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Evaluating the environmental survivability of *Mannheimia haemolytica* on various potential fomites

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Abstract

Bovine respiratory disease (BRD) is an important cause of morbidity and mortality in beef and dairy cattle in North America. Of the significant bacteria implicated in BRD cases, *Mannheimia haemolytica* is the most common microbe isolated in clinical cases, yet little is known about its environmental survivability. The objective of this study was to examine relative survivability of *M. haemolytica* on metal, wood, and plastic under varying environmental conditions. Microenvironments were constructed at approximately 37°F (3°C), 71°F (22°C), and 97°F (36°C) using plastic containers and monitored for temperature and humidity. Stainless steel, polypropylene, and wood spheres were inoculated with *M. haemolytica*, placed in each microenvironment, and cultured for viable bacteria at 0, 2, 6, 24, and 48 h after inoculation. Presence or absence of growth of *M. haemolytica* was confirmed by MALDI-TOF mass spectrometry. Both wood and plastic materials supported the recovery of viable bacteria for a much longer duration than stainless steel. Additionally, increased temperature and decreased humidity lowered bacterial viability. These results demonstrate that both material and environment are factors related to *M. haemolytica* survival, which has potential biosecurity implications when managing BRD in beef and dairy operations.

Key words: *Mannheimia haemolytica*, bovine respiratory disease, survivability, environment, biosecurity, fomite

Introduction

Bovine respiratory disease (BRD), a multifactorial combination of bacterial and viral pathogens, is the most common and costly disease affecting feedlot and stocker cattle in North America and poses a notable challenge in dairy cattle as well.[5,16] Bovine respiratory disease is the second leading cause of mortality in preweaned dairy calves after neonatal diarrhea.[5] In feedlot cattle, BRD is responsible for up to 60% of morbidity.[16] In addition to increased costs associated with treatment and losses, beef cattle affected by BRD have decreased feed conversion and increased time to market.[11] Dairy heifers diagnosed with BRD have been shown to have increased time to first calving and decreased first-lactation milk production.[14]

While there are many clinically relevant pathogens that contribute to BRD, *Mannheimia haemolytica* is considered the most significant in animals with clinical disease.[17] A known component of the normal respiratory microbiota in cattle, *M. haemolytica* is an opportunistic pathogen that presented in 91% of acute pneumonia cases in feedlot cattle in 1 study.[8] Research provides conflicting evidence regarding the importance of between-cattle transmission of *M. haemolytica*,[2,15] and the authors are unaware of published studies that would definitively support or refute the environment/fomites as a significant source of transmission. In 1 study, the survivability of *M. haemolytica* on straw was examined in comparison with various types of media including sterile phosphate-buffered saline, sterile Todd-Hewitt broth, and bacteria-free ewe's milk, but no other potential fomites or materials were included in the study.[3] Additionally, some evidence suggests that dairy calf hutches constructed from a combination of materials (wood, metal, plastic) may increase BRD prevalence as compared to a single material.[7] Overall, there seems to be little evidence in the peer-reviewed literature of the ability of *M. haemolytica* to survive on various types of surfaces and under various environmental conditions.

The objectives of this study were 1) to examine the survivability of *M. haemolytica* on various materials that are commonly used in beef or dairy cattle production settings, and 2) determine if common environmental temperatures and humidity impact relative duration of survival of *M. haemolytica*. A more complete understanding of the survivability of BRD pathogens in the environment would inform disinfection and biosecurity protocols and potentially decrease disease incidence by limiting environmental transmission.

Materials and Methods

An isolate of *M. haemolytica* (serotype A1) from a confirmed case of BRD[10] was procured for use in this study. A bacterial inoculum was produced by incubating the isolate in brain heart infusion[a] (BHI) broth overnight at 98.6°F (37°C) on a shaker table in an air incubator, and then titrated to approximately 2.5 x 10^4 CFU/mL using a timed growth curve from previous work with this isolate and confirmed via quantification. Stainless steel,[b] polypropylene,[c] and wood[d] ¼" balls were used to represent materials commonly encountered in cattle production settings. All 3 materials had a calculated surface area of 1.27 cm^3. All materials were steam sterilized at 250°F (121°C) for 15 min prior to inoculation, and 3 replicates of each material were used. Three microenvironments, termed LOW, MED, and HIGH, were created and maintained at approximately 37°F (3°C), 71°F (22°C), and 97°F (36°C) with average humidity levels of 84%, 52%, and 57%, respectively. Microenvironments were created using opaque polyethylene containers with lids.[e] To maintain consistent humidity at all 3 temperatures, a glass pan containing a saturated sodium chloride solution was placed in each microenvironment. Temperature and humidity for each microenvironment were measured at 1-min intervals for the duration of the study using a data logger system[f] (accuracy: +/- 0.2°C and +/- 2% relative humidity) and analyzed for variance across time.

