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Comparison of bronchoalveolar lavage fluid bacteriology and cytology in calves classified based on combined clinical scoring and lung ultrasonography

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ABSTRACT

Respiratory tract infections are the leading cause of antimicrobial use in calves. Combining clinical examination and lung ultrasonography allows on-farm classification of calves as healthy or suffering from an upper respiratory tract infection (URTI), subclinical or clinical pneumonia. This might help to improve targeted antimicrobial therapy, restricting treatment to pneumonic cases. However, to what extent these diagnostic categories coincide with expected bacteriological and cytological bronchoalveolar lavage fluid (BALf) characteristics is currently unknown. The objective of this study was therefore to compare BALf bacteriology and cytology between healthy calves and calves with URTI, subclinical and clinical pneumonia. The hypothesis was that calves with subclinical and clinical pneumonia would have higher quantitative bacterial counts, bacterial isolation rates and neutrophil counts than URTIs or healthy animals. A cross-sectional study was performed on 305 indoor group-housed dairy and beef calves, from 62 farms. Calves were classified by combining clinical examination and lung ultrasonography. Clinical respiratory disease was defined using the Wisconsin score card and the Healthy Criterion (HC). The HC classified calves as clinically ill if at least one clinical sign was present. Ultrasonographic lung consolidation with a depth of ≥1 cm was considered indicative for pneumonia. Cytology and bacteriology were performed on BALf sampled by non-endoscopic bronchoalveolar lavage. Calves with clinical pneumonia were further subdivided based on culture result and presence of neutrophils phagocytosing bacteria. Combined lung ultrasonography and clinical examination (HC) classified 25.9 % (79/305) of the calves as healthy, 33.1 % (101/305) as URTI, 10.2 % (31/305) as subclinical and 30.8 % (94/305) as clinical pneumonia. Bacterial isolation rates and quantitative BALf culture results did not differ between groups. Calves with clinical pneumonia and neutrophil phagocytosis showed a significantly higher BALf neutrophil percentage compared to healthy calves (59.0 % vs. 37.7 % in healthy calves, $P = .03$). Inversely, lymphocyte percentage was lower in these calves (1.8 % vs. 5.3 % in healthy calves, $P = .003$). Classification of calves using lung ultrasonography and clinical scoring did not correspond with BALf bacteriology and cytology findings, as extrapolated from human and companion animal medicine. Under the current housing conditions of this study high rates of non-infectious airway inflammation or airway colonization by opportunistic pathogens, rather than infection might explain this. Isolation of respiratory pathogens from calves with various signs of respiratory disease or ultrasonographic lesions should be interpreted carefully. Of all cytological features, phagocytosis by neutrophils in BALf might be a useful criterion supporting the diagnosis of bacterial respiratory tract infection.

Abbreviations: URTI, upper respiratory tract infection; BALf, broncho-alveolar lavage fluid; TUS, thoracic ultrasonography; nBAL, non-endoscopic broncho-alveolar lavage; BAL, broncho-alveolar lavage; WI, Wisconsin calf respiratory score; HC, healthy criterion; CFU, colony-forming unit; TNCC, total nucleated cell count; SD, standard deviation; R, range; PPLO, pleuropneumonia-like organism; ELF, epithelial lining fluid

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1. Introduction

To fight antimicrobial resistance and safeguard antimicrobials for future generations, their inappropriate and excessive use needs to be avoided requiring a shift from mass-medication towards individual and targeted treatment (Ungemach et al., 2006; Baptiste and Kyvsgaard, 2017). Recent guidelines for veterinarians encourage use of diagnostic procedures and clinical examination before prescribing antibiotics and perform susceptibility testing as often as possible (FVE, 2019). Respiratory disease is one of the major indications for antimicrobial use in calves (Pardon et al., 2012; Windeyer et al., 2014; Dubrovsky et al., 2019) and in an attempt to follow published guidelines, in Belgium and surrounding countries a non-endoscopic bronchoalveolar lavage (nBAL) is increasingly performed to obtain lower respiratory tract samples for bacteriological analysis (Van Driessche et al., 2017). To enable targeted treatment based on this analysis it is crucial that time between sample collection and pathogen identification and susceptibility results is as short as possible. Using commercially available techniques significant progress has been made in shortening this turnaround time (Van Driessche et al., 2018, 2019). Furthermore, strategic sampling remains important since by sampling calves with clinical pneumonia, without recent history of antimicrobial treatment, chances of isolating causative bacteria are maximized and interpretation of results made easier (Lubbers and Turnidge, 2015; Capik et al., 2017). However, clinical signs indicating a respiratory tract infection, such as fever and cough, are inconsistently present and often inconclusive (Buczinski et al., 2015). On-farm thoracic ultrasonography (TUS) has made a substantial contribution to the diagnostic process, and currently enjoys a status as imperfect near gold standard for pneumonia diagnosis (Buczinski et al., 2015; Berman et al., 2019). By combining TUS with clinical examination calves can be subjected to one of four diagnostic categories: healthy, upper respiratory tract infection (URTI), subclinical or clinical pneumonia (Ollivett and Buczinski, 2016). Calves suffering from an URTI show clinical signs indicative of respiratory infection and no ultrasonographic lesions. Subclinical and clinical pneumonia are both characterized by presence of ultrasonographic lesions, however calves with subclinical pneumonia do not show clinical respiratory signs as calves with clinical pneumonia do. This classification might help practitioners in selecting appropriate animals for sampling and to target (empiric) antimicrobial treatment. Nevertheless, interpretation of qualitative bacterial culture results of lower respiratory tract specimens remains challenging. Since a large part of the conducting airways of healthy cattle are not sterile and opportunistic pathogens reside in the upper respiratory tract bacterial isolation does not necessarily indicate causation (Griffin et al., 2010; Nicola et al., 2017). Opportunistic pathogens can colonize the respiratory tract without causing clinical signs or could be cultured as a contaminant as a result of their presence in the upper respiratory tract during the bronchoalveolar lavage sampling procedure. To overcome this interpretative issue, in human and companion animal medicine quantitative bacterial cultures are sometimes performed and interpreted in combination with cytological findings of BALf to differentiate between contamination and infection (Kahn and Jones, 1987; Peeters et al., 2000; Rasmussen et al., 2002). A high BALf neutrophil percentage and the presence of neutrophils phagocytosing bacteria support the diagnosis of lower respiratory tract bacterial infection in cases of positive bacterial culture (Hawkins et al., 1995; Peeters et al., 2000).

