Animal Health Research Reviews

cambridge.org/ahr

Development and application of molecular diagnostics and proteomics to bovine respiratory disease (BRD)

John Dustin Loy

4040 East Campus Loop, 115Q NVDC, School of Veterinary Medicine and Biomedical Sciences, University of Nebraska-Lincoln, Lincoln, NE 68583-0906, USA

Review

Cite this article: Loy JD (2020). Development and application of molecular diagnostics and proteomics to bovine respiratory disease (BRD). *Animal Health Research Reviews* **21**, 164–167. https://doi.org/10.1017/S1466252320000092

Received: 11 November 2019
Accepted: 25 March 2020
First published online: 2 December 2020

Key words:
BRD diagnosis; MALDI typing; MALDI-TOF MS; *Mannheimia haemolytica*; real-time PCR bovine respiratory disease

Author for correspondence:
John Dustin Loy, 4040 East Campus Loop, 115Q NVDC, School of Veterinary Medicine and Biomedical Sciences, University of Nebraska-Lincoln, Lincoln, NE 68583-0906, USA. E-mail: jdloy@unl.edu

Abstract

Advances in molecular and proteomic technologies and methods have enabled new diagnostic tools for bovine respiratory pathogens that are high-throughput, rapid, and extremely sensitive. Classically, diagnostic testing for these pathogens required culture-based approaches that required days to weeks and highly trained technical staff to conduct. However, new advances such as multiplex hydrolysis probe-based real-time PCR technology have enabled enhanced and rapid detection of bovine respiratory disease (BRD) pathogens in a variety of clinical specimens. These tools provide many advantages and have shown superiority over culture for co-infections/co-detections where multiple pathogens are present. Additionally, the integration of matrix-assisted laser desorption ionization time of flight mass spectrometry (MS) into veterinary diagnostic labs has revolutionized the ability to rapidly identify bacterial pathogens associated with BRD. Recent applications of this technology include the ability to type these opportunistic pathogens to the sub-species level (specifically *Mannheimia haemolytica*) using MS-based biomarkers, to allow for the identification of bacterial genotypes associated with BRD versus genotypes that are more likely to be commensal in nature.

Introduction

Bovine respiratory disease (BRD) is a multifactorial disease complex that causes tremendous economic losses to cattle industries (Griffin *et al.*, 2010). BRD is associated with a number of viruses and bacterial pathogens, often simultaneously, making diagnosis and establishment of causal agents involved in outbreaks and clinical cases challenging (Booker *et al.*, 2008; Fulton *et al.*, 2009). Recent applications of molecular technologies such as polymerase chain reaction (PCR) to BRD are helping to elucidate agents involved in these cases with enhanced sensitivity (Tegtmeier *et al.*, 2000; Bell *et al.*, 2014). Identification of the causative agents is critical so that proper prevention and vaccination protocols can be rapidly implemented. Oftentimes, severe outbreaks occur with significant mortality; therefore, rapid diagnosis can be critical. Additionally, the emergence of multi-drug-resistant strains of BRD pathogens has highlighted the need to identify and further characterize these bacteria to enhance judicious and effective treatment (Lubbers and Hanzlicek, 2013; Woolums *et al.*, 2018).

Development of molecular-based diagnostics

Molecular methods can detect very small quantities of target nucleic acid in complex samples and have been used in veterinary diagnostics for decades (Lauerman, 1998). However, recent advancements in technologies and chemistries have enabled robust and cost-effective assays that allow for simultaneous quantitative detection of multiple targets in a single test reaction. With these advances have come efficient nucleic acid extraction chemistries that can be utilized on high-throughput extraction platforms that co-purify RNA and DNA (Berensmeier, 2006). PCR-based approaches were quickly adapted to and utilized to detect viral pathogens associated with BRD. PCR methods were superior in turnaround time and interpretation compared to classical approaches such as cell culture and antibody-based detection, and even singleplexed conventional PCR testing has significant advantages over classic methods (Vilcek *et al.*, 1994; Schmitt *et al.*, 1994; Masri *et al.*, 1996). Highly multiplexed real-time PCR assays (rtPCR) that include reverse transcription to detect RNA and DNA pathogens are now widely used across US diagnostic labs for virus detection (Horwood and Mahony, 2011; Fulton *et al.*, 2016).

