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Association of Lung Consolidation Depth With Pathogens Isolated From Bronchoalveolar Lavage Fluid in Calves With Clinical Signs of Respiratory Disease

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Keywords: bovine respiratory disease | cytology | pneumonia | rational antimicrobial use | thoracic ultrasound

ABSTRACT

Background: The depth of lung consolidation (≥1 and ≥3 cm) on thoracic ultrasonography (TUS) is increasingly used as a criterion for antimicrobial treatment. However, its association with bacterial infections remains unclear.

Objectives: To investigate the associations of clinical and ultrasonographic findings, particularly consolidation depth, with opportunistic bacterial infections (OBI), viral infections, or *Mycoplasma bovis* (also known as *Mycoplasmopsis bovis*) infections. Different definitions of OBI were explored, based on various combinations of bacterial species, with or without a neutrophilic profile on cytology.

Animals: Eighty-six group-housed calves with at least one clinical sign of respiratory disease from 19 herds experiencing a respiratory epidemic.

Methods: Cross-sectional study. A physical examination, TUS, and non-bronchoscopic bronchoalveolar lavage were performed. The definitions of OBI were based on semi-quantitative culture results and cytology.

Results: Calves with consolidations of ≥0.5 cm had higher odds of having an OBI considering most definitions, on *M. bovis* isolation (odds ratio [OR] = 57.3; 95% confidence interval [CI] = 1.5–2300; $p = 0.03$) and isolation of a bacterial agent in general (OR = 15.5; 95% CI = 2.3–100; $p = 0.01$). Animals with consolidation ≥1 cm had higher odds of OBI considering all definitions, virus isolation (OR = 15.6; 95% CI = 1.0–240; $p = 0.05$) and isolation of a bacterial agent in general (OR = 6.9; 95% CI = 1.7–28; $p = 0.01$). Consolidation ≥3 cm, cough, and the California score were not significantly associated with OBI, *M. bovis*, or both.

Conclusion: In herds experiencing a respiratory epidemic, consolidation depths ≥0.5 and ≥1 cm might indicate respiratory disease with a bacterial component.

1 | Introduction

Bovine respiratory disease (BRD), which refers to respiratory tract infections, affects calves in all cattle sectors, causing decreased animal health, decreased animal welfare, and production losses [1, 2]. Bovine respiratory disease is one of the primary causes of widespread antimicrobial use in cattle, and mass treatment strategies are routine practices in some

Abbreviations: BALf, bronchoalveolar lavage fluid; BCV, bovine corona virus; BRD, bovine respiratory disease; BRSV, bovine respiratory syncytial virus; nBAL, non-bronchoscopic bronchoalveolar lavage; OBI, opportunistic bacterial infection; PI3, parainfluenza 3; qTUS, quick thoracic ultrasound; TUS, thoracic ultrasonography.

sectors [3–7]. Meanwhile, rational antimicrobial use is essential to mitigate antimicrobial resistance, an increasing threat to animal and human health and food safety. For respiratory tract infections, targeted treatment of individuals with bacterial bronchopneumonia is advocated for the rational use of antimicrobials [8].

Thoracic ultrasonography (TUS) is a promising technique for identifying calves requiring treatment because it allows visualization of subpleural consolidations [9]. Consolidation depth thresholds of ≥1 cm (lobular pneumonia) and ≥3 cm (lobar pneumonia) are valuable because they have shown fair diagnostic accuracy in Bayesian latent class analyses and have been associated with production losses [10–15]. However, it is unclear whether these thresholds for lung consolidation depth are truly associated with bacterial infection, either with the primary pathogen *Mycoplasma bovis* (also known as *Mycoplasmopsis bovis*) or opportunistic bacteria such as the *Pasteurellaceae* family. Consolidations <1 cm were poorly examined in previous studies, resulting in an unknown relevance of these small lesions.

In pediatric medicine, multiple and bilateral consolidations <1.5 cm were significantly more common in viral pneumonia, whereas single deeper consolidations have been more commonly observed in bacterial pneumonia [16]. In a separate study, the threshold of 21 mm was found to be most optimal in the differentiation of bacterial and viral pneumonia in children [17]. In a study of 24 calves, bacterial infection was confirmed on histopathology in all (5) cases with lung consolidations ≥3 cm [18]. These findings could indicate that deeper lung consolidations on TUS have higher odds of bacterial involvement.

Currently, a consensus definition of opportunistic bacterial infection (OBI) based on laboratory analysis of the lower respiratory tract is lacking in bovine medicine. Interpretation of cultures of respiratory tract samples is difficult because there is no good framework for differentiating infection from contamination or colonization. To aid in this differentiation, a semi-quantitative description of the culture results has been proposed [19]. Furthermore, considering indicators of a neutrophilic inflammatory response of the host could be useful, as they have been associated with infectious disease in human and equine medicine [20–22]. Although little information is available on cytologic thresholds in cattle, characteristics of a neutrophilic profile have been described after a cluster analysis of non-bronchoscopic bronchoalveolar lavage (nBAL) samples of group-housed calves [23].

Therefore, our main objective was to investigate the associations of clinical and ultrasonographic findings, particularly lung consolidation depth, with the presence of OBI, *M. bovis*, or viruses of specific interest. These viruses were bovine respiratory syncytial virus (BRSV), bovine coronavirus (BCV), and parainfluenza 3 (PI3) virus. Our hypothesis was that calves with deeper lung consolidations would have higher odds of OBI and *M. bovis*. Because a consensus definition of OBI is lacking, the second objective consisted of exploring different definitions based on semi-qualitative culture results and cytological data.

2 | Materials and Methods

2.1 | Animals and Study Design

Our cross-sectional study was conducted in herds experiencing a respiratory epidemic. The clinical and ultrasonographic data and the nBAL samples collected from a previous study on biomarkers were used [24]. Sampling occurred in Flanders (Belgium)in the winter of 2021–2022. Dairy, beef, and veal calf farms were selected based on admission by their local veterinarian and willingness of the owner to participate (convenience selection). For inclusion of farms, the following criteria had to be met: one or more clinical signs (cough, rectal temperature ≥39.4°C, respiratory rate ≥44 breaths/min, nasal and eye discharge) affecting a minimum of five animals and a minimum of 15% of the animals in the same air space within 48 h. The availability of calves determined the number of calves sampled, the number of calves sampled per herd, and the total sample size.

All sampling techniques and the study design were approved by the local ethical committee of the Faculty of Veterinary Medicine, Ghent University under experimental license number EC2021–079.