To improve the interpretation of bacterial culture results of nBAL samples, the objective of our study was to explore to what extent combined clinical and ultrasonographic case definitions correspond with bacteriological and cytological BALf findings. We hypothesized that calves with subclinical and clinical pneumonia would have higher BALf quantitative bacterial culture results and neutrophil percentage compared to healthy and URTI calves.

2. Materials and methods

A cross-sectional study was performed. All sampling techniques and the study protocol were revised by the local ethical committee and permitted under experimental licence number EC2016–89.

2.1. Sample size calculation and study population

The sample size required to detect a 30 % difference (40 % versus 10 %) in bacterial isolation rate between healthy calves and calves suffering from upper respiratory disease, subclinical or clinical pneumonia under field conditions was 30 animals in each of the four test groups (with 95 % confidence and 80 % power) (Winepiscope 2.0). To account for sample loss due to polymicrobial culture results and laboratory processing, sample size was set at 400 animals. Between January and April 2017 sampling was performed on 62 conveniently selected commercial herds (23 dairy, 23 beef, 14 mixed and 2 veal) in West and Eastern Flanders (Belgium), aiming to sample 6–8 calves per farm. All preweaned and weaned calves, group-housed in the same, randomly selected pen were sampled. Herd selection was done with help of different local veterinary practices based on willingness to cooperate and informed consent was obtained from all cooperating farmers. Exclusion criterion for herds was presence of an epidemic episode of respiratory disease in the last two months prior to sampling. The presence of an epidemic episode of respiratory disease was defined as 20 % new cases of respiratory disease in the same stable or age category in a 24 -h period. Beef and dairy calves were both group-housed on straw and received milk replacer, concentrates and roughage with substantial variation between farms. Veal calves were group-housed on a slatted floor and fed milk replacer, concentrates and roughage according to European legislation (EC2008-119).

2.2. Clinical examination and thoracic ultrasonography

The following clinical signs were evaluated by the same investigator for each calf: position of the ears (normal; unilaterally drooped or bilaterally drooped), nasal discharge (absent; unilateral; bilateral), ocular discharge (absent; unilateral; bilateral), type of ocular or nasal discharge (serous; seromucous; mucopurulent; purulent), spontaneous cough (present; absent), breathing frequency (in breaths per minute), rectal temperature (°C), induced laryngeal cough reflex (positive; negative) and induced tracheal cough reflex (positive; negative). A positive induced laryngeal or tracheal cough reflex was defined as a single induced cough following manual compression of the larynx or trachea, respectively. Additionally, a respiratory score was assigned to all calves using the Wisconsin calf respiratory scoring system (WI) (McGuirk and Peek, 2014). Following the clinical examination, each calf was subjected to a thoracic ultrasonographic examination visualizing both cranial and caudal lung lobes on both sides of the thorax. The procedure was as previously described (Ollivett et al., 2015), using a linear probe with a frequency of 7.5-MHz (Tringa Linear Vet®, Esaote, the Netherlands), set at 8 cm of depth, using isopropyl alcohol (70 %) as a transducing agent. Depth of ultrasonographic consolidation was measured in a dorso-ventral plane using the grid on the screen of the ultrasound.

2.3. Bronchoalveolar lavage fluid collection and analysis

Non-endoscopic bronchoalveolar lavage (nBAL) was performed in standing, unsedated calves by the same veterinarian, using a new sterilized catheter for each calf as described previously (Van Driessche et al., 2016). One aliquot isotonic sterile saline was instilled using a volume of approximately 0.6 ml/kg body weight. The weight of the calves was estimated by using weight tape measurements. After instillation the fluid was immediately aspirated and if no fluid was recovered, another 20 ml of saline was injected. Macroscopic presence of

erythrocytes and volume of instilled saline and recovered BALf were recorded for each animal. Samples were transported in plastic tubes on ice, stored at 4 °C after transport and processed within 12 h after sampling.

Bacterial culture for *Pasteurellaceae* and aerobic bacteria was performed by plating 5 µl and 50 µl of undiluted BALf on Columbia blood agar enriched with 5 % sheep blood (Oxoid™, Hampshire, UK). For the isolation of *Mycoplasma bovis* 50 µl of undiluted BALf was inoculated on modified pleuropneumonia-like organism agar (PPLO) (Difco™, Becton Dickinson and Company, Franklin Lakes, NJ, USA), supplemented with 25 % inactivated horse serum (Gibco™, Waltham, USA) and 0.7 % technical yeast extract (Bacto™, Franklin Lakes, USA). After incubation at 35 °C and 5 % CO_2 for 12–24 -hs and 5 days for *Pasteurellaceae* and *M. bovis* respectively, plates were examined for growth and the number of colony-forming units (CFU/ml) for *Pasteurellaceae* and anaerobic bacteria was calculated. Representative colonies were subcultured and species confirmation of *Pasteurellaceae,* aerobes and *Mycoplasma bovis* was done by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) (Brüker Daltonik GmbH, Bremen, Germany), according to the manufacturer's guidelines. Bacterial cultures were qualitatively interpreted as negative, polymicrobial, dominant or pure cultures as described previously (Van Driessche et al., 2017).