Each sterile material was briefly dipped in the bacterial inoculum, shaken slightly to remove excess inoculum, placed in a sterile wide-mouth glass jar, and then placed in the appropriate microenvironment. One jar was used for each material type within each microenvironment. An aliquot of the inoculum was simultaneously collected for bacterial quantification. A 10-fold dilution series was completed, and colonies were counted to calculate the number of colony-forming units (CFUs). First, 100 µL of the bacterial inoculum was transferred into 900 µL of sterile saline solution and vortexed. Next, 100 µL was transferred from the first dilution into a second tube containing 900 µL of sterile saline and vortexed. This was repeated for an additional dilution. One hundred µL of each dilution was plated on a TSA blood agar plate and incubated overnight at 98.6°F (37°C). Counts were performed for each plate containing between 30 and 300 colonies. The number of CFUs were determined according to the following formula: CFU/mL = number of colonies * (1000 µL/volume plated) * 10^{dilution}.

Samples were analyzed for presence of *M. haemolytica* at 0, 2, 6, 24, and 48 h. Descriptive statistics of the environmental conditions were calculated over each time period in a spreadsheet program.[g] Each material, environment, and time point combination were examined in triplicate, except for the 0-h time point, for which triplicates were examined for material only and kept at room temperature. At each of the respective time points, 1 sphere of each of the material types was removed from the jar and placed in a conical tube containing 500 µL of sterile saline. Each sample was vortexed briefly, and a 100 µL aliquot was plated and spread evenly on a TSA blood agar plate. The plates were then incubated in 5% CO_2 at 98.6°F (37°C). At 24 h, the presence or absence of growth was recorded, but the identity of individual colonies was not verified. If bacterial growth was present, all colonies present on that material/environment/timepoint-specific plate were transferred to a cryovial with 80% BHI broth and 20% glycerol, frozen at -112°F (-80°C), and shipped to Kansas State University for definitive identification of *M. haemolytica* via matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). This method analyzes bacterial proteins and compares them to known "fingerprints" within a database to allow for rapid, reliable, and inexpensive identification of bacteria, including Pasteurellacea.[9,12]

Results

Variations in survivability were observed in respect to material, time, and environment. Table 1a and Table 1b provide descriptive statistics for both temperature and humidity over the duration of each time point analyzed. No viable bacteria were isolated at any time point, including time 0, from stainless steel spheres. In addition, no bacteria were recovered from any of the materials by 48 h. Furthermore, no bacteria were recovered from any materials in the HIGH temperature environment except for 1 replicate of the plastic material at 24 h. This growth was later identified as *Staphylococcus epidermidis* and attributed to contamination. Viable *M. haemolytica* was recovered on wood in the LOW environment at 2 (2/2; 1 LOW sphere was lost to follow up) and 6 h (3/3) and at MED temperatures at 2 (2/3) and 6 h (3/3). However, no viable bacteria remained on wood at 24 h (Table 2). For plastic, bacteria were recovered from at least 2/3 of the replicates in the LOW temperature environment at 2, 6, and 24 h. For the MED temperature environment, viable bacteria were recovered from 2/3 materials at 2 h and 1/3 materials at 6 h.

