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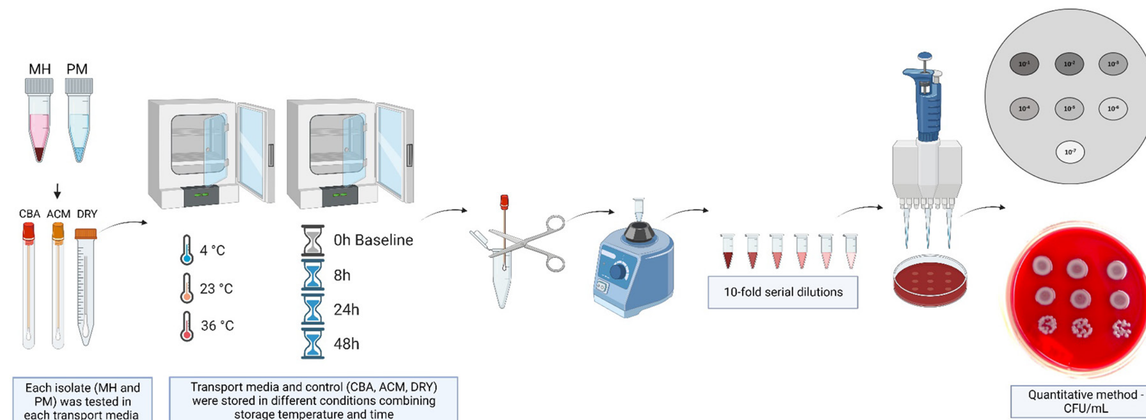
Short Communication
Health, Behavior, and Well-being

In vitro evaluation of the effect of transport medium, temperature, and time on the recovery of *Mannheimia haemolytica* and *Pasteurella multocida*

Adriana Garzon,[1] Alejandro Hoyos-Jaramillo,[1] Stephanie Hustad,[1] Barbara A. Byrne,[2] Heather M. Fritz,[3] Terry W. Lehenbauer,[1,4] Sharif Aly,[1,4] and Richard Pereira[1]*

Graphical Abstract



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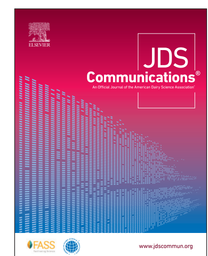
Summary

This article describes the effects of transport medium, time of storage, and storage temperature on *Mannheimia haemolytica* (MH) and *Pasteurella multocida* (PM). Our results support the use of transport media for increasing detection of PM and MH in samples, especially when samples are exposed to high temperatures and longer intervals from collection to diagnostic evaluation.

Highlights

- This study is a framework to evaluate transport media at different times and temperatures.
- For PM, Cary-Blair transport agar (CBA) and Amies culture medium with charcoal (ACM) were found to benefit the isolate's recovery when samples were not refrigerated.
- For MH, CBA and ACM were successful in recovering isolates, especially after 24-h storage.
- ACM and CBA are adequate options to use as transport media under the conditions evaluated.



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In vitro evaluation of the effect of transport medium, temperature, and time on the recovery of *Mannheimia haemolytica* and *Pasteurella multocida*

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Abstract: Appropriate sample collection, storage conditions, and time for transport to the laboratory are important for an accurate diagnostic result. We evaluated the effects of transport storage medium type, time of storage, and storage temperatures on *Mannheimia haemolytica* (MH) and *Pasteurella multocida* (PM) recovery using an in vitro model simulation. A quantitative culture method, using colony-forming units per milliliter, was used to recover MH or PM by an in vitro model with cotton swabs. Three independent trials were conducted, in which cotton swabs were inoculated with MH or PM and placed in either (1) a sterile 15-mL polypropylene tube without transport medium (dry), (2) Amies culture medium with charcoal (ACM), or (3) Cary-Blair transport agar (CBA). Swabs were evaluated for recovery of MH or PM when stored at 3 temperatures (4°C, 23°C, or 36°C) and after storage for 8 h, 24 h, or 48 h. From all study group combinations, a total of 162 individual independent swabs were evaluated. The nonparametric Dunn all-pairs approach was used to compare the proportion of culturable bacteria, between the various storage media, temperature, and time point combinations. The proportion of MH in samples stored at 4°C was significantly higher for ACM and CBA than dry storage at 24 and 48 h. The MH samples stored at 36°C had a significantly higher proportion for ACM and CBA than dry storage at 24 h. The proportion of PM in samples stored at 4°C was significantly lower for ACM compared with dry at 8 h but significantly higher at 48 h. The PM samples stored at 23°C in ACM had a significantly higher proportion than dry samples at 24 h, and, at 48 h, ACM and CBA had a significantly higher proportion than the dry group. All swabs stored at 36°C for 48 h had a proportion close to zero, indicating decreasing diagnostic efficacy. These results support the use of transport media such as ACM and CBA for increasing the detection of PM and MH from samples, especially when samples are exposed to high temperatures. The combination of longer periods from collection of samples to diagnostic evaluation (>24 h) and higher storage temperatures (>23°C) were shown to significantly impair diagnostic accuracy.