However, widespread implementation of these methods for the detection of bacterial pathogens of BRD has lagged. There are several challenges to the development and implementation of these assays. These include culture-based approaches that labs have existing capacity for, the need for isolated pathogens for downstream testing (typing, susceptibility, etc.), and

relative cost. In contrast to culture, PCR-based testing is also inherently narrow in scope, in that the test is limited to the sequence of the assay targets. However, newer technologies using 16S rRNA amplification and sequencing may hold promise for a more comprehensive and broad-based molecular diagnostics tool (Johnston *et al*., 2017; Timsit *et al*., 2018). Additionally, only recently have high-quality complete whole-genome sequences from diverse sources become available, greatly improving the ability to identify and select robust targets for assay design (Clawson *et al*., 2016; Harhay *et al*., 2017).

Another challenge is that diagnostically, interpretation of results from molecular testing may be misleading. Many pathogens are opportunistic in nature, and are present in both normal and diseased animals; therefore, direct detection of pathogens through culture and antibody-based approaches was preferred by some as more interpretable (Fulton and Confer, 2012). However, the combination of being readily able to assess the relative abundance of bacteria using real-time platforms, combined with rapid cycling rotary-based real-time platforms and robust enzyme mixes, has enhanced the utility of molecular methods for the detection of bacterial pathogens of BRD (Reynisson *et al*., 2006; Loy *et al*., 2018*a*).

We developed a real-time-based assay for the most frequently detected bacterial BRD pathogens. Newly available BRD pathogen genome sequences were used to establish robust targets, and the assay was developed and validated on multiple instrument platforms (Clawson *et al*., 2016; Loy *et al*., 2018*a*). As the advantages over culture-based approaches were not immediately apparent, an extensive comparative analysis was done to evaluate PCR-based detection compared to culture on samples that are routinely submitted to diagnostic labs. Both antemortem (nasal and nasopharyngeal swabs) and postmortem diagnostic samples (lungs) were included. Limits of detection for the assay are quite low (1.2–12 CFU ml^{-1}) and had a near-perfect agreement with culture for lung tissues with high overall levels of specificity and sensitivity. One large advantage over culture is the number of co-detections found. Co-detections were extremely underrepresented when relying on culture alone, with only 25 found in the data set, with 125 co-detections using PCR-based approaches, indicating a fivefold increase in the detection of these types of infections (Loy *et al*., 2018*a*). Agreement between culture and rtPCR was found to be highest in lungs and lowest in nasal swabs, likely due to the limitations of culture on samples more likely to contain environmental bacteria, such as nasal swabs. The instrumentation used did not adversely affect overall method sensitivity and specificity; however, the rotary-based instrument had significantly lower Cq thresholds, indicating more efficient PCR reactions. The overall conclusions of this work indicate the multiplexed rtPCR panels are rapid, sensitive, and diagnostically useful in multiple relevant sample types and have several advantages over classical methods.

Development of proteomic-based diagnostics

Another emerging technology, matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry (MS) has revolutionized clinical microbiology labs and veterinary diagnostics (Seng *et al*., 2009; Clark *et al*., 2013). These instruments enable the accurate identification of a single colony of bacterial growth in minutes. Many platforms are also flexible allowing users to add locally relevant strains and species to their databases. Mass spectrum data can be examined for biomarkers that may enable differentiation of phenotypes or genotypes (Mani *et al*., 2017; Pérez-Sancho *et al*., 2018). Recently, we have demonstrated the utility of MALDI-TOF MS, using a bioinformatics approach for biomarker discovery, to distinguish amongst two major genotypes of *M. haemolytica* (Loy and Clawson, 2017). This method enables near-real-time typing of these isolates by mining data collected during the MS identification process. This method has been validated on whole-cell bacteria 'direct smears' so no extraction or other processing is required and isolates can be rapidly screened and selected for downstream testing.