Total nucleated cell count (TNCC) of the recovered lavage fluid was determined manually using a haemocytometer. The sample was vortexed and 1 µl of BALf was diluted with 10 µl Türk's solution (Merck KGaA, Darmstadt, Germany) and counted manually using a Bürker counting chamber (Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany). Diff-Quick (Merck KGaA, Darmstadt, Germany) stained cytocentrifuge (Shandon Scientific, London, UK) preparations of BALf (1200 rpm for 10 min) were made and a total of 400 nucleated cells was counted at x100 magnification to calculate the differential cell count (Hoffman, 2008). All specimens were counted by the same trained observer. For each slide the following cellular characteristics were assessed: presence of immature neutrophils or toxic neutrophils, phagocytosing neutrophils (bacteria; cells; organic particles; inorganic particles), activation stage of macrophages (mild; moderately; severe), presence of giant cells, phagocytosing macrophages (bacteria; cells; organic particles; inorganic particles) and presence of epithelial cells (ciliated epithelial cells; bronchial epithelial cells). Slides were additionally evaluated for presence of extracellular bacteria (bacilli; cocci).

2.4. Animal selection procedure and case definitions

In Fig. 1 a flow chart of the selection procedure is shown. Inclusion criteria were age between 1 and 6 months and no treatment with antimicrobials in the past two weeks. Primary exclusion criteria were a polymicrobial BALf result (questionable interpretation for the purpose of this study) and an incomplete clinical exam. Secondary exclusion criteria were incomplete cytology data due to staining errors, damaged cells or cells unidentifiable due to artefacts.

First, calves were divided into four diagnostic categories based on the combination of clinical signs (present/absent) and ultrasonographic findings (no consolidation/consolidation). In a first analysis, the Wisconsin calf respiratory score was used to classify animals as clinically healthy or ill (McGuirk and Peek, 2014). In a second analysis, a more strict Healthy Criterion (HC) was used. This HC defined healthy calves as not showing nasal discharge or spontaneous cough and having a negative laryngeal and tracheal cough reflex, normal ear position, rectal temperature < 39.3 °C and breathing rate < 44 bpm. Any animal showing at least one of these signs mentioned above, was considered clinically ill. The ultrasonographic criterion for pneumonia was a lung consolidation depth of ≥ 1 cm. Using these clinical and ultrasonographic criteria four diagnostic categories were defined: healthy, upper respiratory tract infection (URTI), subclinical pneumonia and clinical pneumonia. Healthy animals were defined as clinically normal and no lung consolidation. Calves with upper respiratory tract infection (URTI) were clinically abnormal, but did not have lung consolidation. Subclinical pneumonia was defined as presence of lung consolidation without presence of clinical signs. Last, clinical pneumonia was defined as presence of clinical signs in combination with lung consolidation.

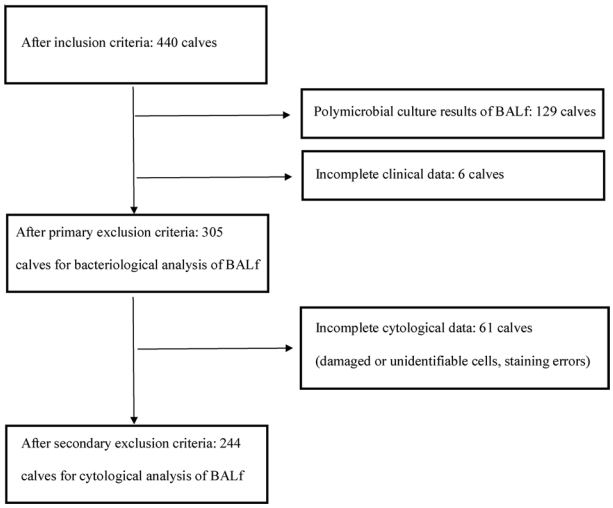

Fig. 1. Flow diagram showing inclusion and exclusion criteria for enrolment of calves in the study.

For a second analysis, a subdivision was made in the group of calves classified with clinical pneumonia based on presence or absence of intracytoplasmatic bacteria in BALf neutrophils (indicative of phagocytosis) and bacterial culture result, as recommended in other species (Hawkins et al., 1995; Peeters et al., 2000). With these additional criteria three subgroups within the clinical pneumonia category were created: (1) clinical pneumonia with positive culture result and presence of neutrophils phagocytosing bacteria, (2) clinical pneumonia with positive culture result and absence of neutrophils phagocytosing bacteria and (3) clinical pneumonia with negative culture result. This approach brought the total number of categories to 6.

2.5. Statistical analysis

All data were entered in a spreadsheet (Excel, Microsoft Inc. Washington, USA) and transferred to SAS 9.4 (SAS Institute Inc., Cary, N.C, USA) for statistical analysis. The experimental unit was the calf. To determine whether statistically significant differences in continuous outcome variables existed between the categories linear mixed effects models (PROC MIXED) were made. Outcome variables were TNCC, neutrophil percentage, macrophage percentage, lymphocyte percentage, percentage of epithelial cells and quantitative bacterial count (cfu/ml). Outcome variables were checked for a normal distribution. Log(x + 1) transformation was needed for TNCC, lymphocyte percentage, epithelial cell percentage and quantitative bacterial culture result. In each model, herd was added as a random effect to account for clustering of calves within a herd. All models were univariable, because the interest was in studying the effect of the classification category (healthy, URTI, subclinical and clinical pneumonia). Pairwise comparisons between different categories of significant effects were made using Bonferroni corrections. Model fit was assessed by visual inspection of residual plots and normality testing of residuals. The herd effect was not significant for any of the models.