Bovine respiratory disease (**BRD**) is a major cause of morbidity, mortality, economic loss, and concern for animal welfare affecting the cattle industry in the United States (USDA 2012, 2016), leading to increased use of antimicrobials in cattle (USDA, 2018) and thus a potential risk for antimicrobial resistance (DeDonder and Apley, 2015; Grissett et al., 2015). Despite advancements in prevention efforts such as vaccines, the use of antimicrobials, and the implementation of management practices to improve animal welfare (housing and stock density), the impact of BRD has remained essentially unchanged over the last few years (Chigerwe et al., 2015; Ollivett, 2020). Several pathogens are involved in BRD, leading to a variety of clinical signs (Gershwin et al., 2015), therefore increasing the challenge for an accurate diagnosis of BRD at the farm. Some of the most common bacterial pathogens involved in BRD include *Mannheimia haemolytica* (**MH**) and *Pasteurella multocida* (**PM**; Ames et al., 2002; Confer and Step, 2009). Both are gram-negative facultative anaerobes belonging to the family *Pasteurellaceae*, and are commonly found as commensal microorganisms in the nasal cavity and oropharynx of healthy cattle (Crosby et al., 2018; Snyder et al., 2019). Because of the multiple clinical manifestations involving BRD, laboratory and diagnostic tools are required for appropriate diagnosis to find the main causal agent(s) and for the establishment of prevention strategies and therapeutic protocols (Caswell et al., 2012; Fulton and Confer, 2012). Isolation of causative bacteria is required for epidemiological data, genome sequencing, and treatment protocols based on antimicrobial susceptibility (Capik et al., 2017; Cummings et al., 2020). Optimal storage conditions en route to the laboratory are vital to avoid bacterial overgrowth in the sample and accurate diagnosis (Boonyayatra et al., 2010). Aerobic culture of samples should be performed as soon as possible after collection to enable reliable recovery of disease-causing bacteria (Fulton and Confer, 2012); however, under field conditions, external variables such as time elapsed between sample collection and laboratory submission, transport medium type, and storage temperature can potentially affect the quality of the results obtained. Furthermore, false negatives or irrelevant results due to overgrowth of contaminant bacteria in samples can lead to inappropriate therapy selections and mislead preventive herd-level efforts (Van Driessche et al., 2020). Although transportation medium, storage temperature, and storage time are essential variables to be considered, few studies have generated evidence-based data to increase knowledge on this topic

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(Chanter et al., 1989; van Rensburg et al., 2004; Van Driessche et al., 2020). The primary objective of this study was to determine potential best practices for sample transportation to the laboratory that will maximize the recovery of PM and MH from cattle with BRD. Thus, the effects of transport medium, time from sample collection to conducting diagnostic testing, and storage temperature on the yield of MH and PM were evaluated using an in vitro simulation model with cotton swabs. We hypothesized that an in vitro approach using MH and PM inoculated onto cotton swabs and placed in a transport medium would result in higher recovery, as measured by colony-forming units per milliliter, than samples stored at room temperature and with no transport medium.

