Storage time and temperature affect the isolation rate of Mannheimia haemolytica and Pasteurella multocida from bovine bronchoalveolar lavage samples

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Abstract

Background A microbiological diagnosis is essential to better target antimicrobial treatment, control and prevention of respiratory infections in cattle. Under field conditions, non-endoscopic bronchoalveolar lavage (nBAL) samples are increasingly collected. To what extent the highly variable turnaround time and storage temperatures between sampling and cultivation affect the isolation rate of bacterial pathogens is unknown. Therefore, the objective of this experimental study was to determine the effect of different storage temperatures (0°C, 8°C, 23°C and 36°C) and times (0,2,4,6,8,24,48 hours) on the isolation rate and concentration of Pasteurellaceae in nBAL samples from clinically affected animals.Results Storage at 36°C resulted in a reduced isolation rate already 2 hours after sampling for Mannheimia haemolytica and 24 hours after sampling for Pasteurella multocida . When samples were stored at 23°C, a decrease in M. haemolytica and P.multocida isolation rate was noticed, starting at 24 and 48 hours after sampling, respectively. The presence of microbial contamination negatively affected the isolation of P. multocida in clinical nBAL samples. An increase in concentration of contaminating bacteria was noticed after 24 hours of sampling at a temperature of 36°C and after 48 hours of sampling at a temperature of 23°C.Conclusion Optimal M. haemolytica and P. multocida isolation rates from clinical nBAL samples are obtained after storage at 0°C or 8°C, provided that the sample is cultivated within 24 hours after sampling. The maximum period a sample can be stored without an effect on the M. haemolytica and P. multocida isolation success varies and is dependent on the storage temperature and the degree of microbial contamination.

Background

Respiratory tract infections (bovine respiratory disease (BRD)) have a major impact on farm economics and animal welfare [1]. Furthermore, they are the main indication for antimicrobial use in calves [2]. In order to rationalize antimicrobial use, diagnostic techniques need to be optimized. Non-endoscopic bronchoalveolar lavage (nBAL) is a practical and economical technique, increasingly used in Western European countries to sample the lower airways of cattle [3]. Although this technique obtains more pure cultures compared to a deep nasopharyngeal swab, microbial sample contamination can occur, partly depending on the experience of the veterinarian [3]. In order to minimize microbial contamination and bacterial overgrowth, cultivation of samples needs to be performed as soon as possible after sampling. However, due to the centralization of veterinary laboratories and the limited operating hours of these laboratories (not 24/7 as in some human clinics), the turnaround time between sampling and cultivating of the samples can take 24 hours to even several days. Optimal storage conditions, supporting survival of causal pathogens and limiting growth of contaminants, are needed to obtain relevant bacterial analysis results [4]. False negative or irrelevant results may lead to therapy failure, resulting in increased antimicrobial use, antimicrobial resistance and mortality. Although storage conditions of clinical samples in the field are important, only few studies addressed this subject. Two studies are available comprising the effects of long term survival of *Pasteurellaceae*, namely in swabs from bears [5] or ovine and bovine tracheobronchial washings [6]. In the latter experiments, however, sterile lung fluids were spiked, and

therefore possible contaminant effects were not taken into account. To what extent nBAL field samples can be stored until analysis, without influencing the isolation rate of clinically import pathogens, is currently unknown. Therefore, the objective of the present study was to determine the effect of storage temperature and duration on the isolation rate of *Pasteurellaceae* from bovine nBAL field samples.

Results

Animals that met the inclusion criteria aged 1 week to 7 months. In total, 13 nBAL samples were collected, of which the initial culture results at T0 showed 6 dominant cultures with 1 clinically relevant pathogen (4 *M. haemolytica*, 2 *P. multocida*) and 7 mixed cultures with both *M. haemolytica* and *P. multocida* (3 pure cultures containing only *M. haemolytica* and *P. multocida* and 4 dominant cultures with also some contaminants present). In total, *M. haemolytica* was isolated from 11 samples (84.6%) with an average log concentration of 4.0 at T0, and *P. multocida* from 9 samples (69.2%) with an average log concentration of 3.7 at T0. *Trueperella pyogenes* was isolated from one sample and *Moraxella bovis* from two samples at T0, both in mixed cultures. *H. somni* was not isolated. Contaminants present in the dominant cultures were *Streptococcus* spp., *Staphylococcus* spp., *Bacillus* spp., *Escherichia coli* and *Rothia nasimurium* with an average log concentration of 2.8 at T0.