Discussion

As was expected, both material and environment had notable effects on the survivability of *M. haemolytica* over 48 hours. The stainless steel material had the smoothest, most polished surface, which possibly decreased the number of bacteria that inoculated onto the surface compared to the other materials. Indeed, the stainless steel seemed to support the least amount of growth with no viable *M. haemolytica* being grown from any microenvironment at any time point. The longer survival on plastic in comparison to wood could possibly be attributed to the increased absorptive capacity of wood, which may have limited the ability for the material to release any remaining viable bacteria at later time points. Also, the surface of the plastic was not as smooth as the other materials and might have limited the drying rate, which could have allowed for longer survival time. When comparing en-

Table 1a. Descriptive statistics for temperature in each microenvironment for the duration of environmental exposure for each time point. Temperature was recorded every minute via a data logger system.[f]

Temperature (°F)						
Sample treatment	**Time**	**Mean**	**Median**	**Standard deviation**	**Min**	**Max**
Low 37°F (3°C)	2h	40	39.9	1.52	37.9	42.6
	6h	38.2	37.6	2.58	34.8	43.5
	24h	36.9	36.7	1.77	34.8	43.5
	48h	37	36.8	1.62	34.8	43.5
Med 71°F (22°C)	2h	71.3	71.3	0.14	71.1	71.6
	6h	71.1	71.1	0.24	70.6	71.6
	24h	71	71	0.16	70.6	71.6
	48h	71.1	71.1	0.16	70.6	71.6
High 97°F (36°C)	2h	96	96	0.44	95.3	96.6
	6h	96.8	96.9	0.62	95.3	97.4
	24h	97.1	97.3	0.45	95.3	97.4
	48h	97.3	97.4	0.36	95.3	97.5

Table 1b. Descriptive statistics for humidity in each microenvironment for the duration of environmental exposure for each time point. Humidity was recorded every minute via a data logger system.[f]

Relative humidity (%)						
	Time	**Mean**	**Median**	**Standard deviation**	**Min**	**Max**
Low 37°F (3°C)	2h	67.5	70.4	15.7	35	100
	6h	65.9	68.3	16.2	35	100
	24h	82.9	87.4	14.4	35	100
	48h	84.1	87.5	11.7	35	100
Med 71°F (22°C)	2h	50.8	51.1	1.18	48.5	52.7
	6h	50.6	50.7	0.89	48.1	52.7
	24h	52	51.9	1.16	48.1	55.3
	48h	51.8	51.8	1.22	48.1	55.3
High 97°F (36°C)	2h	49.5	49.1	2.12	45.7	53.2
	6h	51.6	52.2	4.79	36.2	58
	24h	55.5	56.8	3.58	35.1	58.5
	48h	56.6	57.3	2.84	35.1	59.3

Table 2. Presence or absence of *Mannheimia haemolytica* confirmed by MALDI for each microenvironment (LOW: 37°F [3°C]; MED: 71°F [22°C] ; High: 97°F [36°C]) and material at various time points. Note: No *M. haemolytica* was isolated from stainless steel at any time point nor any of the materials at 48 h.

	Time									
	0h	**2h**			**6h**			**24h**		
		Low	**Med**	**High**	**Low**	**Med**	**High**	**Low**	**Med**	**High**
Wood	+	N/A[†]	+	-	+	+	-	-	-	-
	+	+	+	-	+	+	-	-	-	-
	+	+	-	-	+	+	-	-	-	-
Plastic	+	+	+	-	+	-	-	-	-	-
	+	-	-	-	+	+	-	+	-	-
	+	+	+	-	+	-	-	+	-	-*

* Growth was observed, but identified as *Staphylococcus epidermidis* contamination via MALDI-TOF.
† One LOW wood sphere was dropped and thus lost to follow up.

vironmental conditions, the decreased survival as temperature increased was expected because desiccation increases as temperature increases. However, it is difficult to directly compare the direct effect of temperature as the humidity was not held constant over the 3 temperatures. The combination of low temperature and high humidity likely contributed to the longest survival in the low temperature environment. *Mannheimia haemolytica* naturally lives in the moist, humid respiratory tract of cattle and it is possible that once dry, the bacteria may not survive as well.

Our inability to more strictly control humidity was a function of the way we created our microenvironments and managed temperature. First, we did not use airtight containers to create the microenvironments, and we had to enter each microenvironment several times to remove samples. Although we made every effort to make sample removal times as quick and efficient as possible, there was most certainly brief mixing of ambient air with the microenvironment air. For the LOW microenvironment, we placed the container in a standard refrigerator. Refrigerators naturally have higher ambient humidity levels, often near 100%, which likely caused the larger fluctuations in the LOW microenvironment humidity. The ambient air surrounding the containers held in MED (within a fume hood in the laboratory) and in the HIGH environment (an incubator) were much lower in humidity and thus had less of an impact on the humidity within the microenvironments. However, without replicates of different humidity levels within the different temperature levels, we are unable to analyze the impact of humidity directly. Future studies should be designed to more tightly control humidity and compare the impact of different humidity levels within different temperatures.