Applications using molecular-based diagnostics

One application of rtPCR BRD panels has been epidemiological investigations to examine the contributions of emerging pathogens to BRD. Workman *et al*. have utilized rtPCR to estimate BRD pathogen shedding throughout the beef production cycle to examine the role respiratory coronaviruses play in increasing the risk of BRD (Workman *et al*., 2017; Workman *et al*., 2019). One challenge to interpreting results from these panels is unlike viruses, where any detection of virus shedding may be clinically significant, the detection of bacteria that normally resides in the nasopharynx may not be clinically relevant. Further work to determine Cq cutoffs or levels of relative abundance that may be clinically significant is important to enable these tests so they be more readily interpreted from antemortem samples. In one study, neither the Cq level nor the numbers of animals classified as cases or controls which had a detected pathogen were significantly different at and following feedlot entry for bacterial pathogens. However, nasal shedding of bovine coronavirus (BCV) both in Cq values and the number of animals shedding was higher in those that were classified as cases. A follow-up study using these same diagnostic tools following BRD in preweaned beef calves was able to determine in one longitudinal study, *H. somni*, in addition to BCV, was potentially contributing to clinical cases, as *H. somni* was detected during BRD outbreaks and not at other time points (Workman *et al*., 2019). These observational studies demonstrate that molecular detection tools may be useful to examine risk factors and contributions of pathogens during BRD outbreaks.

Molecular workflows also provide for any number of culture-independent nucleic acid tests in addition to pathogen detection. One approach is to evaluate the extracted clinical samples for the presence of antimicrobial resistance genes to develop a rapid and culture-independent resistance detection method. Recent work has shown that the detection of macrolide and tetracycline-resistant genes in BRD clinical samples have a high agreement with the isolation of *M. haemolytica* with increased MIC values to these antimicrobials (Loy *et al*., 2018*b*). Such an approach could provide clinicians with information about the presence of potential resistant pathogens in samples more rapidly than culture and susceptibility testing.

Applications using proteomic-based diagnostics

Another challenge with the interpretation of detection results from antemortem samples is the presence of mixed intra-species populations that may not be representative of the causative organisms deeper in lung tissues. Capik *et al*. have found using pulsed-field gel electrophoresis that *M. haemolytica* populations in the nasopharynx do not always match those found in the lungs of cattle with BRD (Capik *et al*., 2015). One potential diagnostic

https://doi.org/10.1017/S1466252320000092 Published online by Cambridge University Press

approach to assist microbiologists in finding those populations most likely associated with disease from NP samples is to use MALDI-TOF MS profiles to screen isolates for downstream testing. Genotype 2 *M. haemolytica* is more likely to be associated with lung invasion and contain AMR genes and ICE elements, which can carry antimicrobial resistance genes. Therefore, preferential selection of these genotypes using MALDI-TOF prior to MIC testing and other downstream assays may be useful.

Conclusions

Emerging technologies and methods developed for the detection of etiologic agents associated with BRD have enabled further understanding of the role of microbes in BRD. Application of these technologies will help further elucidate the role of these opportunistic pathogens and will enable more effective disease prevention and treatment strategies.

References

Bell CJ, Blackburn P, Elliott M, Patterson TI, Ellison S, Lahuerta-Marin A and Ball HJ (2014) Investigation of polymerase chain reaction assays to improve detection of bacterial involvement in bovine respiratory disease. *Journal of Veterinary Diagnostic Investigation* **26**, 631–634.

Berensmeier S (2006) Magnetic particles for the separation and purification of nucleic acids. *Applied Microbiology and Biotechnology* **73**, 495–504.

Booker CW, Abutarbush SM, Morley PS, Jim GK, Pittman TJ, Schunicht OC, Perrett T, Wildman BK, Fenton RK, Guichon PT and Janzen ED (2008) Microbiological and histopathological findings in cases of fatal bovine respiratory disease of feedlot cattle in western Canada. *The Canadian Veterinary Journal* **49**, 473–481.