2.2 | Examination and Sampling

On each participating farm, three to seven animals that met the individual inclusion criteria were selected. The inclusion criterion was at least one clinical sign associated with BRD: rectal temperature ≥39.4°C, purulent or mucopurulent nasal discharge, ocular discharge, cough (induced or spontaneous), increased respiratory rate (≥44 breaths/min), and decreased mental state. Exclusion criteria were clinical signs indicative of other infectious diseases: swollen or painful joint(s), swollen or painful umbilicus, and diarrhea. In addition, calves treated with antibiotics and non-inflammatory drugs within a month before sampling were excluded. A physical examination was performed. Examination consisted of an evaluation following the California score [25] with the addition of the elements previously stated in the exclusion criteria (palpation of umbilicus and joints, and evaluation of fecal consistency). Scores ≥5 were considered to indicate BRD [25]. Subsequently, TUS was performed according to the quick thoracic ultrasound technique (qTUS), previously described in detail [26] (see Figure 1A). A portable ultrasound unit (KX5200 VET, Kaixin, Bila Tserkva, Ukraine) with a linear probe of 5.5 to 5.7 MHz and isopropanol alcohol as a transducing agent were used. Depth was set to 7 cm. The consolidation depth was measured using the grid of the ultrasound screen, perpendicular to the pleural line. The maximum consolidation depth was noted per lung region. Lung regions were defined (left cranial, left caudal, right cranial, and right caudal, with the heart as reference) and are presented in Figure 1A. Physical examination and ultrasound assessment throughout the study period were conducted by several veterinarians of the research group, all trained and experienced in qTUS and clinical scoring. Lastly, an nBAL was performed as described elsewhere [27]. This procedure was done by inserting a sterile polytetrafluorethylene catheter into the medioventral nasal cavity and gently advancing it until a wedge position was reached. A volume

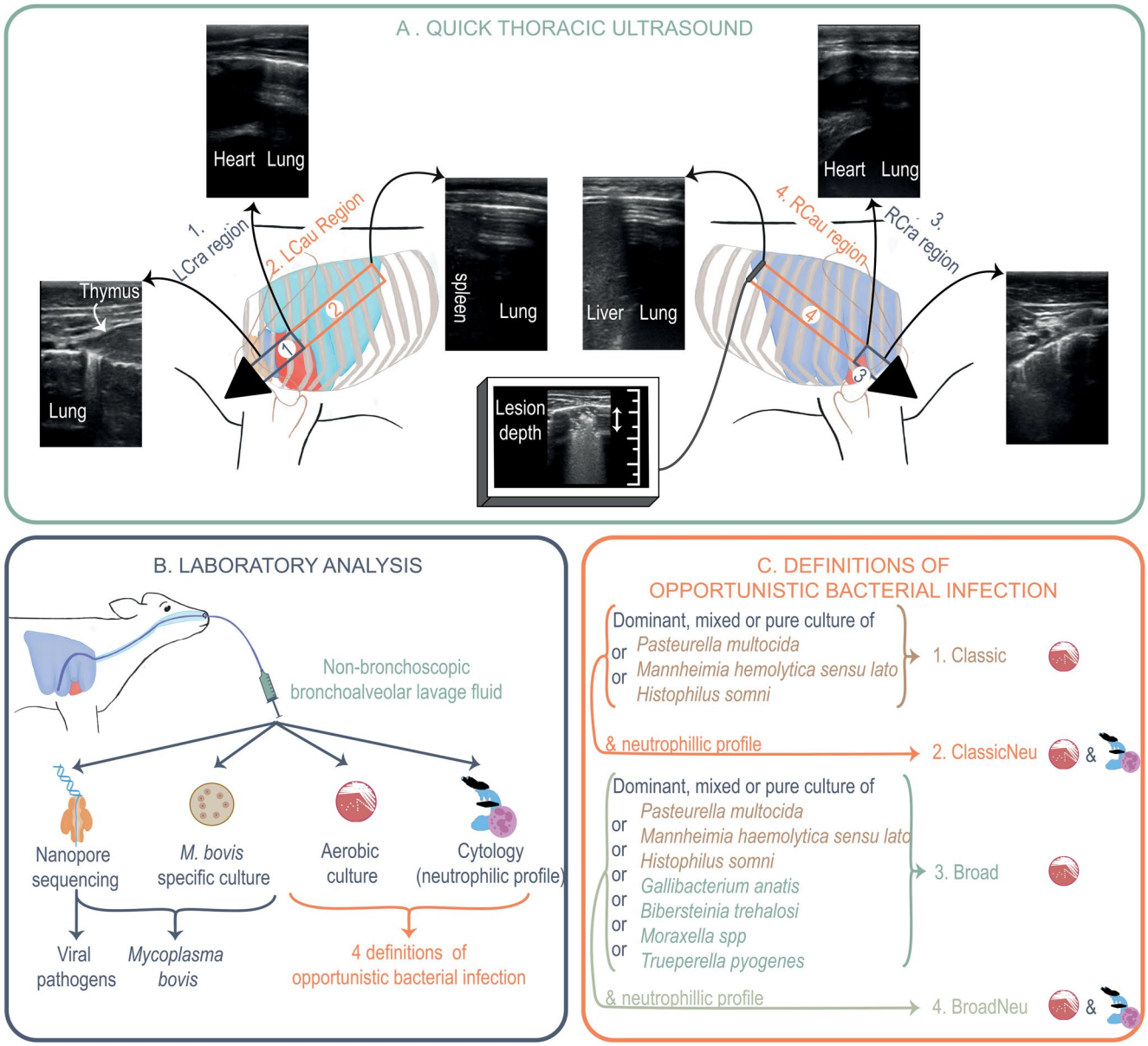

FIGURE 1 | Schematic representation of key aspects of the material and methods from this study. (A) Quick thoracic ultrasound: this part shows the method, and the landmarks used for guidance and division of the scan regions. Consolidations were recorded as maximal depth per region with 1. LCra = Left cranial, 2. LCau = Left caudal, 3. RCra = right cranial, and 4. RCau = right caudal. (B) Laboratory analysis: This part shows the performed analysis on the non-bronchoscopic bronchoalveolar lavage. (C) Definitions of opportunistic bacterial infection: This part shows the criteria used to define an opportunistic bacterial infection following four definitions.

of 0.5 to 1 mL/kg estimated body weight was instilled and re-aspirated. When an insufficient volume was recovered, another 0.5 to 1 mL/kg bolus was used to repeat this step. Samples were divided into an aliquot for pathogen identification, which was transferred to a sterile falcon tube, and into a 1 mL aliquot for cytology, which was transferred to a 1 mL EDTA tube (Vacutest Kima, Azergrande, Italy). The samples subsequently were transported under climate-controlled conditions (±5°C).

2.3 | Laboratory Analysis of Bronchoalveolar Lavage Fluid

Cytological analysis was performed. Cytospin preparations of the bronchoalveolar lavage fluid (BALf) were made using a cytocentrifuge (Shandon Scientific, London, UK) centrifuged for 10 min at 129 relative centrifugal force, and stained with Hemacolor (Merck KGaA, Darmstadt, Germany). These preparations were made by the first author (Justine Clinquart) or the same laboratory technician within 6 h of sampling. All cytospin preparations were evaluated by an experienced operator (Justine Clinquart) for quality and density using a low- and high-power field before analysis, and excluded when unsuitable. Differential counts were performed by the same operator. If available, 500 cells were counted, evenly spread on two cytospin preparations from the same sample. If only one preparation of sufficient density was present, two counts of 250 cells were performed, each starting at the opposite side of the preparation, and overlap was not allowed. Counts were performed at 1000× magnification (Euromex Iscope, Arnhem, The Netherlands)

and were performed using a custom-made counter application (Thunkable, San Francisco, US). If insufficient cells were present, the maximum number of cells was counted; if < 350 cells were present, the sample was excluded. Finally, a sample was considered to have a neutrophilic cytological profile if at least 44.5% neutrophils, < 1.6% eosinophils, and < 11.5% lymphocytes were present as described elsewhere [23].