The effect of the various temperatures and storage periods after sampling on the number of positive samples for *M. haemolytica* is presented in Figure 1. In general, the number of samples from which *M. haemolytica* could be isolated, decreased gradually over time. The higher the storage temperature, the earlier the number of positive samples started to decrease, i.e. at 2 hours of storage at 36°C, at 24 hours of storage at 23°C and at 48 hours of storage at 0°C and 8°C. During storage at 0°C and 8°C, the number of positive samples remained stable up to 24 hours after sampling, varying between 10/11 and 11/11 in this period, whereas at 23°C, only 7/11 samples remained positive after 24 hours of storage. When samples were stored at 36°C for 48 hours, *M. haemolytica* could be isolated from only 1 sample, while this was 4/11 for 23°C and 8/11 for both 0°C and 8°C (Figure 1).

An overall slight decrease in *M. haemolytica* concentration occurred over time (Figure 2). At a storage temperature of 36°C, the average log concentration of *M. haemolytica* decreased after 2 hours of storage from 4 to 3.5 and remained stable until 48 hours after sampling. No difference in concentration of *M. haemolytica* was noticed between each sampling time between a temperature of 0°C, 8°C and 23°C. Starting from 8 hours after sampling, the different temperatures did not affect the concentration of *M. haemolytica*. (Figure 2). No statistical significant difference was seen between the different temperatures and the time after sampling.

The effect of storage temperature and time on the number of samples from which *P. multocida* could be isolated is presented in Figure 3. A decline in the number of positive samples from which *P. multocida* was isolated was observed throughout the experiment for storage at 36°C, where only 4/9 positive samples were retrieved at 24 hours of storage and no positive samples could be retrieved at 48 hours (Figure 3). This decline in the number of positive samples was due to both contaminant overgrowth and a

decreased viability. This decreased viability of *P. multocida* was noticed at a storage temperature of 36°C starting from 24 hours after sampling and at a storage temperature of 23°C starting from 48 hours after sampling. When samples were maintained for 48 hours at 23°C, only 3/9 samples were found positive. At a storage temperature of 0°C or 8°C, the number of positive samples remained stable until 24 hours after sampling, with an isolation rate of 8/9 to 9/9. At 48 hours of storage at a temperature of 0°C, 1 initially *P. multocida* positive sample was negative due to decreased viability. When stored at 8°C for 48 hours, 2 initially *P. multocida* positive samples were negative, one due to decreased viability and one due to contaminant overgrowth.

Compared with *M. haemolytica*, the average concentration of *P. multocida* remained more stable until 24 hours of storage at a temperature of 0°C, 8°C and 23°C (Figure 4). At a storage temperature of 36°C, the *P. multocida* concentration slightly decreased starting from 6 hours after sampling, with a concentration below detection limit at 48 hours after sampling. Accordingly, no positive samples of *P. multocida* were retrieved after storage at 36°C for 48 hours, even in the absence of contamination overgrowth. When samples were stored at 0°C or 8°C, only a slight decrease in log concentration was noticed, from 3.7 at TO to 3.2-3.3 after 48 hours. At 48 hours after sampling, the higher the storage temperature, the lower the concentration of *P. multocida* (Figure 4).

From the 13 samples collected, 10 samples contained microbial contamination at T0. Of the 3 initial samples that were not contaminated at T0, 1 sample showed microbial contamination starting from 2 hours after sampling. The other two initially negative samples showed sporadically microbial contamination with a concentration closely to the detection limit. Results of the influence of bacterial contamination on the isolation rate of *M. haemolytica* and *P. multocida* in the clinical nBAL samples are presented in Table 1. A statistically significant negative association was seen between the presence of contaminants and the presence of *P. multocida*. This negative association was also noticed for *M. haemolytica*, although not statistically significant. An odds ratio of 0.32 was obtained for *P. multocida* (P=0.04), meaning that the presence of contaminants reduced the odds of isolating *P. multocida* for 68%. For *M. haemolytica* this odds ratio was 0.7 (P=0.651), resulting in a reduced odd of isolating this pathogen with 30% when contaminants were present. An average initial contaminants log concentration of 2.8 CFU/mL was observed (Figure 5). This concentration remained stable during the first 8 hours of storage, regardless of storage temperature. However, after 24 hours of storage at 36°C, the average contaminants log concentration increased to 4.4. After 48 hours of storage, an average contaminants log concentration of 4.7 and 5.1 was reached for storage at 23°C and 36°C, respectively (Figure 5). No statistically significant difference was seen between the different temperatures and the hours after sampling.