For eosinophil and basophil percentage no transformation to a normal distribution was possible, hence it was opted to analyse them as binary outcomes in a multivariable logistic regression. Samples containing > 1 % eosinophils were considered positive (increased), as in humans and for basophils, a sample was considered positive if any of

these cells were seen in the 400 cells counted (Meyer, 2007; Olsen et al., 2012). Other categorical outcomes (n = 19), related to bacteriology and cytology of BALf, were immature neutrophils (absent; present), toxic neutrophils (absent; present), neutrophils phagocytosing bacteria (absent; present), neutrophils phagocytosing cells (absent; present), neutrophils phagocytosing organic particles (absent; present), neutrophils phagocytosing inorganic particles (absent; present), moderately activated macrophages (absent; present), extremely activated macrophages (absent; present), multinuclear giant cells (absent; present), macrophages phagocytosing bacteria (absent; present), macrophages phagocytosing cells (absent; present), macrophages phagocytosing organic particles (absent; present), macrophages phagocytosing anorganic particles (absent; present), bronchial epithelial cells (absent; present), ciliated epithelial cells (absent; present), erythrocytes (absent; present), extracellular bacteria (absent; present), extracellular bacilli (absent; present), extracellular cocci (absent; present).

Six bacterial isolation characteristics were tested: percentage of positive cultures in each group for *Pasteurella multocida* (0/1), *Mannheimia haemolytica* (0/1), *Histophilus somni* (0/1) and *Mycoplasma bovis* (0/1), percentage of cultures > 10^3 cfu/ml (0/1) and percentage of cultures > 10^4 cfu/ml per group (0/1).

All models were univariable, only evaluating the effect of classification category. Herd was added in each model as a random factor to account for clustering of the calves within a herd.

Model fit was evaluated using the Hosmer-Lemeshow goodness-of-fit test for logistic models. In all models significance was set at $P < .05$. All possible and biologically relevant interactions between significant main effects were tested. The herd effect was not significant for any of the models.

3. Results

3.1. Animals, clinical signs and ultrasonographic lesions

On 62 farms a total of 440 calves were sampled. After primary and secondary exclusion criteria (Fig. 1), samples of 305 and 244 animals were available for bacteriological and cytological analysis, respectively. Of the 305 calves, 2.3 % (7/305) were aged 4 weeks or less, 63.3 % (193/305) aged between 4 and 8 weeks and 34.4 % (105/305) were older than 8 weeks.

Lung consolidations ≥ 1 cm depth were present in 41.0 % (125/305) of the calves. Using the WI and the stricter healthy criterion (HC), 19.7 % (60/305) and 63.6 % (194/305) of the calves were considered clinically abnormal, respectively.

Based on the WI score in combination with ultrasonographic lung consolidation, 51.1 % (156/305) of the calves were considered healthy, 8.2 % (25/305) classified as URTI, 28.5 % (87/305) as subclinical pneumonia and 12.1 % (37/305) as clinical pneumonia. With the stricter HC combined with lung consolidation 25.9 % (79/305) of the calves were classified as healthy, 33.1 % (101/305) as URTI, 10.2 % (31/305) as subclinical and 30.8 % (94/305) as clinical pneumonia.

3.2. Qualitative and quantitative BALf bacterial culture

The average volume instilled to perform the BALf varied between 25–80 ml. In the majority of the calves (83.4 %) a flushing volume of 40 ml was used (367/440), in 13 % (57/440) 30 ml was used, in 1.4 % (6/440) 80 ml, in 1.1 % (5/440) 60 ml, in 0.7 % (3/440) 25 ml and 2 calves received a volume of 45 and 50 ml, respectively. The average percentage of instilled saline that was recovered after BAL was 32.2 % (standard deviation [SD] = 10.7; range [R] = 3.8–73.8).

A pure culture was obtained in 23.6 % (72/305) of the samples, a dominant culture in 49.8 % (152/305) and 26.6 % (81/305) were negative. An overview of the isolated bacteria is shown in Table 1. Multiple pathogens were present in 6.6 % (20/305) of the samples with the following combinations: *P. multocida* and *M. haemolytica* (30 % [6/20]), *M. bovis* and *P. multocida* (30 % [6/20]), *M. bovis* and *M. haemolytica* (20 % [4/20]), *P. multocida* and *H. Somni* (10 % [2/20]), *M. haemolytica* and *H. somni* (10 % [2/20]). In Tables 2 and 3, differences in isolation rates and quantitative culture results between the studied categories are shown, using the WI score and the HC, respectively. No significant differences were present for isolation rates nor quantitative cultures between the different diagnostic groups.

Table 1
Bacterial pathogens isolated from 305 BALf samples of indoor group-housed calves after exclusion of polymicrobial results.

Pathogen	Number of BAL samples with isolate (%)	Number of pure cultures/ number of positive cultures (%)
Pasteurella multocida	141/305 (46.2)	50/141 (35.5)
Mannheimia haemolytica	62/305 (20.3)	17/62 (27.4)
Histophilus somni	15/305 (4.9)	4/15 (26.7)
Mycoplasma bovis	11/305 (3.6)	1/11 (9.1)
Streptococcus suis	5/305 (1.6)	0
Moraxella spp.	6/305 (2.0)	4/6 (66.7)
Trueperella pyogenes	2/305 (0.7)	0
Staphylococcus xylosus	1/305 (0.3)	0
Gallibacterium anatis	2/305 (0.3)	0
Staphylococcus epidermidis	1/305 (0.3)	0
Streptococcus pluranimalium	1/305 (0.3)	0

3.3. Cytological findings in BALf

For a total of 244 calves complete cytological data was available for analysis. Macroscopic blood staining was present in 9 % (22/244) of the samples. When the four initial groups (healthy, URTI, subclinical and clinical pneumonia without subdivision) were compared no significant differences were found for TNCC or differential cell count between groups. When the second analysis was performed, using the WI score definition, the clinical pneumonia subgroup with positive culture results and phagocytosis by neutrophils showed a significantly higher neutrophil percentage (66.4 %) than healthy calves (37.1 %; $P = .015$), URTIs (32.5 %; $P = 0.013$) and calves with subclinical pneumonia (38.4 %; $P = 0.019$). However, no differences in neutrophil percentage were found when this group was compared to the other groups of clinical pneumonia (positive culture without phagocytosis and negative culture) (Table 4). Inversely, calves with clinical pneumonia, positive culture and phagocytosis showed lower lymphocyte percentages (1.0 %) than healthy calves (5.1 %; $P = 0.0017$), URTI (8.0 %; $P = 0.0040$) and subclinical pneumonia (6.1 %; $P < .001$). Macrophage percentage was lower (23.2 %) in the group of clinical pneumonia with positive culture and phagocytosis compared to URTI (46.4 %; $P = .038$) (Table 4).