Bacterial identification was performed by inoculating BALf on Columbia blood agar enriched with 5% sheep blood (Oxoid, Hampshire, UK) and incubated for 24–48 h in a 5% CO_2-enriched atmosphere at 37°C. The result of these cultures was recorded as negative, pure culture, dominant, mixed, or polymicrobial following methods previously described [28]. For the identification of *M. bovis*, BALf was cultured on a previously described selective indicative agar and incubated for 5–7 days in the same atmosphere [29]. Bacterial identification, including *M. bovis*, was done by phenotypic characteristics followed by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS, Brüker Daltonik GmbH, Bremen, Germany) [29]. In addition, nanopore sequencing was conducted by an external laboratory (PathoSense, Merelbeke, Belgium) [30, 31]. This third-generation sequencing using MinION (Oxford Nanopore Technologies, ONT) is based on metagenomics and enables rapid identification of pathogens. In contrast to PCR-based techniques, prior specification of pathogens is unnecessary, and a wide range of pathogens can be detected and identified [30, 31]. Only viral pathogens and *M. bovis* isolated by this technique were considered in our study. For other bacterial agents, only culture results were considered because the method had not yet been validated by the laboratory at the time of sampling. In addition, the evaluation of this method was outside the scope of our study. A schematic representation of the laboratory analysis performed on the BALf is presented in Figure 1B.

2.4 | Definitions of Opportunistic Bacterial Infections and Variables

Four definitions of OBI were explored. Only dominant, mixed, or pure cultures with abundant growth were considered positive in all definitions [28]. Polymicrobial cultures were not considered positive. These definitions are presented in Figure 1C. The first (1. Classic) and second (2. ClassicNeu) only considered calves with *Pasteurella multocida*, *Mannheimia haemolytica*, and *Histophilus somni* to have an OBI. The third (3. Broad) and fourth (4. BroadNeu) considered a broader range of bacteria, which also included less common bacteria (*Gallibacterium anatis*, *Bibersteinia trehalosi*, *Moraxella* spp., *Trueperella pyogenes*) associated with respiratory disease [32–34]. In the second (2. ClassicNeu) and fourth (4. BroadNeu), an additional criterion of having a neutrophilic cytologic profile was needed to be considered positive.

For further analysis, binary variables were created to facilitate statistical analysis. For the outcome factors, pathogens (OBI, virus, *M. bovis*, bacteria in general) were categorized separately as absent or present. For OBI, above-mentioned definitions were used (Classic, ClassicNeu, Broad, BroadNEU). A virus was considered present if BRSV, BCV, or PI3 was isolated. A bacteria in general was considered present if *M. bovis* was isolated, if the sample was positive for the BroadNeu OBI definition, or both. Binary predictor variables were sex (female vs. male), cough (present vs. absent), clinical BRD defined as

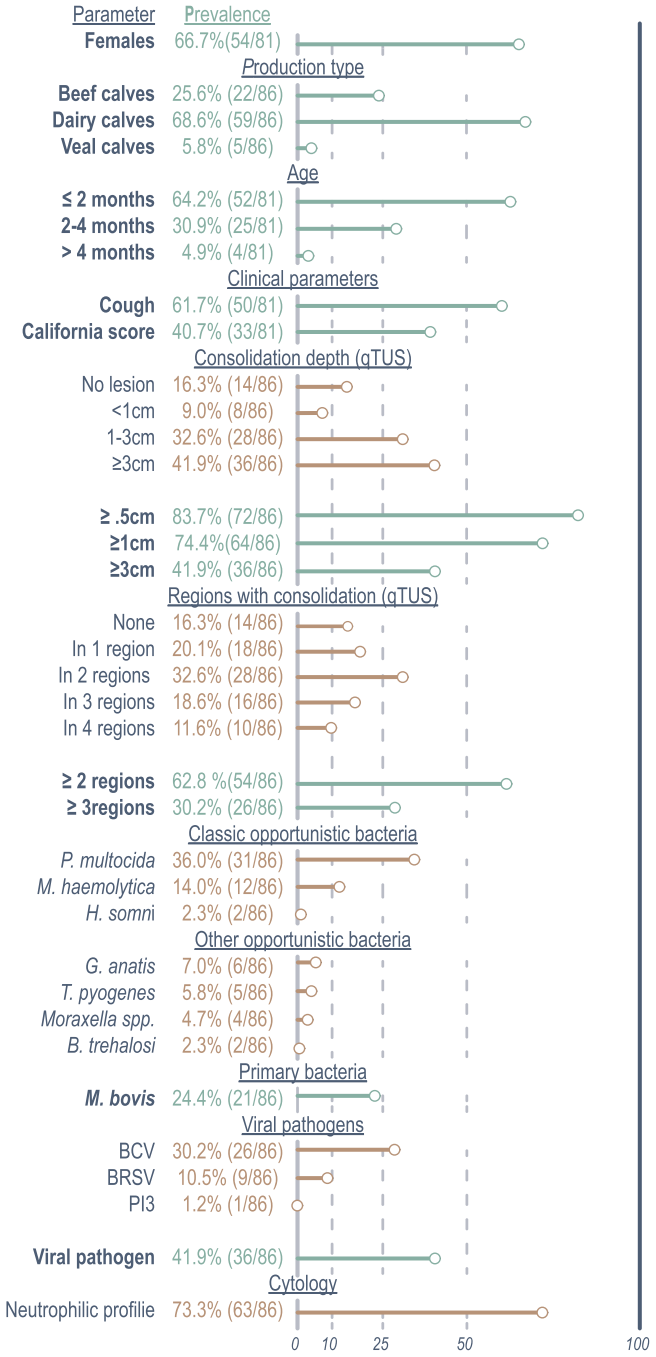

FIGURE 2 | Descriptive information from bronchoalveolar lavage fluid of 86 calves from 19 herds experiencing an epidemic of respiratory disease. Parameters used in subsequent statistical analysis are shown in green. Full names of shown pathogens (method of isolation): *Mannheimia haemolytica* sensu lato, *Pasteurella multocida*, *Histophilus somni*, *Gallibacterium anatis*, *Trueperella pyogenes*, *Bibersteinia trehalosi (aerobic culture)*, *Mycoplasma bovis* (specific culture and nanopore sequencing), BCV = bovine coronavirus, BRSV = bovine respiratory syncytial virus, PI3 = parainfluenza 3 (nanopore sequencing).

California score positive (≥ 5 vs. < 5), maximal lung consolidation depth (≥ 0.5 cm vs. < 0.5 cm, ≥ 1 cm vs. < 1 cm, ≥ 3 cm vs. < 3 cm), number of lung regions affected by any lesion (≥ 2 regions vs. < 2 regions, ≥ 3 regions vs. < 3 region), isolation of a viral pathogen, and isolation of *M. bovis*. Consolidation depth thresholds were chosen based on previous research and the research gap regarding small consolidations of < 1 cm. Three-categorical predictor variables were production type (dairy, beef, veal) and age(≤ 2 months, 2–4 months, > 4 months).

2.5 | Statistical Analysis

All analyses were performed using R (version 4.4.0) [35], and figures were created using a combination of the "ggplot2" package [36] and Adobe Illustrator (version 16.0.0, Adobe, San Jose, California). To investigate the associations between clinical and ultrasonographic findings with the different pathogen outcomes (virus, *M. bovis*, OBI, bacteria in general), univariable mixed effects logistic regression was conducted using the "lme4" package [37]. The herd was modeled as a random factor in each analysis. Analyses were performed separately for each combination of outcomes and predictor variables. Because of the low number of veal calves, the analysis of production type consisted of dairy calves versus beef calves. To analyze the predictor factor age, which consisted of three categories, similar mixed effects logistic regression was performed with an additional post hoc test, the Holm test, using the "multcomp" package [38]. Statistical significance was set at < 0.05.