One isolate of each MH and PM was used in the present study, previously isolated from cows with pneumonia, and submitted to MALDI-TOF for bacterial confirmation at the species level (MALDI-TOF score values ≥2; MH = 2.61; PM = 2.37). Pure isolates were stored at −80°C in Microbank beads (Pro-Lab Diagnostics Bacterial Preservation System) at the University of California, Davis, William R. Pritchard Veterinary Medical Teaching Hospital Microbiology Laboratory. Isolates were revived on 5% sheep blood agar (**SBA**; Hardy Diagnostics) and incubated at 37°C in 5% CO_2, then maintained fresh by subculturing one plate per trial using 5% SBA (3 trials in total).

To prepare the inoculum from each fresh subculture, 12 to 17 colonies of each MH and PM were picked and inoculated separately into 40 mL of sterile PBS to obtain a bacterial suspension with an absorbance of 0.08 to 0.1 at 600 nm by a NanoDrop OneC Microvolume UV-Vis spectrophotometer (Thermo Fisher Scientific Inc.), which is equivalent to yield concentration of 5×10^{-4} to 5×10^{-5} cfu/mL.

A quantitative culture method (using cfu/mL) was used to evaluate the initial concentrations of MH and PM inocula and subsequent quantification at each combination of time points and storage temperatures. This procedure was previously described by Van Hecke et al. (2017). Briefly, these steps were followed. (1) A sterile cotton swab with a plastic shaft (Medline Vitality Medical) was dipped into the prepared inoculum and then transferred into a 2-mL microcentrifuge tube with 1 mL of sterile PBS. (2) The swab tip was then cut with sterile scissors and vortexed for 30 s. (3) Serial 10-fold dilutions in sterile PBS of up to 10^{-6} were prepared from the 1 mL of the PBS solution, where the swab was vortexed, using a medium binding polystyrene microplate (96 deep wells with rounded bottom; Nest Scientific USA Inc.). (4) A total of 15 µL of each dilution was transferred via multichannel pipette onto 5% SBA plates using the drop plating technique (Brun-Buisson et al., 1987). (5) Even (10^0, 10^{-2}, 10^{-4}, and 10^{-6}) and odd dilutions (10^{-1}, 10^{-3}, and 10^{-5}) were plated using a 5% SBA plate for each dilution. (6) After the liquid drops on the agar were absorbed, the plates were incubated in an inverted position at 37°C with 5% CO_2. (7) Baseline (0 h) quantification of each inoculum (cfu/mL) was conducted after 24 h of incubation. (8) The dilution considered for the study was the one that resulted in at least 5 colonies per 15 µL of the drop sample dispensed. The total count of cfu/mL was recorded from the countable dilution, and the total count was calculated by enumerating the number of colonies found, using the formula [(#colonies) × (1/dilution factor) × 1,000 µL/mL]/(15 µL of volume plated). The viable cell count was expressed as cfu/mL.

Each trial was repeated 3 times independently for both MH and PM, to assess potential variations in the plating technique. For each trial, a fresh subculture (MH and PM) was made, and a new sterile PBS solution (40 mL) was inoculated with MH or PM to achieve an optical density at 600 nm between 0.08 and 0.1, allowing consistent evaluation for cfu/mL between trials. Sterile cotton swabs with plastic shafts were immersed in freshly prepared inoculum with MH or PM and placed in either (1) a 15-mL polypropylene tube (Globe Scientific) without transport medium (dry); (2) Amies medium with charcoal (**ACM**, BD BBL CultureSwab Plus Transport Systems); or (3) Cary-Blair transport agar (**CBA**, BD BBL CultureSwab Transport Systems Cary-Blair agar, single swab). Swabs were evaluated for recovery of MH or PM after storage at 3 temperatures: 4°C (stored in a refrigerator), 23°C (stored at room temperature), and 36°C (stored in an incubator at 36°C). This was intended to reflect the different scenarios that normally happen when transporting samples, which are refrigeration temperatures (4°C), no refrigeration in temperate weather (23°C), and no refrigeration in high temperatures (36°C). Samples were also evaluated at 4 time points for each of the 3 temperatures evaluated: 0 h (baseline) and 8 h, 24 h, and 48 h post-baseline. Each trial had a total of 54 swabs (3 transport media, 3 storage temperatures, 2 plates per method), resulting in a total of 162 individual swab samples evaluated (Figure 1). Fresh cultures were prepared at each trial on SBA 5% and incubated overnight at 37°C with 5% CO_2. The baseline (0 h) was considered the initial quantification, performed by direct plating with a multichannel pipette and quantifying after 24 h of incubation using the cfu/mL method described above.

