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Note

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A B S T R A C T

Genotype 2 *Mannheimia haemolytica* associate with the lungs of cattle with bovine respiratory disease more frequently than genotype 1 strains. Different colony colors and morphologies were identified between genotype 1 and 2 solid media cultures. Genotype of strains, and frequency differences between them in mixed cultures are discernable by visual inspection.

Mannheimia haemolytica is a gram-negative bacterium often found in the upper respiratory tract of cattle (Rice et al., 2008). When cattle are stressed, *M. haemolytica* can invade their lungs and cause bovine respiratory disease (BRD) (Griffin et al., 2010). Not all strains associate equally with BRD (Klima et al., 2014). Two major genotypes of *M. haemolytica* have been identified in cattle (1 and 2). While both are found in the upper respiratory tract, genotype 2 strains are more frequently isolated from the lungs of cattle with BRD than genotype 1 strains (Clawson et al., 2016).

Accurate identification of *M. haemolytica* genotypes can facilitate investigations into their distribution, transmission, and pathogenicity. Recently, a matrix-assisted laser desorption/ionization- time of flight mass spectrometry (MALDI-TOF MS) assay was developed that distinguishes the two genotypes based on MS biomarkers (Loy and Clawson, 2017). However, the assay is typically performed on cultured bacterial colonies in the absence of preliminary genotype information. If a primary culture from a clinical specimen were to contain both genotypes, it is possible that colonies representing only a single genotype would be selected for the assay. Consequently, there is a need for additional screening methods that assess the genotypic diversity of *M. haemolytica* strains.

We observed colony phenotypic differences between genotype 1 and 2 *M. haemolytica* laboratory strains grown on chocolate and Brain Heart Infusion (BHI) blood agar plates (Hardy Diagnostics, Santa Maria, CA, USA). To further investigate this observation, a diverse collection of *M. haemolytica* strains was cultured on the two plate types and visually examined. The collection consisted of 21 genotype 1 strains including subtypes b, c, e, f, and i, and 17 genotype 2 strains including subtypes b, c, d, and e (Supplemental file 1, subtypes described in Clawson et al., 2016). Included in this collection were the same isolates originally used to develop the MALDI-TOF MS assay (Loy and Clawson, 2017). The strains were grown for two generations at 37 °C with 5% CO_2 on both chocolate and BHI blood agar plates. Photographs were taken of each plate at 18 and 24-h growth (Supplemental file 2).

Two colony phenotypic characteristics were identified that could be used in combination to visually differentiate a genotype 1 *M. haemolytica* from a genotype 2; color and three-dimensional shape. Genotype 1 colonies were duller and grayer than genotype 2 colonies, which were a creamy white color (Fig. 1, Supplemental file 2). There was more phenotypic diversity within genotype 1 strains; some were very pale while others were brighter gray. However, all genotype 2 strains examined in this study were brighter and whiter than all genotype 1 strains. These color differences between genotype 1 and 2 strains were discernable on both chocolate and BHI blood agar plates under ambient light.

Genotype 1 colonies had a more complex three-dimensional shape than genotype 2 on both BHI blood and chocolate agar plates. The genotype 1 colonies had a raised outer edge, a depressed inner ring, and a raised center. In contrast, genotype 2 colonies predominantly had a smooth dome shape. As with color, there was more diversity in genotype 1 colony shape; some strains had a more exaggerated raised center and edge than others.

An easy method to quickly assess the three-dimensional shape of a *M. haemolytica* colony was to hold the agar plate at a 45-degree angle to a light source and observe the reflection of the light off of the colony (Fig. 2, Supplemental file 2). Due to their more complex three-dimensional shape, genotype 1 colonies reflected the light with a distortion near the edge of the colonies. For 11 of the 21 genotype 1 strains