3 | Results

3.1 | Herd and Animals

The number of sampled calves was 89, of which three were excluded, two because of inadequate quality of the cytological preparation, and one because of a missing maximal depth of ultrasonographic consolidations. Information on sex, age, and physical examination was unavailable for five calves, which were excluded from the analyses considering these factors.

Detailed descriptive information is provided in Figure 2. The majority of the calves were dairy calves (68.6%), followed by

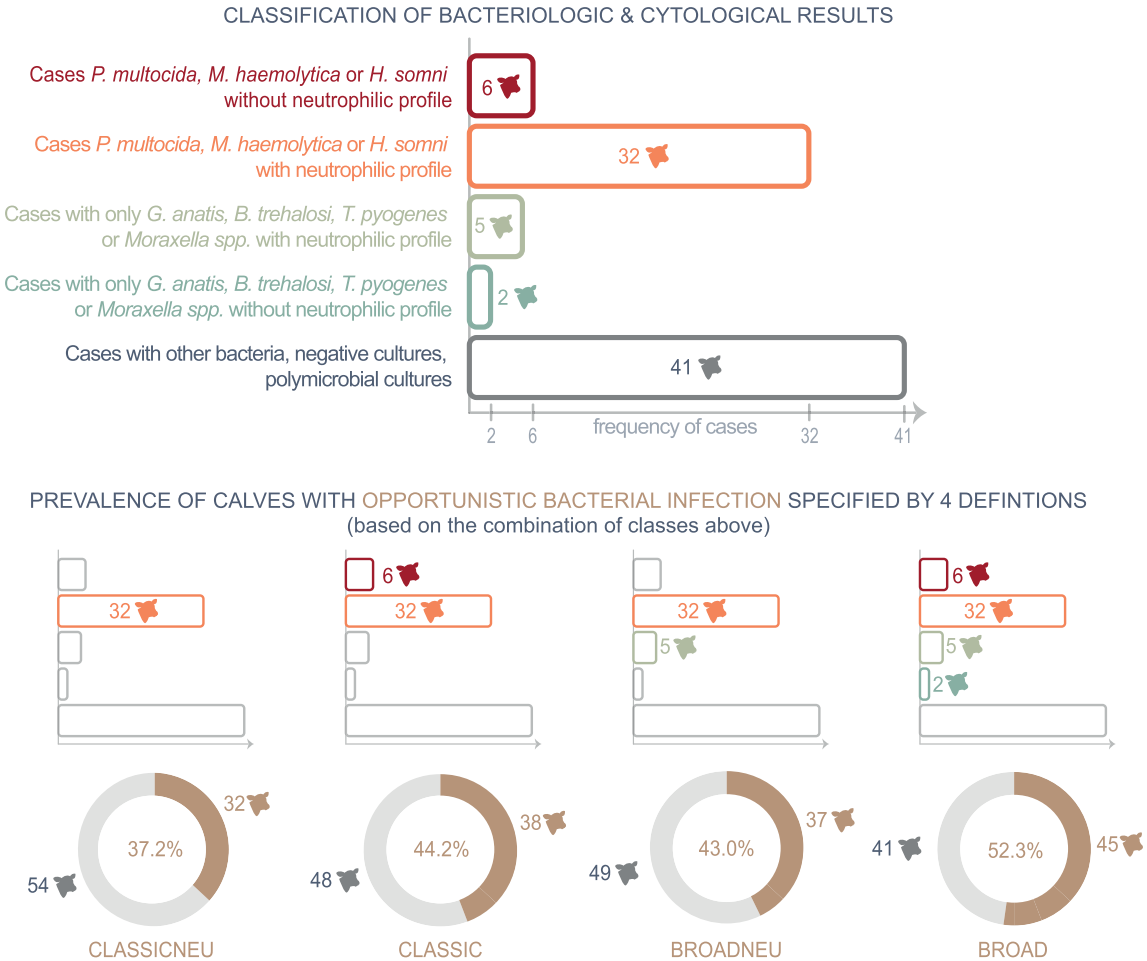

FIGURE 3 | Prevalence of opportunistic bacterial infections in 86 calves from 19 herds experiencing an epidemic of respiratory disease, specified by four proposed definitions. The graph at the top shows the prevalence of the different classes based on bacteriologic and cytologic results of the bronchoalveolar lavage fluid. Numbers before a calf icon represent absolute numbers. The bottom part shows the prevalence of opportunistic bacterial infection as specified by 4 definitions. The combination of the classes used for each definition is colored in the bar plots above the doughnut chart. Classes considered as negative for each definition are represented in gray.

beef calves (25.6%). Calves aged ≤ 2 months were most common (64.2%). Cough was found in 61.7% of the calves, and 40.7% of the animals were positive for the California score. Consolidations were present in 83.7% of the calves with 74.4% being at least 1 cm deep and 41.9% at least 3 cm deep. Consolidations in at least 2 regions were present in 62.8% of the cases, of which 30.2% had consolidation in at least 3 regions. *Pasteurella multocida* (36.0%) and *M. haemolytica* (14.0%) were the most frequently isolated opportunistic bacteria, whereas *G. anatis*, *T. pyogenes*, *Moraxella* spp., *H. somni* and *B. trehalosi* were only present in a minority of the calves (in order of most prevalent to least prevalent, 2.3%–7.0%). Bovine coronavirus was the most frequently isolated virus (30.2%) followed by BRSV (1.5%). In only one calf was PI3 isolated (1.2%). *M. bovis* was isolated in 24.4% of the calves. Cytological examination identified a neutrophilic profile in 73.3% of calves.

3.2 | Four Definitions of an Opportunistic Bacterial Infection

Following the criteria of the Classic definition of OBI (i.e., mixed or pure cultures of *P. multocida*, *M. haemolytica*, and *H. somni*), 44.2% (38/86) of the calves were affected. When the additional criterion of a neutrophilic profile was required in the ClassicNeu definition, this percentage decreased to 37.2% (32/86). Using the Broad definition, which also includes less common bacteria such as *G. anatis*, *B. trehalosi*, *Moraxella* spp., and *T. pyogenes*, 52.3% (45/86) of the calves had OBI. The requirement for a neutrophilic profile in these cases resulted in a prevalence of 43.0% (37/86). The prevalence is shown in Figure 3.

3.3 | Prevalence of Bacterial and Viral Pathogens According to Lung Ultrasonographic Findings

Figure 4 shows the prevalence of different pathogens (virus, *M. bovis*, and OBI) according to consolidation depth and the number of affected regions. The prevalence of bacterial infections (OBI as defined by BroadNeu or *M. bovis*) increased markedly from a consolidation depth of 0.5 cm (smallest consolidation recorded). Detailed information on co-infections per sampled calf is provided in Figure 5. Because of the relatively small sample size, statistical analyses were performed on binary variables for ultrasound predictors, and pathogens were analyzed separately based on presence or absence (see "Definitions of opportunistic bacterial infections and variables" in materials and methods).