To reduce variability when comparing cfu/mL recovery due to variations in the initial (baseline) cfu/mL between trials, the cfu/mL data were converted to a proportion, where 0 h (baseline) was the reference denominator and the cfu/mL counts for the specific time point and treatment combination was the nominator; under these circumstances, 0 h would refer to 100% proportion of cfu/mL. Normality and homogeneity of variance assumptions were rejected, and the nonparametric Dunn all-pairs for joint ranks test in JMP (Version 16) was used to evaluate the effect of storage media and storage time on the proportion of MH and PM from swabs kept at 3 different storage temperatures, by using a pairwise approach. A P-value <0.05 was considered to indicate statistical significance. The least squares means were represented graphically with their respective confidence intervals of 95% (CI 95%).

The effects of transport medium type and storage time for MH proportion are presented in Figure 2. The MH samples stored at 4°C differed between transport medium types at 24 h and 48 h, whereas the dry group had a lower cfu/mL proportion than CBA and ACM transport media ($P < 0.01$). For MH samples stored at 23°C for 24 h, the ACM transport medium had a higher cfu/mL proportion than the dry transport tube ($P = 0.05$). For MH samples stored at 36°C for 24 h, the dry transport tube had a lower cfu/mL proportion than CBA and ACM transport media ($P < 0.01$).

The effects of transport medium types and storage times for PM proportion are shown in Figure 3. For those PM samples stored at 4°C for 8 h, the ACM medium had a lower cfu/mL proportion than the dry transport tube ($P = 0.05$), and at 48 h, dry had a lower cfu/mL proportion compared with ACM transport medium ($P < 0.01$). For those PM samples stored at 23°C for 24 h, the ACM medium had a higher cfu/mL proportion than dry ($P < 0.01$), and at 48 h,

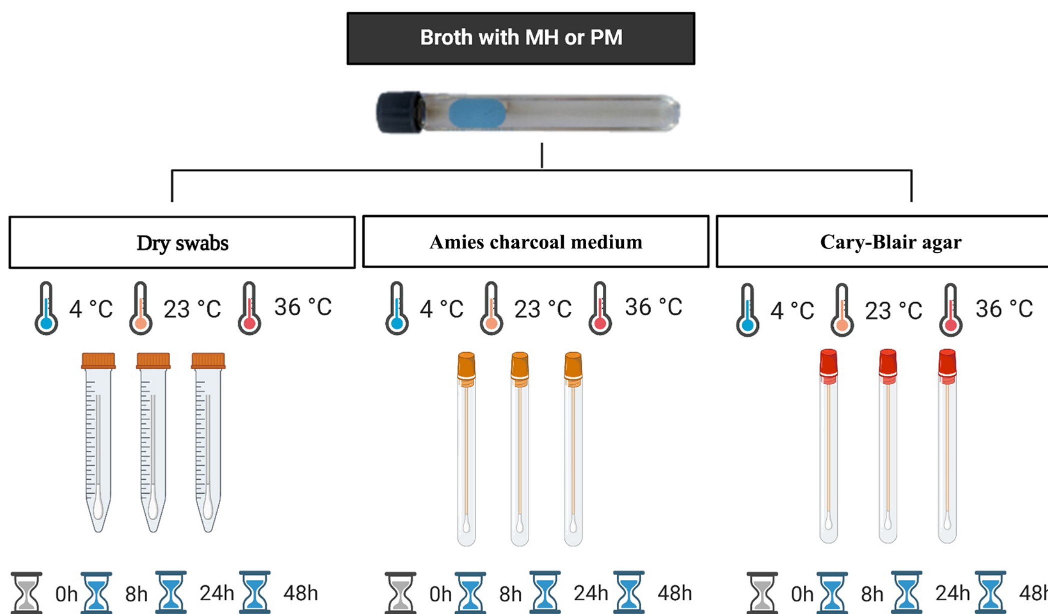

Figure 1. Outline of the sampling protocol for *Mannheimia haemolytica* (MH) and *Pasteurella multocida* (PM) for each type of transport medium, storage temperature, and time stored. Each swab represents an independent sampling point. For each trial, a fresh inoculum was prepared, and triplicates were performed to account for technical variation. Colonies were counted via a quantitative culture method (cfu/mL). Time 0 h was used as baseline to calculate the proportion of culturable bacteria.