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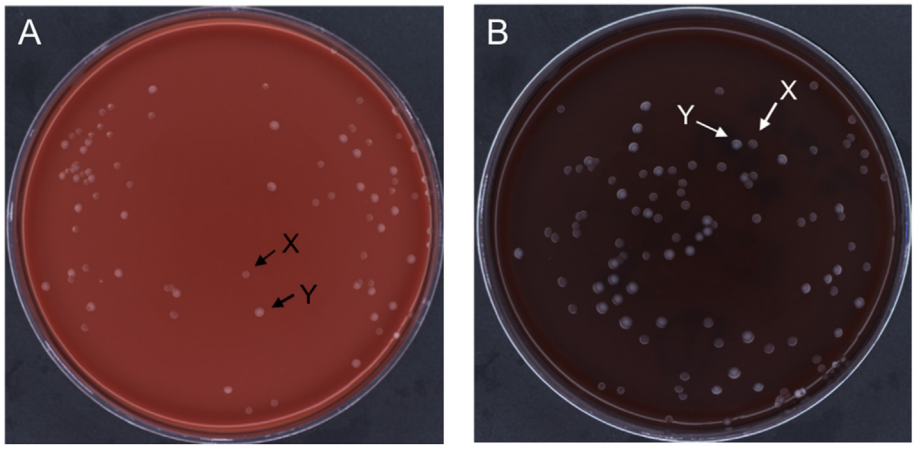

Fig. 1. Genotype 1 and 2 colony differences on chocolate (A) and BHI blood agar plates (B). The X and Y arrows point to genotype 1 and 2 colonies, respectively. The genotype 2 colonies are slightly larger, whiter and more opaque than genotype 1 colonies on chocolate agar. On BHI blood agar, the genotype 2 colonies are much larger, whiter and creamier than genotype 1 colonies.