3.4 | Associations of Isolated Pathogens With Clinical and Ultrasonographic Findings

Multilevel logistic regression showed no association between age and the presence of OBI according to different definitions, the

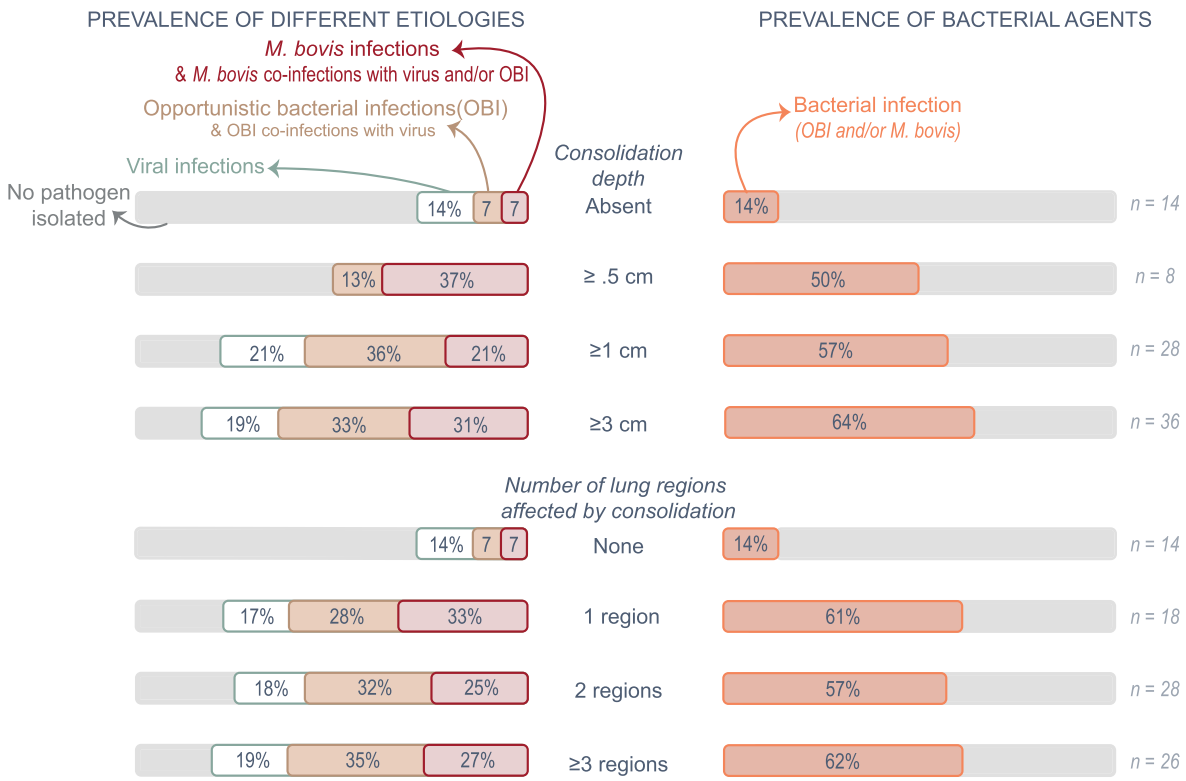

FIGURE 4 | Percentages of isolated etiologies by ultrasonographic findings in 86 calves from 19 farms experiencing an epidemic of respiratory disease. Etiologies are based on isolated pathogens in bronchoalveolar lavage fluid with viral pathogens determined by nanopore sequencing and *M. bovis* on a specific culture and nanopore sequencing. Opportunistic bacterial infections were based on semi-quantitative aerobic culture and the presence of a neutrophilic profile on cytology (BroadNeu definition). Etiologies are shown in different colors on the left side: red represents *M. bovis* infections with or without co-infections with viral or opportunistic bacteria, brown represents opportunistic bacterial infections with or without viral coinfections, green represents pure viral infections and gray represents cases where no pathogen was isolated. On the right side, the prevalence of bacterial infections is shown in orange.

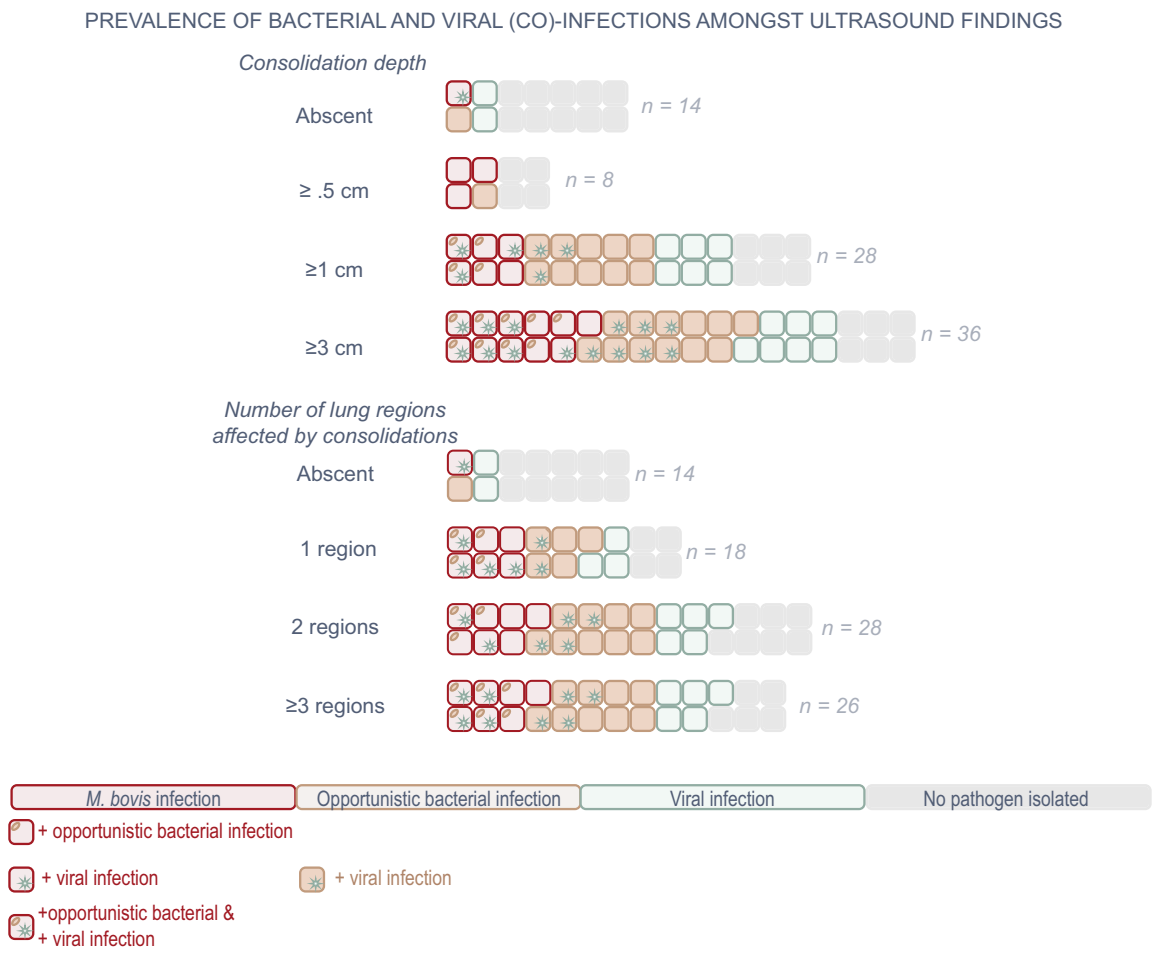

FIGURE 5 | Distribution of isolated pathogens among the studied ultrasonographic findings in 86 calves from 19 farms experiencing an epidemic of respiratory disease. Etiologies are based on isolated pathogens in the bronchoalveolar lavage fluid, with viral pathogens determined by nanopore sequencing and *M. bovis* identified through specific culture and nanopore sequencing. Opportunistic bacterial infections were based on semiquantitative aerobic culture and the presence of a neutrophilic profile on cytology (BroadNeu definition).

presence of a virus, or the presence of *M. bovis*. The detailed results of the univariable mixed effect logistic regressions of the binary descriptive, clinical, and etiological predictor factors are shown in Figure 6. The figure contains the relative prevalence, odds ratios, *p* values, and intraclass correlation coefficient. Dairy cattle had higher odds of having an OBI, as defined by Classic, ClassicNeu, and BroadNeu. No significant associations were found with sex, cough, and California score. Similarly, no associations were found among different pathogen outcomes themselves.