Using the HC, the subgroup of clinical pneumonia with positive culture results and phagocytosis by neutrophils showed a significantly higher neutrophil percentage (59.0 %) than the healthy (37.7 %; $P = .0038$) and URTI (35.4 %; $P = .015$) group (Table 5). Within the clinical pneumonia group, percentage neutrophils was significantly higher in the phagocytosis group (59.0 %) compared to the no-phagocytosis group (36.5 %; $P = .021$). Inversely, calves with clinical pneumonia, positive culture and phagocytosis showed lower lymphocyte percentages (1.8 %) than healthy calves (5.3 %; $P = 0.0051$), subclinical pneumonia (7.1 %; $P < 0.001$) and clinical pneumonia without phagocytosis (5.2 %; $P = 0.011$) (Table 5).

The cellular characteristics for each of the categories studied are presented in Supplementary Tables 1 and 2. Significant differences between groups could only be demonstrated for the presence of macrophages phagocytosing bacteria, which were more frequently present in the group of calves with clinical pneumonia, a positive culture and evidence of intracellular bacteria in neutrophils (57.1 %) compared to healthy calves (6.9 %; $P = 0.043$), clinical pneumonia without

Table 2
Isolation rates and quantitative culture results of 305 indoor group-housed calves categorized according to the Wisconsin score card[a] and the presence of lung consolidation[b].

	Healthy[c]	URTI[d]	Subclinical pneumonia[e]	Clinical pneumonia[f]		
				Positive culture Phagocytosis	Positive culture No phagocytosis	Negative culture
n	156	25	87	7	26	4
% Pm	44.9	48.0	42.5	71.4	65.4	
% Mh	17.9	28.0	18.4	28.6	30.8	
% Hs	4.5	16.0	4.6	0	0	
% Mb	3.8	4.0	2.3	0	7.7	
Log cfu/ml mean ± SD (min-max)	4.5 ± 5.3 (1.3 – 6.2)	3.3 ± 3.5 (1.6–4.1)	4.7 ± 5.4 (1.3 – 6.2)	2.7 ± 2.8 (1.9–3.2)	5.2 ± 5.6 (2 – 6.3)	
% > 10^3 cfu/ml	39.8	33.3	40.4	20.0	54.5	
% > 10^4 cfu/ml	15.9	5.6	10.4	0	27.3	

URTI = upper respiratory tract infection, Pm = *Pasteurella multocida*, Mh = *Mannheimia haemolytica*, Hs = *Histophilus somni,* Mb = *Mycoplasma bovis*.
Values with different superscript are statistically different at $P < 0.05$.
[a] Clinically healthy = Wisconsin score < 5.
[b] Pneumonia = presence of lung consolidation with a depth of ≥ 1 cm.
[c] Healthy = clinically healthy + no lung consolidation.
[d] URTI = clinically ill + no lung consolidation.
[e] Subclinical pneumonia = clinically healthy + pneumonia.
[f] Clinical pneumonia = clinically ill + pneumonia.

Table 3
Isolation rates and quantitative culture results of 305 indoor group-housed calves, categorized according to clinical signs[a] and lung ultrasonographic findings[b].

	Healthy[c]	URTI[d]	Subclinical pneumonia[e]	Clinical pneumonia[f]		
				Positive culture Phagocytosis	Positive culture No phagocytosis	Negative culture
n	79	101	31	13	60	21
% Pm	45.6	44.6	51.6	76.9	56.7	
% Mh	20.3	18.8	9.7	23.1	33.3	
% Hs	5.1	6.9	6.5	0	3.3	
% Mb	3.8	4.0	3.2	0	5.0	
Log cfu/ml mean ± SD (min-max)	4.3 ± 5 (1.3 – 5.8)	4.6 ± 5.3 (1.3– 6.2)	3.8 ± 4.1 (1.6 – 4.7)	5.2 ± 5.7 (1.9 – 6.2)	5.9 ± 5.5 (1.3 – 6.3)	
% > 10^3 cfu/ml	39.5	38.7	37.5	45.5	43.8	
% > 10^4 cfu/ml	14.0	14.5	12.5	9.1	16.7	

URTI = upper respiratory tract infection, Pm = *Pasteurella multocida*, Mh = *Mannheimia haemolytica*, Hs = *Histophilus somni*, Mb = *Mycoplasma bovis*.
Values with different superscript are statistically different at P < 0.05.
[a] Healthy Criterion = normal ear position, absence of nasal and ocular discharge, no spontaneous cough, breathing frequency < 44 bpm, rectal temperature < 39.3 °C, negative laryngeal and tracheal cough reflex.
[b] Pneumonia = presence of lung consolidation with a depth of ≥ 1 cm.
[c] Healthy = clinically healthy + no lung consolidation.
[d] URTI = clinically ill + no lung consolidation.
[e] Subclinical pneumonia = clinically healthy + pneumonia.
[f] Clinical pneumonia = clinically ill + pneumonia.

phagocytosing neutrophils (0 %; $P < .001$) and clinical pneumonia with negative bacterial culture (0 %; $P < .001$) using the WI score (Supplementary Table 1). Using the HC, phagocytosing macrophages were more frequently present in the group clinical pneumonia, positive culture and intracellular bacteria in neutrophils (46.2 %) compared to URTI (6.5 %; $P = 0.023$) (Supplementary Table 2).