Table 1: The effect of the presence of microbial contamination on the isolation of *M. haemolytica* and *P. multocida*

		Contaminants		Odds ratio	Confidence Interval	*P*-value
		Negative	Positive			
Mannheimia haemolytica	Negative	37.4%	62.2%	0.70	0.58 -1.41	0.651
	Positive	45.8%	54.2%			
Pasteurella multocida	Negative	21.2%	78.8%	0.32	0.37 -0.83	0.04
	Positive	45.7%	54.3%			

Discussion

This study describes the effects of different storage temperatures and times on the isolation rate and concentration of *Pasteurellaceae* from nBAL samples. When nBAL samples were stored at a temperature of 0°C or 8°C, high isolation rates of *P. multocida* and *M. haemolytica* were obtained until 24 hours of storage. Since storage at 0°C has low feasibility in routine practice, storage of clinical nBAL samples in a refrigerator is a readily available alternative for most practitioners. Also in previous studies low temperatures are recommended for storage [7, 8].

Even though there was no statistically significant decrease in the isolation rate after 24 hours of storage at room temperature (23°C) for both *P. multocida* and *M. haemolytica*, a probably relevant decrease in *M. haemolytica* isolation rate (from 11/11 to 7/11) was observed. The reason for this decreased isolation rate was both due to a decreased viability (2/4 samples) and to microbial contamination (2/4 samples). According to Tano et al. [9], clinically important bacteria can stay viable for 24 hours at room temperature, but not in polymicrobial samples. However, these samples were spiked with high pathogen concentrations (10^6 CFU/mL). When lower concentrations, comparable with concentrations obtained in the present study, were used (10^4 CFU/mL and 10^5 CFU/mL), results in viability varied. In another study the viability of *M. haemolytica* remained for a long period of up to 156 days [6]. However samples were spiked with high concentration (10^6 – 10^7 CFU/mL) and no bacterial contamination was present. These results stress the importance of a high initial pathogen concentration and avoiding microbial contamination during and after the sampling procedure.

When samples were stored at 36°C, the isolation rate started to decline already 2 and 24 hours after sampling for *M. haemolytica* and *P. multocida*, respectively. When samples were maintained for 48 hours at these temperatures, isolating clinically relevant pathogens was rare or no longer possible. This was mainly, but not exclusively, due to the increased concentration of contaminants, leading to uninterpretable samples when a concentration of $\geq 1x10^6$ CFU/mL of contaminants was reached. These results stress

the importance of not leaving samples above room temperature, for example in a closed car or in a tropical environment.

The concentration of *M. haemolytica* slightly decreased over time independent of the storage temperature, though not statistically significant, while for *P. multocida*, this concentration remained more stable. Currently, no information is available on the survival rate of latter 2 bacteria in nBAL samples. One recent study describes the survival rate and density range of *Pasteurellaceae* in the nasopharyngeal microbiota in healthy calves [10]. This study showed a longer duration of carriage in the nose and higher concentration for *P. multocida* compared to *H. somni*, however rates of *M. haemolytica* were too low for meaningful survival modelling. Retaining high concentrations of relevant bacteria in clinical samples can be an added value to diagnostics, both for clinical interpretation as for direct detection methods using for example matrix-assisted laser desorption/ionization-time-of- flight mass spectrometry (MALDI-TOF MS) [11]. A higher negative association between the presence of contaminants and the isolation rate of *P. multocida* was found compared to *M. haemolytica*. This might be partially due to the fact that the average initial load of *M. haemolytica* in the samples was higher compared with the initial *P. multocida* load. Contaminant overgrowth might therefore negatively impact *P. multocida* isolation rate more quickly than the *M. haemolytica* isolation rate. A previous study showed that the growth of *M. haemolytica* can be inhibited by contaminants like *Escherichia coli* rather than *Staphylococcus* spp. or *Streptococcus* spp. [12]. To what extent different bacterial contaminants had an inhibitory effect on *P. multocida* and/or *M. haemolytica* in this study is unclear, considering the limited number of samples and since different bacterial contaminants were often combined at different concentrations within one clinical sample.

A limitation of current study is the limited sample size. When using 8 positive samples per test group, only 60% difference in isolation rate could be detected. One of the reasons for the limited number of used samples are the strict inclusion and exclusion criteria applied. Indeed, only samples obtained from untreated calves, well-characterized as clinically affected at the level of the lower respiratory tract were included. In addition, only samples from which clinically relevant bacteria could be isolated at T0 were included in the experiment, resulting in the exclusion of various samples. Nevertheless, we feel that the current experimental set-up with a limited number of well-chosen samples and in-depth analysis of the obtained results allows drawing conclusions that are relevant for the practitioner. Another limitation of this study is that, considering the cultivation conditions used in this study, other relevant bacterial pathogens such as *Histophilus somni* and *Mycoplasma bovis* could not be isolated from the current clinical nBAL samples. However, *H. somni* is only rarely isolated because of poor viability and the fact that it is easily overgrown by other bacteria, either clinically relevant or not. Although different studies are available describing the effect of storage conditions on the recovery of *M. bovis*, this was only investigated at low temperatures in milk samples [13,14] or colostrum samples [15]. Therefore, further research into the effect of storage conditions on the recovery of *M. bovis* from nBAL samples is encouraged. Currently, the gold standard technique for identifying these fastidious bacteria is polymerase chain reaction (PCR). Since viability is not mandatory with this technique, it can be expected that the effect of storage conditions for identifying these pathogens will be less important in most veterinary labs using PCR to identify the latter pathogens. Moreover, maximizing the chance of isolating *P. multocida* and