The detailed results of a similar analysis with ultrasonographic findings as predictive variables are shown in Figure 7. Significant associations were found between consolidations of ≥0.5 cm and ≥1 cm (qTUS) and almost every definition of OBI (except the Classic definition with consolidation ≥0.5 cm). Calves with consolidation of ≥0.5 cm had higher odds of isolation of *M. bovis* (OR = 57.3; 95% CI = 1.5–2300; $p = 0.03$). When a consolidation ≥1 cm was present, there were significantly higher odds of isolation of viral pathogens (OR = 15.6; 95% CI = 1.0–240; $p = 0.05$). Both consolidations ≥0.5 cm and ≥1 cm were associated with bacterial agents in general (BroadNeu, *M. bovis* or both; ≥0.5 cm [OR = 15.5; 95% CI = 2.3–1000; $p = 0.01$], ≥1 cm [OR = 6.86; 95% CI = 1.7–28; $p = 0.01$]). For consolidations ≥3 cm, no significant associations were found with any of the outcome factors.

The number of regions affected by consolidation on qTUS (≥2 and ≥3 regions) was not significantly associated with any definition of OBI. Consolidations in ≥2 regions were associated with the presence of a bacterial agent in general (BroadNeu and/or *M. bovis*).

4 | Discussion

In recent years, TUS has been proposed as a better alternative to inaccurate and variable clinical signs for treatment guidance and rationalization of antimicrobial use for pneumonia in calves [11, 13]. We explored the hypothesis that deeper lung consolidations are more likely to be associated with OBI or *M. bovis* infections. Associations of ultrasonographic lung consolidations of different depths with OBI, *M. bovis*, and viruses isolated from BALf have not been explored previously. In our study, consolidations ≥0.5 and ≥1 cm, were associated with OBI (as assessed by different definitions) in farms experiencing a respiratory epidemic. Additionally, ultrasonographic consolidations ≥0.5 cm were associated with the isolation of *M. bovis*, and consolidations ≥1 cm were associated with the isolation of viruses. When considering cases of OBI or *M. bovis* infection or both, the cases targeted by individual antimicrobial treatment, associations

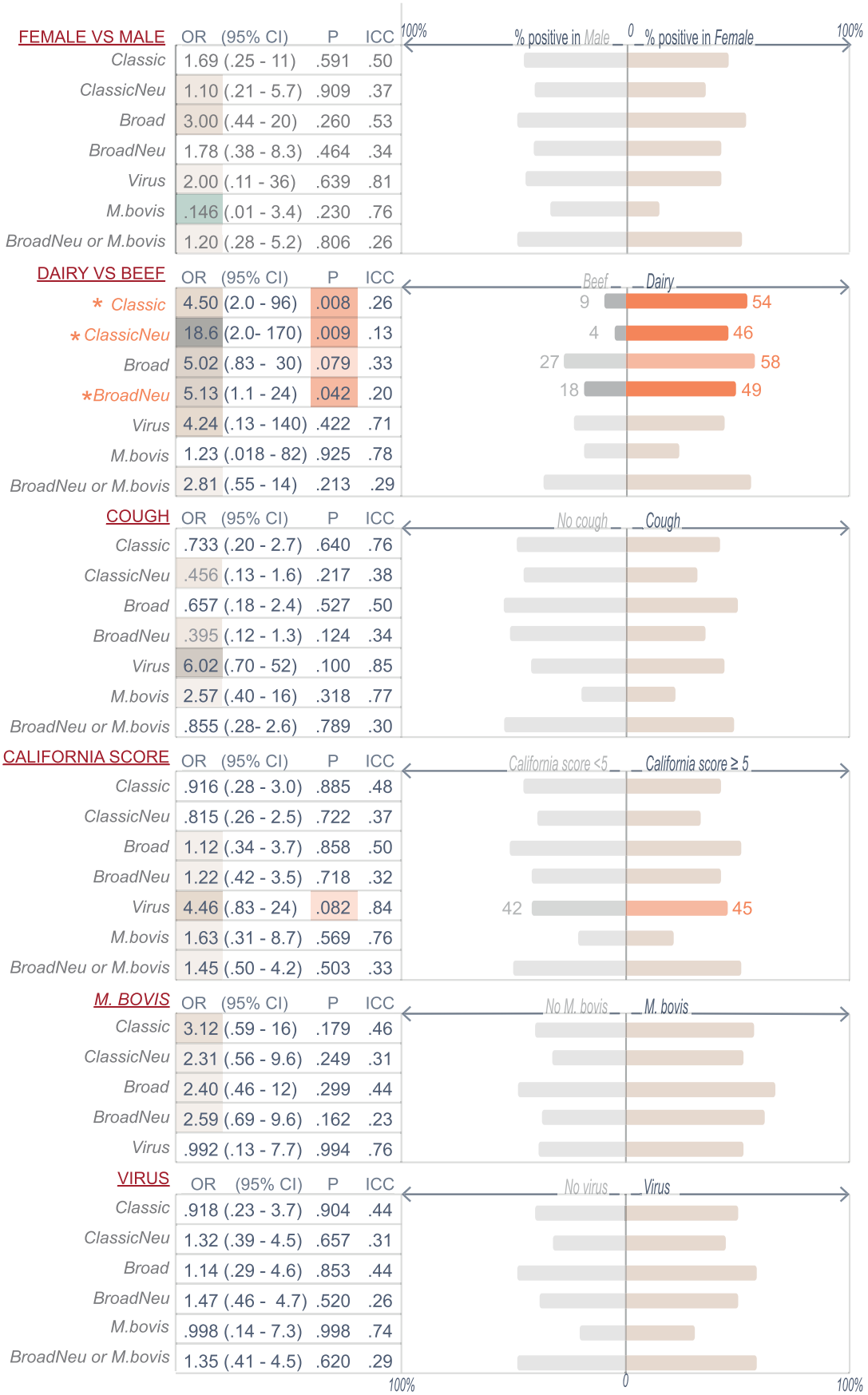

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FIGURE 6 | Results of univariable mixed effects logistic regression exploring the associations between isolated pathogens and animal characteristics, clinical factors, and pathogens among each other. Analysis of 86 herds from 19 herds experiencing an epidemic of respiratory disease. The herd was modeled as random effect. Isolated pathogens were determined by semi-qualitative interpretation of aerobic culture and cytology, combined into 4 different definitions for opportunistic bacterial infection, nanopore sequencing for viruses, and a combination of nanopore sequencing and specific culture for *M. bovis*. Significant associations are marked with an asterisk (*) in addition to being colored dark orange. *p* values between 0.05 and 0.1 are colored light orange. Odds ratios with value ≥2 are color-coded with larger values being colored darker brown. Values <0.5 are colored in green. ICC = intraclass correlation coefficient.

were found with consolidations ≥0.5 cm, consolidations ≥1 cm, and consolidations in at least two lung regions.