4. Discussion

In herds with endemic pneumonia several diagnostic modalities can be combined to optimize treatment regimens and detect causative organisms. Since a certain lag period exists between sample collection and microbiological results there is a need for on-site techniques to determine which calves need first intention antimicrobial treatment. Prudent antimicrobial use requires that pathogen isolation and susceptibility analysis is performed and first intention treatment is adjusted accordingly. To maximize the diagnostic potential of BALf analysis in this process, selection of the animals used for sampling is important. We performed a cross-sectional study to identify to what extent a clinical and ultrasonographic diagnosis of healthy, URTI, subclinical or clinical pneumonia corresponds with BALf bacteriology and cytology.

This is the first study comparing isolation rates and quantitative bacterial culture results between different diagnostic groups of calves. A first major finding was that bacterial isolation rate and quantitative bacterial count of both *Pasteurellaceae* and *M. bovis* did not differ between groups. Although different diagnostic techniques can be used to collect lower respiratory tract samples in calves, to date no gold standard is available. The small volume non-endoscopic BAL used in this study is an inexpensive and practical technique increasingly used in the field in Western Europe. Given this widespread use in the authors' and neighbouring countries, studying BALf obtained by this technique was preferred to guide practitioners in interpretation of its bacteriology and cytology results. Respiratory pathogens were cultured from BALf of healthy calves and calves with subclinical and clinical pneumonia. These results support the hypothesis that positive bacterial culture results from lower respiratory tract specimens do not necessarily represent pulmonary infection, and need to be differentiated from

Table 4
BALf cytological results of 244 indoor group-housed calves categorized according to the Winsconsin score card[a] and the presence of lung consolidation[b].

	Healthy[c]	URTI[d]	Subclinical pneumonia[e]	Clinical pneumonia[f]		
				Positive culture Phagocytosis	Positive culture No phagocytosis	Negative culture
n	119	20	72	7	23	3
TNCC (x 10^9 cells/L)	1.8 ± 1.6	1.8 ± 1.8	2.3 ± 2.3	2.1 ± 1.3	1.8 ± 1.2	.5 ± .2
Neutrophil %(mean ± SD)	37.1 [a] ± 25.1	32.5 [a] ± 26.7	38.4 [a] ± 22.6	66.4 [b] ± 10.6	41.7 [a,b] ± 18.8	22.1 [a,b] ± 19.0
Macrophage % (mean ± SD)	41.6 [a,b] ± 19.0	46.4 [b] ± 22.3	40.7 [a,b] ± 17.8	23.2 [a] ± 8.9	39.6 [a,b] ± 14.4	59.9 [a,b] ± 18.7
Lymphocyte % (mean ± SD)	5.1 [a] ± 4.6	8.0 [a] ± 12.6	6.1 [a] ± 4.4	1.0 [b] ± 0.8	3.8 [a,b] ± 3.0	3.4 [a,b] ± 2.0
Eosinophil % (mean ± SD)	.31 ± .82	.26 ± .46	.34 ± 1.2	.10 ± .19	.27 ± .4	.09 ± .16
Basophil % (mean ± SD)	.03 ± .11	.06 ± .13	.02 ± .09	.10 ± .17	.02 ± .08	.08 ± .14
Epithelial cell % (mean ± SD)	15.9 ± 14.1	12.8 ± 10.8	14.5 ± 11.7	9.3 ± 6.0	14.5 ± 15.1	14.4 ± 17.4
Eosinophil positive (%)	15.1	20.0	12.5	0	9.5	0
Basophils present (%)	9.2	25.0	5.6	28.6	9.5	33.3

TNCC = total nucleated cell count, URTI = upper respiratory tract infection.
Values with different superscript are statistically different at $P < 0.05$.
[a] Clinically healthy = Wisconsin score < 5.
[b] Pneumonia = presence of lung consolidation with a depth of ≥ 1 cm. Values with different superscript are statistically different at $P < 0.05$.
[c] Healthy = clinically healthy + no lung consolidation.
[d] URTI = clinically ill + no lung consolidation.
[e] Subclinical pneumonia = clinically healthy + pneumonia.
[f] Clinical pneumonia = clinically ill + pneumonia.

colonization of the lower respiratory tract, since this is not a sterile environment, and positive cultures can also be found in healthy calves (Griffin et al., 2010; Nicola et al., 2017). Therefore, results of bacteriological analysis, without considering the clinical status of the animal cannot be recommended as the sole decision criterion to initiate antimicrobial treatment. The isolation rates in healthy calves were comparable to previous studies showing isolation rates of 43 % for *P. multocida,* 12–16 % for *M. haemolytica* and 4–5 % for *H. somni* (Allen et al., 1991; Angen et al., 2009). Isolation rates for *M. bovis* were low in all groups, this is in contrast to other studies, demonstrating isolation rates ranging from 20 % up to 50 % in bronchoscopic and blind bronchoalveolar lavage samples (Pringle et al., 1988; Allen et al., 1991). These differences might be explained by variation in sampling and culture techniques, age of sampled calves, rearing conditions and preventive antimicrobial treatment regimes, or might be the result of misclassification of calves due to the use of imperfect diagnostic techniques.

To differentiate lower respiratory tract colonization from infection, mainly to exclude infectious bronchopneumonia when suggestive clinical signs and positive culture results are present, quantitative BALf bacterial counts are suggested in many other species such as horses (Hodgson, 2006) and dogs (Peeters et al., 2000). The underlying hypothesis for the use of this cut-off values is that the number of bacteria cultured form the lower respiratory tract is higher in infected animals compared to a colonization of the lower respiratory tract. Cut-off values proposed range from $> 1.0 \times 10^3$ cfu/ml in horses (Hodgson, 2006) to $> 1.3 \times 10^3$ cfu/ml BALf in dogs (Peeters et al., 2000). Yet, in our study no clear cut-off value could be established since differences between diagnostic groups were absent and large variation in bacterial counts was present, even within diagnostic groups. These results could be related to several factors. First, the time of sampling during the disease process could have had an influence. An early stage pneumonia is characterized by presence of a small number of micro-organisms which increase rapidly in numbers during the course of the disease

Table 5
BALf cytological results of 244 indoor group-housed calves, categorized according to clinical signs[a] and ultrasonographic findings[b].