M. haemolytica is more critical since performing antimicrobial susceptibility testing in these species can be of major importance for appropriate antimicrobial treatment, while antimicrobial resistance is less prevalent in *H. somni* [16], or even not routinely tested for in *M. bovis.*

Conclusion

This study demonstrates that optimal *M. haemolytica* and *P. multocida* isolation rates from clinical nBAL samples are obtained after storage at 0°C or 8°C, provided that the sample is cultivated within 24 hours after sampling. The maximum period a sample can be stored without an effect on the *M. haemolytica* and *P. multocida* isolation success varies and is dependent on the storage temperature and the degree of microbial contamination.

Methods

The sample size required to determine a 60% difference in isolation rate (80% vs 20%) with 80% power and 95% confidence for a 2-sided test was 8 positive nBAL samples per test group (Winepiscope 2.0, Zaragoza, Spain). Each test group comprises the presence of a clinically relevant bacterial pathogen, namely *Pasteurella multocida*, *Mannheimia haemolytica* or *Histophilus somni*. Therefore samples were taken until a minimum number of 8 positive culture results per test group was reached. An experimental study design was performed on 4 unrelated farms (3 beef, 1 dairy) between March and April 2018. Farms suffering from an acute outbreak of BRD were reported by local veterinarians and subsequently visited by the research staff. Animals to be sampled were selected based on previously described inclusion criteria [17]. Additionally, thoracic ultrasound examination was performed with a 7.5-MHz linear probe (Tringa Linear Vet, Esaote, the Netherlands) as previously described [18]. The definition for a case was the presence of a consolidated zone in the lung of ≥1 cm depth [19]. Animals that were treated with antimicrobials within 14 days prior to sampling were excluded from the study.

Cattle that met the inclusion criteria were sampled with the nBAL procedure as previously described [20]. Briefly, after disinfecting the nostril with 70% alcohol, a reusable home-made polytetrafluorethylene catheter adjusted with a 12-G catheter stylet was inserted in the nasal cavity and gently advanced, through larynx and trachea, into the bronchi. Next, 60 mL of sterile 0.9% NaCl was injected into the lungs and immediately aspirated (recovery of 30-50% of the fluid). Samples were transported at ambient temperature and further processed within 30 minutes after sampling.

Twenty mL of each nBAL sample was used for further analysis and was divided equally over four different 50mL Falcon tubes after vortexing for 1 minute (5mL each). Each Falcon tube was incubated at a different temperature, all monitored with a thermometer, i.e. 0°C +/-1°C (ice), 8°C +/- 1°C (refrigerator), 23°C +/- 1°C (room temperature) and 36°C +/- 1°C (incubator) for 0, 2, 4, 6, 8, 24 and 48 hours. After each incubation period, the sample was vortexed for 30 seconds and 100 µL was transferred to an Eppendorf tube, already containing 900µL phosphate buffered saline (PBS). Ten-fold dilutions were made of each sample for quantitative analysis as previously described [21]. From each dilution, 100µl was inoculated

on Columbia agar with 5% sheep blood (blood agar; Oxoid, Hampshire, UK) and incubated overnight at 35°C +/-2°C in a 5% CO_2 atmosphere. All macroscopically different colonies were counted and identified with MALDI-TOF MS as previously described [22]. A positive culture result was defined as the macroscopically visible presence of one or more clinically relevant *Pasteurellaceae* (*P. multocida*, *M. haemolytica* and *H. somni*) colonies in pure, dominant or mixed cultures as previously described [3]. Only samples with a positive culture at time point 0 hours (T0) were included in the experiment. When no clinically relevant pathogen could be isolated at time points 2, 4, 6, 8, 24 or 48 hours, the concentration of the pathogen isolated at T0 in this sample was set at 100 Colony forming units (CFU)/mL (being the detection limit of this plating procedure).

The association between the different conditions for the isolation of *Pasteurellaceae* was determined by means of a multivariable logistic regression model with repeated measures (PROC GLIMMIX). Bonferroni corrections were used to compare between 4 groups. To determine the association between the presence of contaminants and isolation of *M. haemolytica* or *P. multocida* logistic regression was used (PROC LOGISTIC). Model validity was evaluated by the Hosmer-Lemeshow goodness-of-fit test for logistic models. Significance was set at $P<0.05$. All data were collected in Microsoft Excel and statistical analysis was performed in SAS 9.4 (SAS Institute Inc., Cary, NY).