the dry transport tube had a lower cfu/mL proportion than CBA and ACM media ($P < 0.01$). For those PM samples stored at 36°C at all time points (8, 24, and 48 h), the dry transport tube had a lower cfu/mL proportion than CBA and ACM media ($P < 0.05$).

Few studies have focused on analyzing the effect of transport medium, storage temperature, and time elapsed on the outcome of bacterial recovery (Carter, 1983; Chanter et al., 1989; Tefera and Smola, 2002). Therefore, the main objective of this study was to fill the gap in understanding the optimal conditions for storage and transportation of samples for optimal bacterial recovery.

Storage of PM samples at refrigeration temperatures (4°C) for 24 h resulted in a higher cfu/mL recovery in both transport media (CBA and ACM) as well as in the control transport container without transport medium. Similarly, previous studies have observed higher recovery rates of PM when samples are transported at refrigeration temperatures before submission to the laboratory (Tefera and Smola, 2002; van Rensburg et al., 2004). In the case of MH, both transport media (CBA and ACM) had a consistently higher cfu/mL recovery at refrigeration temperature (4°C), with a higher cfu/mL proportion at 8 h, but low at 24 h, and again a higher cfu/mL proportion at 48 h. The variability in MH recovery at the different time points was also reported by Van Driessche et al. (2020), who argued that a lower concentration of other microorganisms after 48 h can hinder the detection of clinically essential isolates. Furthermore, a higher bacterial concentration at initial culture can support faster colonization of the culture plates (Van Driessche et al., 2020). Another study reported a different outcome, in which PM and MH were not recovered shortly after being stored at lower

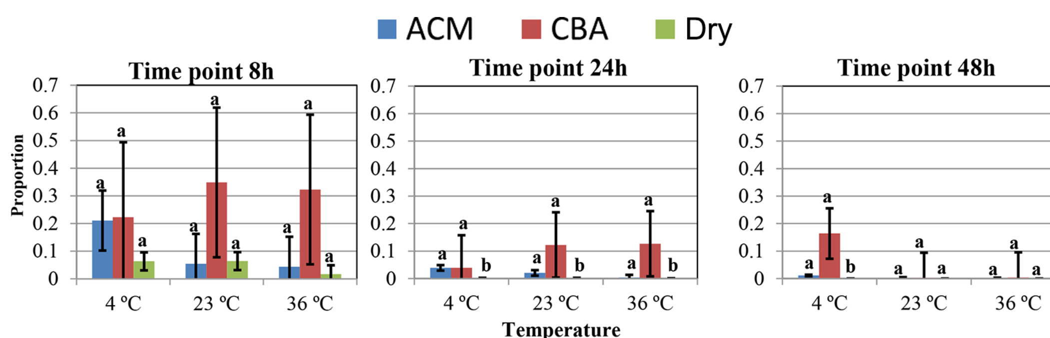

Figure 2. Evaluation of *Mannheimia haemolytica* proportion for each transport medium, broken down by time point and temperature. Error bars correspond to the 95% CI of the mean. Letters reflect the results for Dunn all-pairs for joint ranks nonparametric test, and different letters indicate a significant difference between media within each time point and temperature. ACM = Amies culture medium with charcoal; CBA = Cary-Blair transport agar; Dry = sterile 15-mL polypropylene tube without transport medium.