	Healthy[c]	URTI[d]	Subclinical pneumonia[e]	Clinical pneumonia[f]		
				Positive culture Phagocytosis	Positive culture No phagocytosis	Negative culture
n	59	80	27	13	54	11
TNCC (x 10^9 cells/L)	1.9 ± 1.5	1.8 ± 1.6	2.8 ± 3.2	2.4 ± 2.3	1.8 ± 1.2	1.5 ± 1.3
Neutrophil % (mean ± SD)	37.7 [a] ± 26.3	35.4 [a] ± 24.6	41.0 [a,b] ± 24.9	59.0 [b] ± 18.8	36.5 [a] ± 17.7	38.8 [a,b] ± 30.1
Macrophage % (mean ± SD)	41.3 ± 20.7	43.0 ± 18.7	36.7 ± 16.9	31.0 ± 16.0	42.4 ± 15.2	45.0 ± 25.8
Lymphocyte % (mean ± SD)	5.3 [a] ± 4.7	5.7 [a,b] ± 7.4	7.1 [a] ± 5.7	1.8 [b] ± 1.1	5.2 [a] ± 3.3	4.3 [a,b] ± 3.0
Eosinophil % (mean ± SD)	.28 ± .72	.31 ± .82	.18 ± .40	.11 ± 0.19	.45 ± 1.3	.08 ± .12
Basophil % (mean ± SD)	.04 ± .11	.04 ± .11	.02 ± .07	.05 ± .13	.02 ± .11	.02 ± .07
Epithelial cell % (mean ± SD)	15.3 ± 12.8	15.6 ± 14.4	15.4 ± 11.0	8.1 ± 7.1	15.4 ± 13.7	11.8 ± 11.8
Eosinophil positive (%)	13.6	17.5	11.1	0	15.4	0
Basophils present (%)	10.2	12.5	11.1	15.4	5.8	9.1

TNCC = total nucleated cell count, URTI = upper respiratory tract infection.
Values with different superscript are statistically different at $P < 0.05$.
[a] Healthy criterion = normal ear position, absence of nasal and ocular discharge, no spontaneous cough, breathing frequency < 44 bpm, rectal temperature < 39.3 °C, negative laryngeal and tracheal cough reflex.
[b] Pneumonia = presence of lung consolidation with a depth of ≥ 1 cm.
[c] Healthy = clinically healthy + no lung consolidation.
[d] URTI = clinically ill + no lung consolidation.
[e] Subclinical pneumonia = clinically healthy + pneumonia.
[f] Clinical pneumonia = clinically ill + pneumonia.

process (Ackermann et al., 2004). If the lower respiratory tract is sampled in an early stage of infection, bacterial burdens might be too low to detect by means of bacterial culture. This could be avoided using enrichment broths or PCR testing to increase sensitivity of pathogen detection. However, based on the results of our study using these techniques would further increase overdiagnosis of bacterial pneumonia, possibly resulting in unnecessary antimicrobial use. Secondly, these results could indicate that airway colonization by opportunistic pathogens occurs to a far greater extent than previously expected in calves. Possibly this results from environmentally evoked impaired respiratory tract defences, for instance due to exposure to ammonia or other air pollutants. Thirdly, despite the use of a weight-adjusted lavage volume, as is recommended in dogs (Melamies et al., 2011) and humans (Ratjen and Bruch, 1996), differences in quantitative bacterial culture results might be attributed to different dilutions rates of the epithelial lining fluid (ELF) in the sample (Zedtwitz-Liebenstein et al., 2005). Since it was impossible in our study to analyse BALf urea, as suggested in other species as marker of dilution (Rennard et al., 1985), to enable calculation of the possible dilution of ELF these effects could not be estimated. Lastly, nasal passage of the catheter could result in upper respiratory tract contamination of samples, albeit not proven to date that this contamination is that substantial that interpretation of BALf bacterial culture results would be significantly hampered. Nevertheless, to partially avoid this issue polymicrobial culture results were excluded from this study for their possible inconclusive nature. Bacterial culture of BALf yields significantly more negative and less polymicrobial results compared to deep nasopharyngeal swabs (Van Driessche et al., 2017), however, a certain degree of polymicrobial samples is inevitable. Exclusion of these cultures decreased sample size in our study which might have had an impact on the quantitative bacterial culture results.