Abbreviations

BRD: bovine respiratory disease; CFU: colony forming unit; MALDI-TOF MS: matrix-assisted laser desorption/ionization-time-of- flight mass spectrometry; nBAL: non-endoscopic bronchoalveolar lavage; PCR: polymerase chain reaction

Declarations

Ethics approval and consent to participate

All procedures were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC 2016/20). Verbal informed consent was obtained from all participants by telephone (veterinary officers and farmers), which was approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC 2016/20).

Consent for publication

Not applicable

Availability of data and materials

The dataset used and analysed during the current study is available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

LVD designed and conducted all experiments, collected literature data, analysed data, prepared figures and prepared the paper. CDN conducted all experiments, collected literature data, analysed data and prepared figures. FB designed the study, analysed data and supervised the work. BP designed the study, analysed data, prepared figures and supervised the work. FH analysed data. All authors read and reviewed the final manuscript.

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Figures

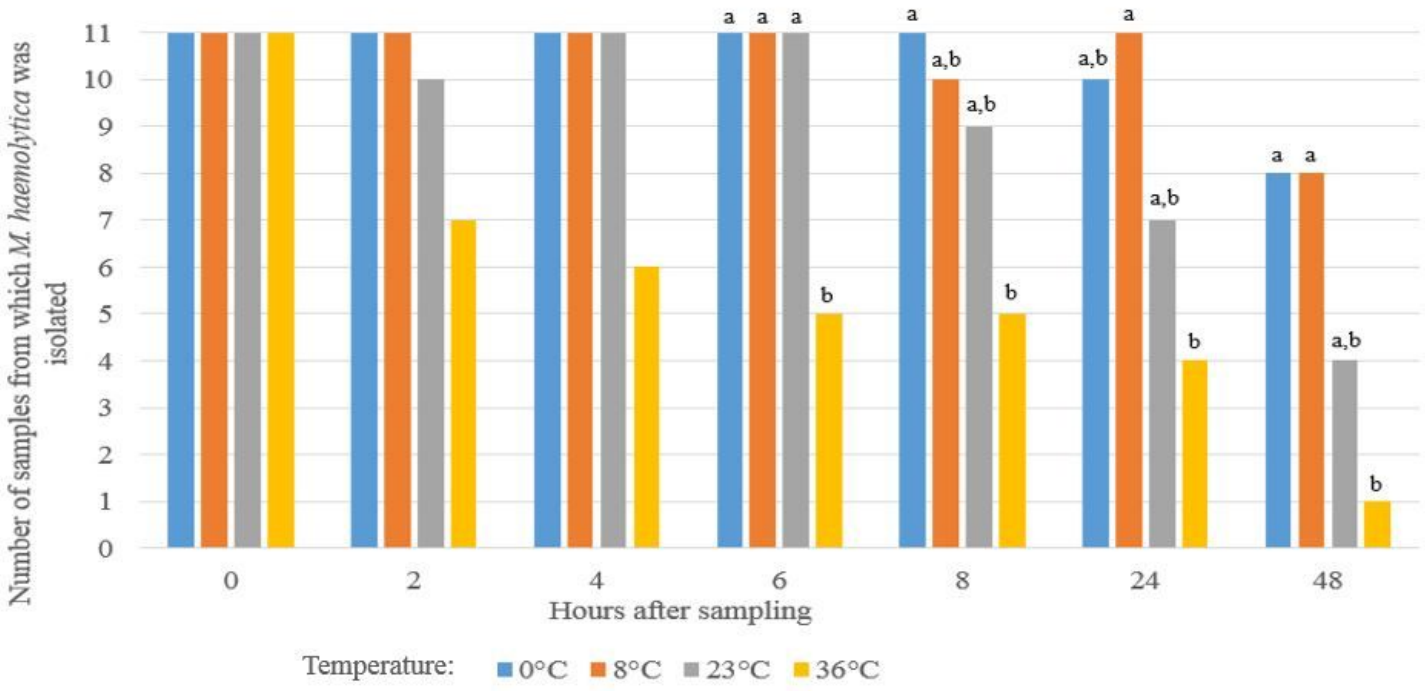

Figure 1

Effect of storage conditions (temperature and time) on the number of bovine nBAL samples from which M. haemolytica could be isolated. Different letters (a-b) indicate a significant difference ($P<0.05$) in temperature within one time after sampling.