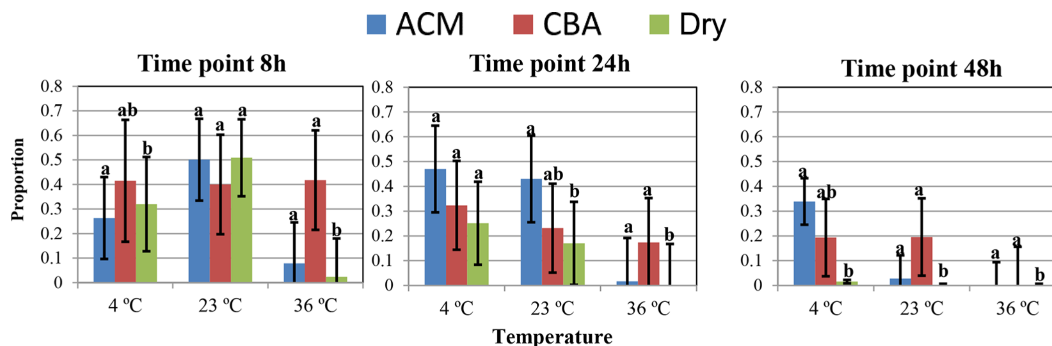

Figure 3. Evaluation of *Pasteurella multocida* proportion for each transport medium, broken down by time point and temperature. Error bars correspond to the 95% CI of the mean. Letters reflect the results for Dunn all-pairs for joint ranks nonparametric test, and different letters indicate a significant difference between media within each time point and temperature group. ACM = Amies culture medium with charcoal; CBA = Cary-Blair transport agar; Dry = sterile 15-mL polypropylene tube without transport medium.

temperatures (4°C) but instead had better recovery when stored for a longer time at room temperature (21°C; Carter, 1983). Yet, in our study, PM and MH were recovered in higher cfu/mL proportions when stored at lower temperatures (4°C) and across time points (8, 24, and 48 h). This indicates the importance of refrigerating samples after collection until arrival at the laboratory, increasing the diagnostic value of the samples. As a caveat, our results were based on pure isolates; thus, the interaction between agonistic or antagonistic bacteria normally present in respiratory samples was not part of the experiment.

In this experiment, PM proportion at 23°C and 36°C at 24 h was higher in both transport media (CBA and ACM) than in the dry group. In the case of MH, the proportion at 24 h was lower than PM but still higher than dry. Bavananthasivam et al. (2012) reported that PM could survive longer than MH in CBA medium, even though both bacteria belong to the same family (*Pasteurellaceae*). Similar results have also been observed in another study, where MH isolates demonstrated shorter survival ability than PM in the same transport medium (Tefera and Smola, 2002). Further, it was also reported that the CBA medium presented high recovery for both MH and PM (Tefera and Smola, 2002). Similar results were found in our in vitro study, where both MH and PM had higher recovery when using the CBA transport medium compared with an empty transport tube (dry).

A study published by Chanter and colleagues (1989) in which nasal swabs were collected from nasal cavities in pigs at the slaughterhouse and stored in a charcoal transport medium reported a 50% recovery decrease of PM after 48 h when specimens were kept at 10°C. Similarly, low cfu/mL recovery proportions in both PM and MH were found in our study in samples exposed to warm temperatures (36°C) and longer time points (48 h). This low recovery proportion was evident at 48 h under temperatures of 23°C and 36°C for MH, and 36°C for PM, limiting the detection of even one isolate, therefore affecting the diagnostic accuracy. Under field conditions, samples left without refrigeration and exposed at higher temperatures (e.g., summer weather) should be cautiously considered when evaluating their reliability for diagnostic purposes.

For PM, CBA and ACM were found to have an overall beneficial outcome for the successful recovery of isolates, especially when samples were not refrigerated or exposed to extremely high temperatures. Our findings also support the importance of refrigerating samples to increase the cfu/mL recovery of PM. For MH, CBA and ACM were demonstrated to significantly benefit the successful recovery of isolates, especially after 24 h of storage. Furthermore, the only advantage between ACM and CBA was observed for a storage temperature of 4°C for 8 h, where ACM was lower than the dry group, perhaps suggesting a slight advantage for CBA. However, no significant difference in cfu/mL recovery between ACM and CBA was observed, indicating that both options would be appropriate for use under the conditions evaluated.

This study establishes the framework to evaluate the effects of transport media and different storage conditions, using an in vitro model, on recovery of MH and PM. Although we chose a representative isolate for each (MH and PM) from clinical cases of pneumonia in adult cows, this could represent a limitation of the study. Future studies should address the use of different isolates at the time of comparing different storage conditions.

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