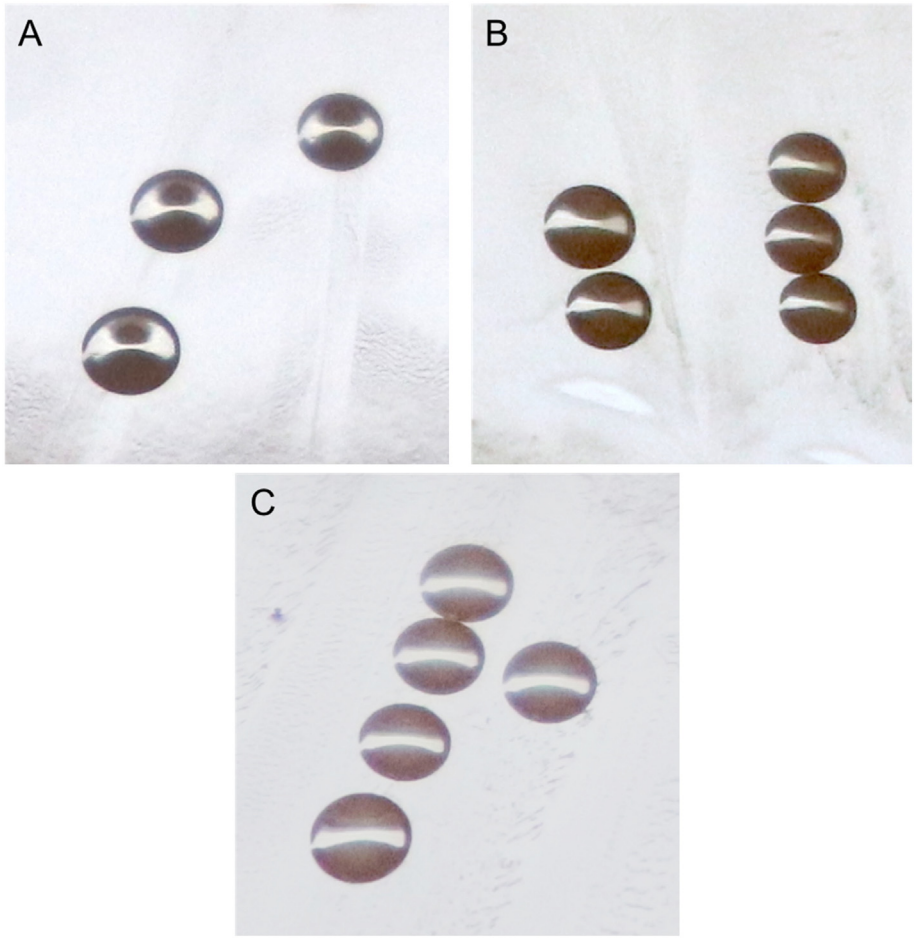

Fig. 2. Colony morphologies of genotype 1 and 2 strains cultured on chocolate agar under angled light. A) Colonies have raised edges and a raised center, with a lower inner ring. This can be seen by the distorting effect on the reflected light near the edges of the colony. This morphology was only observed in genotype 1 strains. B) These colonies still have the same general three-dimensional shape as those in Fig. 2A, but the raised edges are not as extreme, causing the distortion effect of the reflected light to be more subtle. This morphology was observed in genotype 1 and 2 strains. Genotype 1 colonies are shown in the image. C) Colonies are dome shaped. Light reflects off them evenly, yielding solid bands with very little distortion near the edges. This morphology was only observed in genotype 2 strains.

examined at 18 h, this distortion was large on either chocolate or blood agar plates (Fig. 2A, Supplemental files 1–2), while for the other 10 strains this distortion was a subtle bump on the edges of the reflection on both plate types (Fig. 2B, Supplemental files 1–2). Genotype 2 domed colonies predominantly reflected the light as a solid band with very little distortion in shape (Fig. 2C, Supplemental files 1–2). Fifteen out of the 17 genotype 2 strains had this reflection shape on either chocolate or blood agar plates (Supplemental files 1–2). The remaining two strains had a subtle bump on the edges of their reflection on both plate types but were still identifiable as a genotype 2 strain due to a creamy white colony color.

The exact morphology of a colony can vary over time, this was seen between the 18 and 24-h timepoints. As plates aged, the distorting effect of the reflection became more exaggerated. Due to this variation, during the course of this study some genotype 1 strains could have either bump or distorted reflections and some genotype 2 strains could have either smooth or bump reflections between plates. This indicates the ambiguity with the bump reflection as a genotype classifier in the absence additional information. It is important to note that the exact shape of the reflection will depend on the particular light source; in this study a long fluorescent light was used, about 3 ft above the plate. Regardless of the light source, the important feature to note is the distorting effect near the edges of the colony.

The colony color and morphology phenotypes of the collection were

most distinct when strains were streaked for isolation. When colonies were grown densely together, the differences become less distinct and harder to differentiate, especially on BHI blood agar. Using both colony colors and light reflections, the entire collection of *M. haemolytica* was accurately genotyped on chocolate agar by visual inspection (Supplemental files 1–2).

To further test the accuracy of these observations, 31 bovine nasal swab samples were plated on chocolate agar for *M. haemolytica* identification and genotyping by visual inspection from primary culture. The swabs had been stored at -80 °C in buffered peptone and 12% glycerol (McDaneld et al., 2018). Most nasal swab samples contained multiple species of bacteria and *M. haemolytica* DNA had been detected by qPCR (Loy et al., 2018), though the genotypes had never been determined. The samples were thawed to room temperature, and spread plates were grown overnight at 37 °C with 5% CO_2. Two to four individual colonies that were suspected to be *M. haemolytica* were passaged to new chocolate agar plates. To confirm preliminary colony morphology observations, the *M. haemolytica* colonies were passaged one final time to new chocolate agar plates. Thirty-three colonies selected from 14 nasal swab sample cultures were visually genotyped by colony color alone, and 32 colonies from 17 nasal swab cultures were visually genotyped by both color and light reflection. All colonies were determined to be either genotype 1 or genotype 2, except for one colony that was called as ambiguous due to having the color of a genotype 2 and the reflection of a genotype 1 (Supplemental file 1). To determine the accuracy of these visual assessments, species and genotype identification was confirmed by MALDI-TOF MS (Loy and Clawson, 2017). Of the 33 colonies that were inspected solely by colony color, 30 were correctly genotyped (Cohen's kappa, k = 0.79, substantial agreement). The three colonies that were misidentified were both genotype 2 strains that were visually identified as genotype 1. Of the 31 colonies that were determined to be genotype 1 or 2 by inspection of both colony color and light reflection, all were correctly genotyped (Cohen's kappa, k = 1, perfect agreement). The one colony that was called as ambiguous by visual inspection was determined by the MALDI-TOF assay to be a cluster V *Mannheimia* sp. that has not yet been resolved to the species level (Angen et al., 1999).