Capik SF, White BJ, Lubbers BV, Apley MD, Mosier DA, Larson RL and Murray RW (2015) Characterization of *Mannheimia haemolytica* in beef calves via nasopharyngeal culture and pulsed-field gel electrophoresis. *Journal of Veterinary Diagnostic Investigation* **27**, 568–575.

Clark AE, Kaleta EJ, Arora A and Wolk DM (2013) Matrix-assisted laser desorption ionization–time of flight mass spectrometry: a fundamental shift in the routine practice of clinical microbiology. *Clinical Microbiology Reviews* **26**, 547–603.

Clawson ML, Murray RW, Sweeney MT, Apley MD, Dedonder KD, Capik SF, Larson RL, Lubbers BV, White BJ, Kalbfleisch TS, Schuller G, Dickey AM, Harhay GP, Heaton MP, Chitko-Mckown CG, Brichta-Harhay DM, Bono JL and Smith TPL (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**, 982.

Fulton RW and Confer AW (2012) Laboratory test descriptions for bovine respiratory disease diagnosis and their strengths and weaknesses: gold standards for diagnosis, do they exist? *The Canadian Veterinary Journal = La revue veterinaire canadienne* **53**, 754–761.

Fulton RW, Blood KS, Panciera RJ, Payton ME, Ridpath JF, Confer AW, Saliki JT, Burge LT, Welsh RD, Johnson BJ and Reck A (2009) Lung pathology and infectious agents in fatal feedlot pneumonias and relationship with mortality, disease onset, and treatments. *Journal of Veterinary Diagnostic Investigation* **21**, 464–477.

Fulton RW, D'offay JM, Landis C, Miles DG, Smith RA, Saliki JT, Ridpath JF, Confer AW, Neill JD, Eberle R, Clement TJ, Chase CCL, Burge LJ and Payton ME (2016) Detection and characterization of viruses as field and vaccine strains in feedlot cattle with bovine respiratory disease. *Vaccine* **34**, 3478–3492.

Griffin D, Chengappa MM, Kuszak J and Mcvey DS (2010) Bacterial pathogens of the bovine respiratory disease complex. *Veterinary Clinics of North America: Food Animal Practice* **26**, 381–394.

Harhay GP, Harhay DM, Bono JL, Smith TPL, Capik SF, Dedonder KD, Apley MD, Lubbers BV, White BJ and Larson RL (2017) Closed genome sequences of seven *Histophilus somni* isolates from beef calves with bovine respiratory disease complex. *Genome Announcements* **5**, e01099–17.

Horwood PF and Mahony TJ (2011) Multiplex real-time RT-PCR detection of three viruses associated with the bovine respiratory disease complex. *Journal of Virological Methods* **171**, 360–363.

Johnston D, Earley B, Cormican P, Murray G, Kenny DA, Waters SM, Mcgee M, Kelly AK and Mccabe MS (2017) Illumina MiSeq 16S amplicon sequence analysis of bovine respiratory disease associated bacteria in lung and mediastinal lymph node tissue. *BMC Veterinary Research* **13**, 118.

Lauerman L (1998) Mycoplasma PCR assays: detection by PCR amplification. In Lauerman LH (ed), *Nucleic Acid Amplification Assays for Diagnosis of Animal Diseases*. Visalia, CA, USA: American Association of Veterinary Laboratory Diagnosticians, pp. 1–134.

Loy JD and Clawson ML (2017) Rapid typing of *Mannheimia haemolytica* major genotypes 1 and 2 using MALDI-TOF mass spectrometry. *Journal of Microbiological Methods* **136**, 30–33.

Loy JD, Leger L, Workman AM, Clawson ML, Bulut E and Wang B (2018*a*) 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. *Journal of Veterinary Diagnostic Investigation* **30**, 837–847.

Loy JD, Payne J, Deal C, Dutta E, Bulut E, Clawson ML and Wang B (2018*b*) Moving beyond the MIC: evaluation of a novel multiplex real time PCR assay for detection of antimicrobial resistance genes in clinical bovine respiratory disease samples. 61st Annual Conference of the American Association of Veterinary Laboratory Diagnosticians, October 20, 2018 Kansas, City, MO. 7.