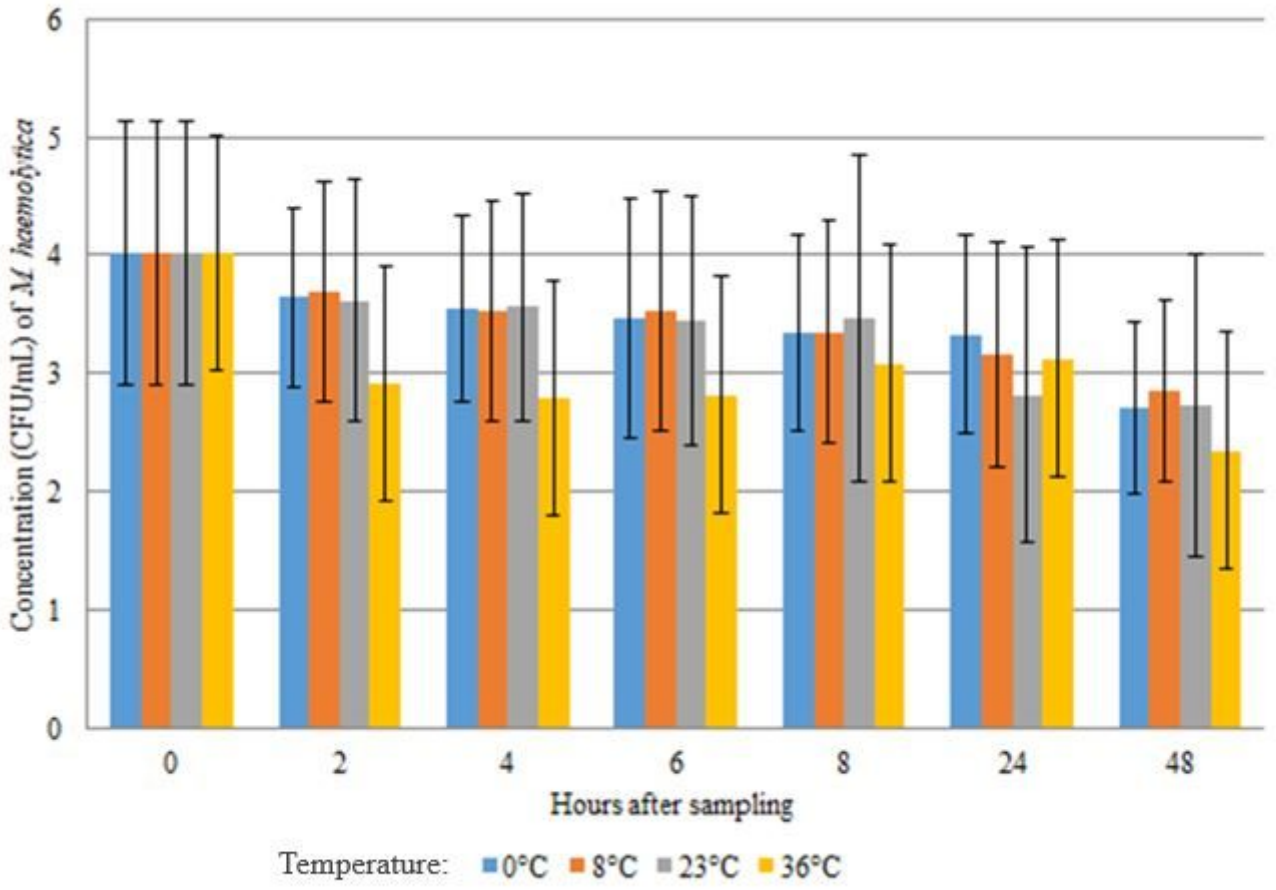

Figure 2

Effect of storage conditions (temperature and time) on the concentration of M. haemolytica in bovine nBAL samples. No statistically significant difference was seen between the different temperatures and the hours after sampling.