When using both colony color and shape morphologies as criteria, *M. haemolytica* can be identified and genotyped with high accuracy (see Supplemental file 3 for a flow chart of colony color and shape morphology use). The ability to genotype *M. haemolytica* by visual inspection can be used as part of a diagnostic pipeline to ensure that the full diversity and frequencies of *M. haemolytica* genotypes is being captured from BRD cases and other samples of interest.

Author statement

ELW conceptualized the project, performed research, developed the reflection methodology, analyzed results, validated methods, and wrote the manuscript. GS performed research, developed the reflection method, validated methods, and edited the manuscript. JDL assisted in the project investigation and validation and edited the manuscript. AMW provided resources and assisted in the project investigation and edited the manuscript. TGM provided resources and assisted in the project investigation and edited the manuscript. MLC conceptualized and supervised the project, validated methods, analyzed results, and edited the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2020.105877.

References

Angen, O., Mutters, R., Caugant, D.A., Olsen, J.E., Bisgaard, M., 1999. Taxonomic relationships of the [*Pasteurella*] *haemolytica* complex as evaluated by DNA-DNA hybridizations and 16S rRNA sequencing with proposal of *Mannheimia haemolytica* gen. nov., comb. nov., *Mannheimia granulomatis* comb. nov., *Mannheimia glucosida* sp. nov., *Mannheimia ruminalis* sp. nov. and *Mannheimia varigena* sp. nov. *Int.* J. Syst. Bacteriol. 49 (1), 67–68.

Clawson, M.L., Murray, R.W., Sweeney, M.T., Apley, M.D., DeDonder, K.D., Capik, S.F., Larson, R.L., Lubbers, B.V., White, B.J., Kalbfleisch, T.S., et al., 2016. Genomic signatures of *Mannheimia haemolytica* that associate with the lungs of cattle with respiratory disease, an integrative conjugative element, and antibiotic resistance genes. BMC Genomics 17 (1), 982.

Griffin, D., Chengappa, M.M., Kuszak, J., McVey, D.S., 2010. Bacterial pathogens of the bovine respiratory disease complex. Vet. Clin. North Am. Food Anim. Pract. 26, 381–394.

Klima, C.L., Alexander, T.W., Hendrick, S., McAllister, T.A., 2014. Characterization of *Mannheimia haemolytica* isolated from feedlot cattle that were healthy or treated for bovine respiratory disease. Can. J. Vet. Res. 78, 38–45.

Loy, J.D., Clawson, M.L., 2017. Rapid typing of *Mannheimia haemolytica* major genotypes 1 and 2 using MALDI-TOF mass spectrometry. J. Microbiol. Methods 136, 30–33.

Loy, J.D., Leger, L., Workman, A.M., Clawson, M.L., Bulut, E., Wang, B., 2018. Development of a multiplex real-time PCR assay using two thermocycling platforms for detection of major bacterial pathogens associated with bovine respiratory disease complex from clinical samples. J. Vet. Diagn. Investig. 30, 837–847.

McDaneld, T.G., Kuehn, L.A., Keele, J.W., 2018. Evaluating the microbiome of two sampling locations in the nasal cavity of cattle with bovine respiratory disease complex (BRDC). J. Anim. Sci. 96 (4), 1281–1287.

Rice, J.A., Carrasco-Medina, L., Hodgins, D.C., Shewen, P.E., 2008. *Mannheimia haemolytica* and bovine respiratory disease. Anim. Health Res. Rev. 8 (2), 117–128.