In these herds experiencing a respiratory epidemic, several consolidation parameters were significantly associated with bacterial infections (OBI or *M. bovis* or both). The investigated consolidation depths of <1 cm (>0–≥0.5 cm), ≥1, and ≥3 cm have already been used in cattle for the diagnosis of bronchopneumonia or targeted treatment [10, 12, 15, 39–41]. In our study, the number of calves with bacterial infections (OBI or *M. bovis* or both) increased markedly in all categories of consolidation depth (Figure 4). Moreover, associations were found for threshold ≥0.5 cm with almost all suggested definitions of OBI. Additionally, this threshold was associated with the presence of *M. bovis*. When consolidation of ≥0.5 cm was encountered, there was a 60% chance that a bacterial agent (OBI defined by BroadNeu or *M. bovis* or both) was present, compared with 14% when such consolidation was not found. Unfortunately, this threshold has not yet been studied for its diagnostic accuracy and is seldom used in research. This discrepancy is likely caused by fear of diagnostic errors (misclassification) because overdiagnosis would result in the overuse of antimicrobials. There is a risk that pleural abnormalities will be misinterpreted as consolidations or that consolidations between 0.5 and 1 cm might be missed. This is especially true in field settings where speed is favored and image interpretation can be hampered by suboptimal lighting. Meanwhile, these small lesions might have a higher rate of self-cure, and hence it might not be rational to initiate antimicrobial treatment at that stage.

The ≥1 cm threshold demonstrated good diagnostic capability for BRD when assessed using a Bayesian latent class model [11]. Furthermore, this threshold was associated with a lower average daily gain [14]. In our study, this threshold was associated with all definitions of OBI and a bacterial agent in general (also including *M. bovis*). Similar to the ≥0.5 cm threshold, 61% of calves with a consolidation of >1 cm had a bacterial agent in general. In comparison, 27% of calves had smaller or no consolidations. However, the differences between ≥0.5 and ≥1 cm should be interpreted cautiously because this difference is only based on eight calves (i.e., those with ≥0.5–1 cm).

On the other hand, a threshold of ≥3 cm was suggested to yield the best diagnostic performance for active bronchopneumonia in one study using a Bayesian latent class model [10]. Meanwhile, these consolidations have been linked to decreased milk production in the first lactation and had a more pronounced effect on growth [15]. Calves with a consolidation ≥3 cm had 3.16 higher odds (95% CI = 1.0–10.1) of being positive by the Broad definition, and 2.82 higher odds (95% CI = 1.0–8.0) of being positive by the BroadNeu definition. However, these had *p* values just above 0.05 and thus were not considered significant. Whether this result is a consequence of a lack of power or a consequence of a lack of an association cannot be concluded. Nevertheless, using this threshold, a relevant number of calves with OBI or *M. bovis* infection would have been missed in this population and would not have been treated. Using a certain number of minimally affected scan regions (2 or 3) seems to provide little additional value in this population. Lung regions were only significantly associated with a bacterial agent in general. Similar to the ≥3 cm consolidation depth, a relevant number of calves with OBI or *M. bovis* would have been missed in this population.

In addition to bacterial agents, TUS consolidation also was associated with viral pathogens. These associations were limited to consolidation depths of ≥1 cm. Additionally, there was no discernible trend of pure viral infections with smaller consolidation depths (Figure 4). Therefore, distinguishing viral and bacterial causes by consolidation depth would be impossible in this population. In contrast, a 21 mm depth was proposed in pediatric medicine to differentiate between bacterial and viral pneumonia [17]. Yet, pure viral infections were relatively uncommon, whereas co-infection was very common in our study. Possibly, because of the stoic nature of calves, strict viral infections lead to mild clinical signs that remain undetected when clinical observation is not continuous. Furthermore, the presence and depth of consolidation also may be pathogen-specific. For example, severe consolidations were observed after the experimental induction of BRSV, which is consistent with the often severe macroscopic pathologic lesions [42, 43]. Bovine coronavirus, on the other hand, might behave similarly to Severe acute respiratory syndrome coronavirus 2 in children and be more associated with subpleural consolidations of <1 cm [44]. The number of affected regions was not associated with viral causes in our population. Similar to what has been suggested in pediatric medicine, other ultrasonographic parameters could be explored for differentiating viral from bacterial pneumonia, such as the presence of vertical artifacts, air bronchograms, fluid bronchograms, and pleural effusion [16]. Conversely, despite being significantly different for viral versus bacterial pneumonia, remarkable overlap still was present in many findings, indicating that diagnosis of purely viral infection by a single TUS might not be achieved. Because of its cross-sectional design, our study did not evaluate the dynamics of lung lesions over time. Although our study aimed to sample calves at early stages of the disease, it is possible that different stages of disease were present. Meanwhile, the time to detection of ultrasound lesions post-experimental infection was longer for BRSV compared with *M. haemolytica* and *P. multocida* [43, 45].

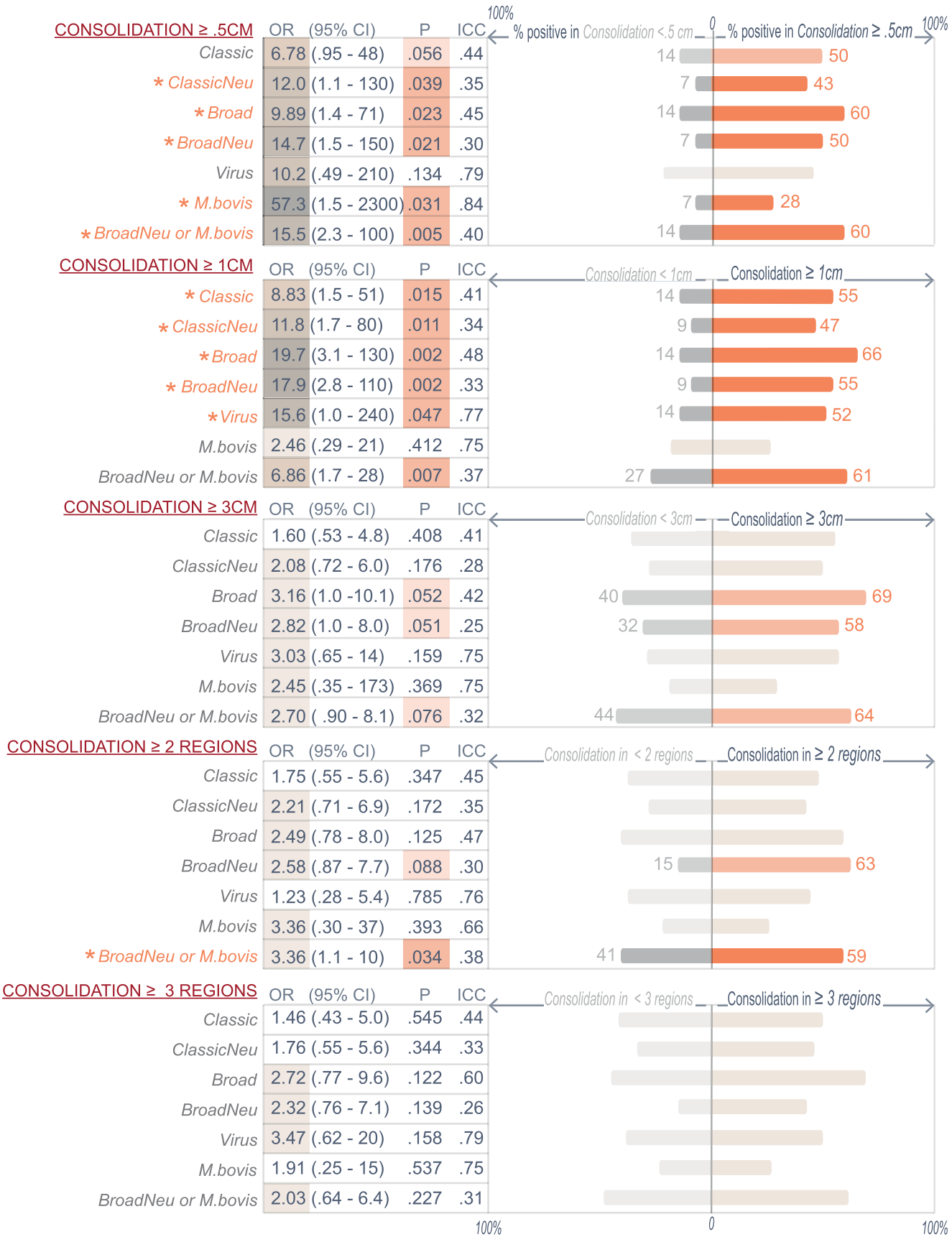

FIGURE 7 | Legend on next page.