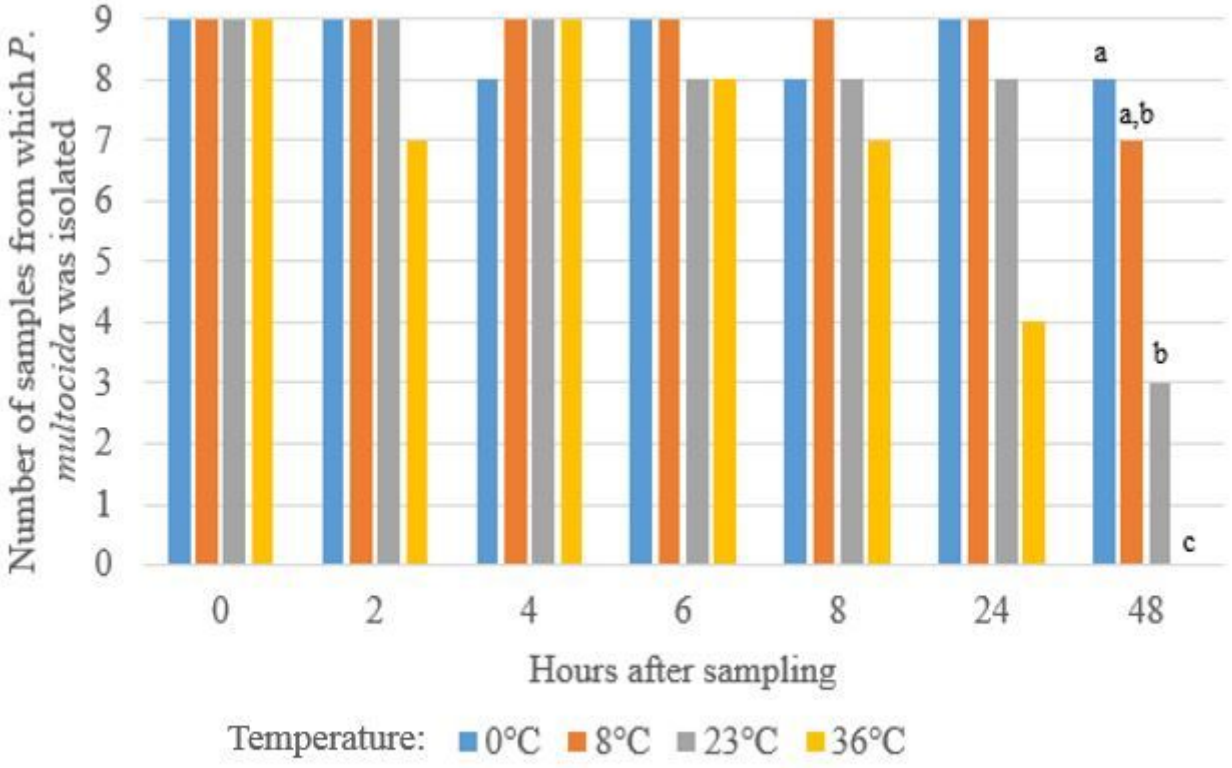

Figure 3

Effect of storage conditions (temperature and time) on the number of bovine nBAL samples from which P. multocida could be isolated. Different letters (a-c) indicate a significant difference ($P<0.05$) in temperature within one time after sampling.

