psittacosis in *Psittaformes* breeding facilities. In: H. W. Saatkamp(ed). Proceedings van het symposium ter gelegenheid van het 4de lustrum van de VEEC, Dutch Society for Veterinary Epidemiology and Economics: 13 December 2007, Wageningen, Nederland, pp. 104.

Companion of student during their thesis

Marijke Van de Walle. (2005-2006). "Moleculair epidemiologisch onderzoek naar het voorkomen van Chlamydophila psittaci bij papegaai-achtigen en de mens". Department of Molecular Biotechnology. Faculty of Bioscience Engineering. Ghent University. Ghent, Belgium.

Omalbanin Pir Ahari. (2001-2002). "Cytogenetic investigation on Sarabi Cows" (in Persian) . Department of Animal Science , Faculty of Agriculture ,Zanjan University, Iran.

Language ability

Language	Fluently	Well	Comment
Kurdish	**x**		Native language
English	**x**		
Persian	**x**		
Turkish		**x**	
Dutch			Understanding and reading

Research Skills

Cell culture, bacterial culture, PCR (nested PCR, Real time PCR), working in biosafety level 3 laboratory, working with hazardous materials and bio-hazardous organisms (*Chlamydiaceae*), familiar with transport of infectious and related substances, Cytogenetic techniques (such as karyotyping, G-banding), recombinant protein production and purification, preparation of frozen tissue sections and fluorescence stainings, DNA extraction [from animal internal tissues(liver, spleen,…), dry swabs, birds' excretions,…], ELISA, Giménez staining, gene cloning, animal experiments.

Computer skills

MS office, statistical analysis: MSTATC, SAS, SPSS

Hobbies

Mountaineering, reading