Because this study did not evaluate the presence of ultraviolet (UV) light, extrapolation of these results to a field setting would likely overestimate the true environmental survivability of *M. haemolytica* in the environment. Ultraviolet light is commonly used in many applications to inhibit growth or kill bacteria. For example, researchers utilized UV light to kill *M. haemolytica* for use in a killed vaccine study.[10] Because many normal environments present in cattle-raising operations receive at least some UV exposure from the sun, further studies are needed to evaluate its effects on *M. haemolytica* survival. Evaluating this aspect could prove especially challenging, as natural UV exposure is not consistent between seasons or in any production system, and some indoor systems may not have any notable direct UV exposure at all.

Additionally, the choice of media could have a different effect on bacterial growth as they can differ in nutrient availability, antimicrobial effects, and desiccation rate compared to oral or respiratory secretions in the live animal. We used brain-heart infusion broth, which contains only nutrients to support growth of the bacteria.[1] In comparison, healthy respiratory secretions contain IgG antibodies, mucins, enzymes, and other locally produced substances that may also inhibit bacterial growth.[6] However, the exact, complete composition of healthy cattle respiratory secretions remains unknown. In sick animals, inflammatory cells such as neutrophils may also be present, which would act to decrease the number of viable bacteria. Saliva, in addition to enzymes, minerals, and antibodies, has an increased pH, typically around 8.5,[13] which could have an adverse effect on bacterial survivability. The viscosity of respiratory and oral secretions is also higher, and often quite variable depending on numerous factors, possibly affecting the amount of time they take to dry and thus impacting the time that bacteria remain viable. Because of these variables, further research is needed to determine if naturally inoculated surfaces support the survivability of *M. haemolytica* and explore the impact of different secretions from healthy and sick animals.

The temperature and humidity combinations evaluated in this study were chosen to represent naturally occurring environments in which cattle are produced. However, these are not the only conditions under which cattle are produced. For example, temperatures are below freezing for a portion of the year in many regions, and humidity varies greatly across North America. Even in the same location, 24-h temperature variations can be in excess of 60°F (33°C). Also, other factors such as wind and precipitation could possibly impact bacterial survival, affecting both adherence to materials as well as desiccation rates. These factors also impact BRD morbidity, which could affect the amount of *M. haemolytica* being introduced to the environment at a given time.[4]

Regardless of these limitations, the information gained in this study remains important for beef and dairy production systems as the role of environmental transmission of *M. haemolytica* in the pathogenesis of BRD is currently unknown. Selection of materials in facilities where cattle are transported, processed, and housed can have not only physical and financial implications, but also may have biosecurity implications as well. Being able to select or manage materials in a way that potentially limits the survivability of 1 of the most significant BRD bacterial pathogens in beef and dairy cattle could provide an opportunity to decrease potential environmental transmission of *M. haemolytica* and improve the biosecurity of cattle operations.

Conclusions

Bovine respiratory disease and *M. haemolytica* pose significant problems for cattle production systems. Understanding the effects of material and environment on the survivability of *M. haemolytica* may allow for improvements in biosecurity on many cattle operations. Even with the information gained in this study, many knowledge gaps remain. Further research on the survival of *M. haemolytica* on other materials, under other environmental conditions and within naturally occurring respiratory secretions, is needed.

Endnotes

[a] BBL™ Brain Heart Infusion, Becton, Dickinson and Company, Sparks, MD
[b] Stainless Steel Corrosion Resistant Precision Ball, 440C Alloy, W.W. Grainger, Inc., Lake Forest, IL
[c] Polypropylene Solid Plastic Ball, W.W. Grainger, Inc., Lake Forest, IL
[d] Wood ball, Craftparts.com LLC, Haltom City, TX
[e] Roughneck 10 Gallon Storage Tote Gray, Rubbermaid, Atlanta, GA
[f] Hobo MX1101 Temp/RH Data Logger, Onset Computer Corporation, Bourne, MA
[g] Microsoft Excel for Microsoft 365, Version 2108, Microsoft, Redmond, WA

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