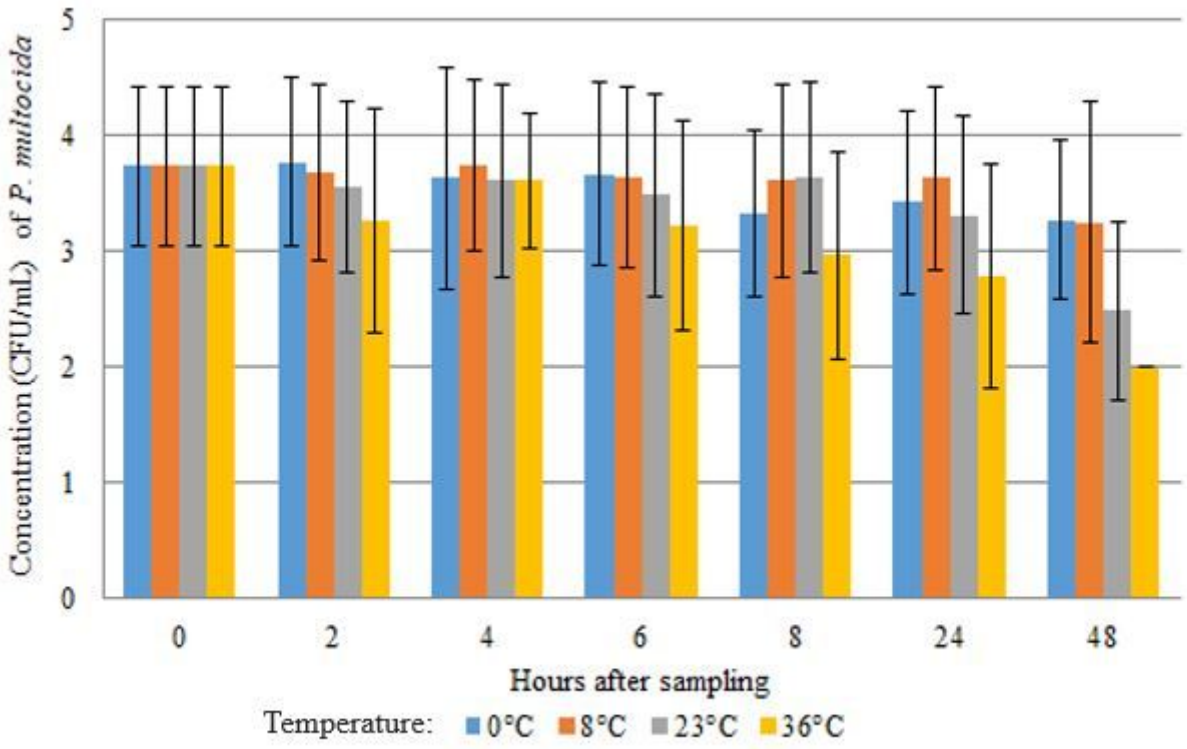

Figure 4

Effect of storage conditions (temperature and time) on the concentration of P. multocida in bovine nBAL samples. No statistically significant difference was seen between the different temperatures and the hours after sampling.

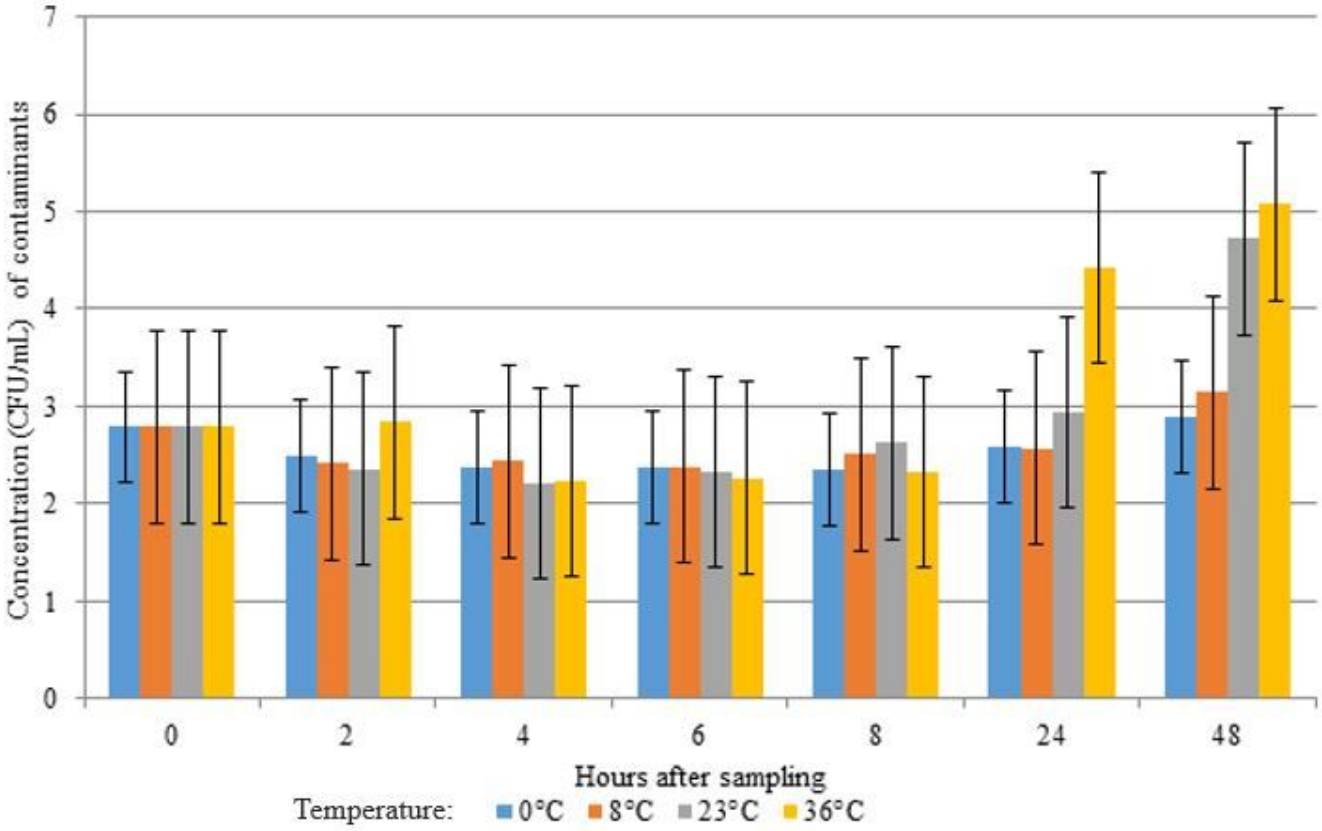

Figure 5

Effect of storage conditions (temperature and time) on the concentration of bacterial contaminants in bovine nBAL samples. No statistically significant difference was seen between the different temperatures and the hours after sampling.