FIGURE 7 | Results of univariable mixed effects logistic regression exploring the associations between isolated pathogens and ultrasonographic findings. Analysis of 86 herds from 19 herds experiencing an epidemic of respiratory disease. The herd was modeled as random effect. Isolated pathogens were determined by semi-qualitative interpretation of aerobic culture and cytology combined into 4 different definitions for opportunistic bacterial infection, nanopore sequencing for viruses, and a combination of nanopore sequencing and specific culture for *M. bovis*. Significant associations are marked with an asterisk (*) in addition to being colored dark orange. *p* values between 0.05 and 0.1 are colored light orange. Odds ratios with value ≥ 2 are color-coded with larger values being colored darker brown. Values < 0.5 are colored in green. ICC = intraclass correlation coefficient.

Despite the increasing popularity of ultrasonography, clinical scoring is still the most widely used method to determine which calves need antimicrobial treatment. However, no associations of cough or the California score with OBI, *M. bovis*, or both were found. Together with the reported poor diagnostic accuracy for BRD [46, 47], using clinical variables as a sole guide for antimicrobial use seems unjustified.

The prevalence of OBI differed remarkably among the proposed definitions. For example, a 15% difference was noted between ClassicNeu and Broad. Meanwhile, all definitions were associated with damaged lung tissue as assessed by ultrasonography. Noteworthy, for calves with a consolidation ≥ 1 cm, the odds of isolation of not only the classic *Pasteurellaceae* but also less common bacteria (Broad and BroadNeu definitions) were higher. This difference included *G. anatis*, *B. trehalosi*, *Moraxella* spp., and *T. pyogenes*, which also were reported or suggested to cause OBI as well [32–34, 48].

To define OBI based on semi-quantitative culture interpretation, however, is controversial because *P. multocida* is isolated from healthy animals, and several of the considered bacteria are present in the normal microbiome of either the nasopharynx or the lung [49–52]. Although commensals do not cause any harm to their host, infective bacteria cause neutrophilic inflammation in the lower airways. In human medicine, the absence of neutrophilia has been described as a good predictor of the absence of bacterial superinfection [53]. However, employing neutrophilia as a criterion of OBI is difficult in cattle because no good reference framework is available for (n)BAL. Furthermore, environmental factors frequently seen in cattle barns, such as higher dust levels (i.e., particulate matter 10), also can trigger neutrophilic inflammation [54]. Notwithstanding, recently a cluster analysis of nBAL cytology of group-housed cattle reported a neutrophilic cytologic profile with the potential to represent neutrophilic inflammation in calves [23]. In our study population, specifically in herds experiencing an epidemic of respiratory disease, requiring the presence of a neutrophilic inflammation did not seem to have additional value compared with the definitions of OBI not including cytologic results. Because cytology is labor intensive and affected by the volume used and retrieved [55], it does not seem necessary to add cytologic criteria in the definitions of OBI in similar situations to the studied population, in calves showing at least one (mild) clinical sign of BRD during an outbreak. Cytology might be more useful in completely subclinical disease and in endemic situations where the isolation of commensal bacteria might be a more relevant problem. In our study, dairy calves had higher odds of OBI infection compared with beef calves. Differences in management, housing factors, genetic predispositions, and behavioral differences between dairy and beef farmers could have caused this result. In the sampled region, beef farms tend to be smaller operations, often with higher individual care, whereas the care provided on dairy farms tends to be more variable.

Our study had some limitations. First, our study had a rather limited sample size, which prevented separate analysis of the different pathogen species. Grouping these pathogens does not account for differences between pathogen species that are likely present. Secondly, qTUS was performed by different assessors, which likely resulted in some variability in the assessment of TUS consolidations. However, all researchers involved used the technique regularly and underwent intensive training, which has been proven to improve inter-rater agreement [56]. Third, one of the individual calf selection criteria consisted of the presence of one clinical sign related to BRD, and no clinically healthy control calves were sampled. These features probably led to an overestimation of the prevalence of certain characteristics studied (presence of cough, California score, ultrasonographic parameters, isolation of pathogens, and cytology) compared with the traditional prevalence. Additionally, associations might differ in these calves compared to calves without any clinical signs. Calves displaying clinical signs of respiratory disease in herds experiencing an epidemic likely already have a higher chance for pathogen isolation. Furthermore, clinically healthy control animals were not sampled because of the estimation that calves present in a herd experiencing an epidemic might already be in an early stage of disease without showing clinical signs or ultrasonographic lesions. To date, no consensus exists on case definitions for respiratory disease in calves [57] and the diagnosis of healthy calves remains difficult. Although TUS can identify lesions in subclinical animals [58], it is not a gold standard [11]. In combination, control animals also were not sampled because of financial constraints. Therefore, our results might not represent the entire population, and conclusions about clinically healthy calves are not possible based on our findings. Fourth, the nBAL technique used in our study is characterized by upper airway passage, which could have led to contamination of the samples with normal flora of the upper airway. This potential contamination could have resulted in false-positive categorization of OBI in some cases. However, this risk was minimized by only considering cultures with abundant growth as positive and excluding polymicrobial cultures. Although contamination is possible, its impact is likely limited considering the findings of a comparison study of a nasal swab with nBAL. In this study, pure or negative cultures of BALf were not influenced by the presence of polymicrobial deep nasal swabs of the same animals [49]. Lastly, for cytology, insufficient cells were present in 2 samples (< 500 and > 350 cells). The influence is expected to be very small because a high reproducibility of neutrophil and eosinophil enumeration was found at a minimum of 200 cells in samples from dogs [59].

In conclusion, in herds experiencing a respiratory epidemic, both TUS consolidations of ≥0.5 and ≥1 cm in depth were most consistently associated with the presence of an OBI or a bacterial agent in general (OBI or *M.bovis* isolation or both). This finding might be of interest for further work on developing guidelines for rational antimicrobial treatment. Nevertheless, consolidations of ≥1 cm additionally were associated with the isolation of a virus, necessitating further clarification on ultrasound findings among different pathogens. Cough and the California score were not associated with OBI or a bacterial agent in general.

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Disclosure

Authors declare no off-label use of antimicrobials.

Ethics Statement

This study was conducted in compliance with the Ghent University rules of animal experiments with the approval of the university's animal experiment ethics committee. Authors declare human ethics approval was not needed.

Conflicts of Interest

The study was supported by funding from the first author's institute. T. Lowie, J. Bokma, S. Jourquin, and B. Pardon have received honoraria as speakers for pharmaceutical companies. T. Lowie, S. Jourquin, and B. Pardon are the founders of qTUS, a training company. The other authors declare no conflicts of interest.

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