A second major finding was that neutrophil percentage was not higher in animals with clinical pneumonia (as defined by positive HC or WI score and lung consolidation), when cases of clinical pneumonia were not further subdivided based on phagocytosis by neutrophils. This is in contrast to the hypothesis and previous work (Ollivett et al., 2015). These discrepancies in results with or without taking phagocytosis into account could be explained as follows. First, for this study calves were selected from herds with endemic pneumonia with various clinical signs and diagnostic classification was performed using clinical examination and on-farm TUS. Sampling of animals in these conditions, with these diagnostic tools reflects current field conditions, improving the external validity of our study. However, these diagnostic tools show a certain degree of imperfection. Interpretation of clinical signs is subject to individual variation, and inter-observer reliability for scoring clinical signs using the Wisconsin Score card is low (Buczinski et al., 2016). Thoracic ultrasonography is a specific and sensitive on-farm tool for the detection of (sub)clinical pneumonia and inter-rater agreement is good for the presence of lung consolidation, comet tails and pleural effusion (Michaux et al., 2018). Yet, the reliability of measuring the depth of consolidation is only moderate (Michaux et al., 2018). Since all sampling and recordings were done by one single observer in our study, misclassification bias could have occurred. In order to overcome these issues we explored several clinical definitions of which the Wisconsin score card was used as an international reference and a very strict clinical scoring system (Healthy Criterion) as an alternative. However, for both clinical scoring systems the same conclusions could be drawn. Second, these results might also indicate that animals with clinical pneumonia, diagnosed by clinical and TUS findings, did not all suffer from an active bacterial infection. Calves could also have been affected by viral infections or TUS lesions could be the result of a previous episode of respiratory tract infection which has not yet completely healed. Our results show that accessible methods to determine respiratory health based on ultrasonographic and clinical examination do not coincide with analyses performed on lung level. Although it was not the objective of this study to provide guidelines for antimicrobial use for respiratory disease in calves, these results might indicate that initiation of antimicrobial treatment based on the four diagnostic categories alone will likely not result in a hundred-percent prudent use of antimicrobials. Therefore, additional parameters in BALf, such as phagocytosis by neutrophils, can be used to identify calves with an active bacterial infection. This is in agreement with studies in dogs using neutrophilic cellular patterns in BALf together with intracellular presence of infectious organisms, as indicators for a lower respiratory tract infection (Peeters et al., 2000; Johnson et al., 2013). In human medicine the presence of intracellular bacteria in BALf neutrophils alone as a diagnostic tool has a reasonable specificity, but lacks sensitivity restricting its use to confirm absence of pulmonary infection in patients with suggestive clinical signs (Papazian et al., 1993; Sirvent, 2003). Our findings were comparable since 15–20 % of the healthy and URTI calves displayed phagocytosing neutrophils in BALf. This presence of intracellular bacteria in neutrophils of healthy and URTI calves could partially be the result of continued phagocytosis of neutrophils during transport and preservation of the samples (Pickles et al., 2002; Cian et al., 2015). Future studies should also take the number of infected cells into account to establish cut-off values defining bacterial infection using this additional cytological parameter in calves.

A third major finding was the presence of high average BALf neutrophil levels, regardless of the diagnostic classification of the calves. This resembles a study in feedlot calves, demonstrating neutrophil levels of more than 20 % in clinically healthy controls (Allen et al., 1992). These results are in contrast to another study showing a neutrophil percentage < 5 % in BALf of ultrasonographically healthy calves (Ollivett et al., 2015). These differences in results might be related to the sampling techniques used. By using a small volume nBAL, as in our study, it is possible that a more proximal part of the lower airways is sampled, resulting in a larger bronchial component in the sample, compared to the alveolar component. This could increase neutrophil percentage of BALf (Sweeney et al., 1992). Notwithstanding, by using a narrow nBAL catheter with an outer diameter of 4 mm which wedges deep into the lungs, the bronchial component of BALf would be limited. Furthermore, given that neutrophil percentage was also high in healthy calves, the presence of pulmonary inflammation induced by non-infectious factors under these indoor housing conditions is likely. This airway inflammation might be triggered by inhalation of dust, ammonia or other toxic components (e.g lipopolysacharide (LPS)) in stable air, as shown in horses (Holcombe et al., 2010; Pirie et al., 2010). Nevertheless, as in infected and non-infected asthmatic children, even when neutrophil percentage is generally high due to exposure to toxic stable climate components, calves suffering from a bacterial pneumonia will display significantly higher neutrophil values compared to baseline values in healthy animals under the same housing conditions, as we demonstrated when taking phagocytosis into account (Najafi et al., 2003).

Another cytological feature we found is the significant difference in lymphocyte percentage between groups. Lymphocyte percentage in BALf was comparable with results of other studies in calves (Pringle et al., 1988; Allen et al., 1992; Ollivett et al., 2015), although being significantly lower in calves with clinical pneumonia, positive culture and presence of phagocytosis compared to healthy calves in our study. This could be due to a relative reduction caused by the increased influx of neutrophils in BALf, or the possibility that lymphocytes are more important in other non-infectious or viral respiratory diseases (Samegai et al., 2002). The role of viral agents triggering pulmonary inflammation cannot be excluded in our study as viral analysis of samples has not been performed for financial reasons.

Finally, to correctly interpret the results of this study some limitations should be mentioned. Although BAL is the reference method to collect lower respiratory tract samples for cytology, some features of the nBAL technique should be taken into account. First, in diffuse, interstitial pulmonary diseases BALf cytological outcome is not affected by the lobe of the lungs that is sampled (Meyer, 2007; Meyer et al., 2012). On the contrary, in animals with pneumonia BALf cytology

might not be extrapolated to the lungs as a whole since it is expected that differences between infected and non-infected lung lobes are present (Ollivett et al., 2015). The nBAL is a blind sampling technique which samples lung lobes randomly in unsedated animals (Van Driessche et al., 2016). As a result non-infected lung lobes could be sampled resulting in false negatives. Techniques which allow selected sampling of infected lung lobes, such as selective bronchoscopic sampling are likely more easy to interpret, yet their use in field conditions remains limited unless cheaper and disposable techniques become available. Secondly, by choosing a cross-sectional study design no information on former respiratory disease episodes in the calves is available. Therefore, ultrasonographic lesions found could represent former disease episodes, are healing lesions or lesions that would be self-cured by the calves.

In conclusion, classification of calves into four diagnostic categories based on clinical and TUS findings alone did not coincide with bacterial isolation rate, quantitative bacterial culture and neutrophil percentage of BALf obtained by nBAL. Bacterial culture results of nBAL samples should preferably be interpreted in combination with cytological findings to confirm causality of cultured pathogens. In calves housed under current indoor-housing conditions, BALf neutrophil percentage and bacterial isolation levels can already be high, independent from clinical disease, potentially due to stable air quality. Neutrophil percentage and phagocytosis of bacteria by neutrophils are potentially valuable indicators to identify individuals requiring antimicrobial treatment as these show potential to be used for confirmation of an active bacterial pulmonary infection.

Ethical committee approval

All sampling techniques and the study protocol were revised by the local ethical committee and permitted under experimental licence number EC2016 - 89.

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Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prevetmed.2020.104901.

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