Lubbers BV and Hanzlicek GA (2013) Antimicrobial multidrug resistance and coresistance patterns of *Mannheimia haemolytica* isolated from bovine respiratory disease cases – a three-year (2009–2011) retrospective analysis. *Journal of Veterinary Diagnostic Investigation* **25**, 413–417.

Mani RJ, Thachil AJ and Ramachandran A (2017) Discrimination of *Streptococcus* equi subsp. equi and *Streptococcus* equi subsp. zooepidemicus using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Journal of Veterinary Diagnostic Investigation* **29**, 622–627.

Masri SA, Olson W, Nguyen PT, Prins S and Deregt D (1996) Rapid detection of bovine herpesvirus 1 in the semen of infected bulls by a nested polymerase chain reaction assay. *Canadian Journal of Veterinary Research = Revue canadienne de recherche veterinaire* **60**, 100–107.

Pérez-Sancho M, Vela AI, Horcajo P, Ugarte-Ruiz M, Domínguez L, Fernández-Garayzábal JF and De La Fuente R (2018) Rapid differentiation of *Staphylococcus aureus* subspecies based on MALDI-TOF MS profiles. *Journal of Veterinary Diagnostic Investigation* **30**, 813–820.

Reynisson E, Josefsen MH, Krause M and Hoorfar J (2006) Evaluation of probe chemistries and platforms to improve the detection limit of real-time PCR. *Journal of Microbiological Methods* **66**, 206–216.

Schmitt BJ, Lopez OJ, Ridpath JF, Galeota-Wheeler J and Osorio FA (1994) Evaluation of PCR for diagnosis of bovine viral diarrhea virus in tissue homogenates. *Journal of Veterinary Diagnostic Investigation* **6**, 44–47.

Seng P, Drancourt M, Gouriet F, La Scola B, Fournier P-E, Rolain JM and Raoult D (2009) Ongoing revolution in bacteriology: routine identification of bacteria by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Clinical Infectious Diseases* **49**, 543–551.

Tegtmeier C, Angen Ø and Ahrens P (2000) Comparison of bacterial cultivation, PCR, *in situ* hybridization and immunohistochemistry as tools for diagnosis of *Haemophilus somnus* pneumonia in cattle. *Veterinary Microbiology* **76**, 385–394.

Timsit E, Workentine M, Van Der Meer F and Alexander T (2018) Distinct bacterial metacommunities inhabit the upper and lower respiratory tracts of healthy feedlot cattle and those diagnosed with bronchopneumonia. *Veterinary Microbiology* **221**, 105–113.

Vilcek S, Elvander M, Ballagi-Pordány A and Belák S (1994) Development of nested PCR assays for detection of bovine respiratory syncytial virus in clinical samples. *Journal of Clinical Microbiology* **32**, 2225–2231.

Woolums AR, Karisch BB, Frye JG, Epperson W, Smith DR, Blanton Jr J, Austin F, Kaplan R, Hiott L, Woodley T, Gupta SK, Jackson CR and Mcclelland M (2018) Multidrug resistant *Mannheimia haemolytica* isolated from high-risk beef stocker cattle after antimicrobial metaphylaxis

https://doi.org/10.1017/S1466252320000092 Published online by Cambridge University Press

and treatment for bovine respiratory disease. *Veterinary Microbiology*, **221**, 143–152.

Workman AM, Kuehn LA, Mcdaneld TG, Clawson ML, Chitko-Mckown CG and Loy JD (2017) Evaluation of the effect of serum antibody abundance against bovine coronavirus on bovine coronavirus shedding and risk of respiratory tract disease in beef calves from birth through the first five weeks in a feedlot. *American Journal of Veterinary Research* **78**, 1065–1076.

Workman AM, Kuehn LA, Mcdaneld TG, Clawson ML and Loy JD (2019) Longitudinal study of humoral immunity to bovine coronavirus, virus shedding, and treatment for bovine respiratory disease in pre-weaned beef calves. *BMC Veterinary Research* **15**, 161.

https://doi.org/10.1017/S1466252320000092 Published online by Cambridge University Press