

Current and emerging diagnostic approaches to infectious diseases of cattle

John Dustin Loy, DVM, PhD, Dipl. ACVM

University of Nebraska-Lincoln, Nebraska Veterinary Diagnostic Center, Lincoln, NE 68583

Abstract

Diagnostic approaches to endemic and emerging diseases of cattle are an area that has seen rapid changes due to advances in technology and development of new methods that utilize these technologies. Although the fundamentals to detection and diagnosis of infectious diseases are little changed (i.e. detection of pathogens, molecules, or markers associated with infectious agents) the approaches and interpretation of diagnostic findings varies greatly depending on the test method. New advances in real time polymerase chain reaction (rtPCR), MALDI-TOF mass spectrometry and next generation sequencing (NGS) have driven development of highly sensitive, specific and rapid pathogen detection for a variety of infectious agents. These tools have also been used to investigate numerous research questions relating to epidemiology and pathobiology in cattle. However, in some cases the interpretation of these results can be challenging and often varies depending on the disease or disease complex involved and herd or population level information. A brief review of fundamentals of these tests and interpretation in some clinical applications will be discussed.

Key words: bovine, MALDI-TOF, PCR, pathogen, detection, NGS

Classically, approaches to diagnosis of infectious diseases were very straightforward. A pathogen was isolated in culture from a sample collected from an animal with corresponding clinical findings, was identified and confirmed, and a diagnosis was achieved. As diagnostic science progressed, methods were developed that enabled detection of molecules that were specifically associated with pathogens (proteins and/or nucleic acids), antibodies generated by an immune response to specific pathogens, or biomarkers associated with disease states. However, most of these tests generated binary results (positive or negative) that could be easily interpreted in a clinical context. Modern detection methods, such as real time PCR (rtPCR) now provide a much higher level of sensitivity, which can confound interpretation, especially for opportunistic organisms associated with disease complexes or agents that may have an environmental presence. These pathogens are present in many normal animals at some level, resulting in detection, or accumulate in the environment and are detected as animals ingest or inhale them. Additionally, new technologies such as next generation sequencing (NGS) have allowed for study of pathogens and opportunistic pathogens at the genomic level. NGS when combined with matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry allows for rapid typing of opportunistic pathogens. Pathogen typing can aid clinicians in determining if a strain isolated from a case is more likely to be pathogenic or commensal in nature and may provide some abilities to compare strain level variation on herds or farms.

Polymerase chain reaction

Rapidly advancing in both refinement and capabilities since its discovery in the 1980s, polymerase chain reaction (PCR) has been one of the most important advancements in diagnostic science.¹ The addition of multiplexable fluorescent probes that allow for nucleic acid amplification to be observed in real time (rtPCR) has had tremendous impacts on veterinary diagnostics.² Additionally, the use of high throughput nucleic acid extraction chemistries enabled both RNA and DNA to be simultaneously extracted from nearly any clinical sample type in a rapid and efficient manner. Together, these technologies allowed for multiple tests to be combined in a single tube (multiplexed), and allowed for quantification (or semi-quantification) of RNA or DNA target level in the original sample to be assessed in a matter of hours. Limits of detection for many assays can be as low as 1-10 copies of target nucleic acid and recent advances using reverse transcription PCR assays to detect RNA targets from pathogens with DNA genomes such as *Tritrichomonas foetus* and *Anaplasma marginale* have greatly increased the sensitivity of PCR-based approaches for detection of these pathogens.^{3,4}

Interpretation of these assay results can be straightforward in primary pathogens that are only present in disease states where herds are usually free (i.e. foreign animal diseases). However, a large number of cattle diseases are opportunistic and exist at some level in the herd or environment continuously. Therefore, mere detection of an organism, or organism related molecules, is not the same as a diagnosis. Previous tests, such as pathogen culture, ensured that A) pathogen is viable and B) there was sufficient pathogen in the sample to enable recovery, which is often 10^5 colony forming units or greater. Given this difference in sensitivity, direct comparison between culture and rtPCR results does not always result in agreement. One distinct advantage to rtPCR is the result is quantifiable, measured in cycle threshold (Ct) of pathogen target. A Ct value is the number of heating and cooling cycles required (typically < 40) to generate sufficient amplification of the pathogen to be detectable and thus is reversely correlated with quantity. Therefore, a low Ct value, such as 12, would be more likely to be associated with disease state than a high Ct value, such as 38. For many tests/sample types, a 2-3 Ct reduction would be equivalent to a log increase in pathogen present in the sample and vice versa. Familiarity with Ct values when combined with clinical observations can help distinguish acute clinical infections with an expected low Ct value, from those that may be due to recent vaccine administration, from environmental contamination, or from carriage of opportunistic pathogens. Such observations are usually driven by the pathogen or disease in question. For example, PCR tests for *Mycobacterium avium* ssp *paratuberculosis* (MAP or Johne's) are extremely sensitive, and false positive high Ct value results may occur when animals are commingled with heavy shedders in contaminated environments. Additionally, use of modified live vaccines may cause false positive results, sometimes for weeks, as has been observed following viral respiratory vaccine administration.^{5,6}

Multiplexed real time PCR syndromic panels

One advantage of highly customizable rtPCR technologies is the ability to multiplex tests based on disease syndromes and sample types. Currently there are numerous bovine specific syndromic PCR panels that enable rapid screening and semi-quantification of the most common pathogens associated with disease syndromes. With modern extraction chemistries that purify both DNA and RNA, these can flexibly be used to detect everything from RNA viruses to protozoa. Frequently ordered panels include those for bovine respiratory disease, infectious bovine keratoconjunctivitis (IBK or pinkeye), neonatal calf diarrhea, and abortions, among others.⁷⁻⁹ Other panels have been developed to aid in pathogen characterization which enable rapid detection of characteristics like antimicrobial resistance in BRD samples and the presences of toxins or virulence factors in enteric bacteria such as *E. coli* or *Clostridium* spp.^{10,11} Other panels to detect frequently found antimicrobial resistance genes in some pathogens can also be applied in conjunction with syndromic PCR panels. One recent example of this is the detection of macrolide and tetracycline resistance genes in bovine respiratory pathogens.¹² Since members of the *Pasteurellaceae*, the most frequent bacteria associated with BRD, have integrative and conjugative elements that allow for sharing of resistance genes, many of these resistant strains, at least in the United States, seem to share the same genes/mechanisms for these drug classes, and therefore can be detected with an assay for these common genes.^{13,14}

Digital PCR

Digital PCR (dPCR) is an emerging application of real time PCR, wherein the partitioning of the PCR reaction randomly into tens of thousands of individual reactions allows for absolute quantification of target in the sample.¹⁵ dPCR relies on the same chemistry as real-time PCR, that is detection of a fluorescence signal that can be multiplexed, however, it utilizes detection after the amplification is complete. The number of individual positive and negative partitions can be assessed, and through application of Poisson distribution, the absolute quantity of target can be calculated. The partitions in many commercial technologies involve generating thousands of droplets in an emulsion or utilizing microfluidic chips/slides to fill microwells or cavities. The advantages of dPCR include enhanced performance in the presence of inhibitors, a common problem with veterinary samples, lack of need for standard curves, absolute quantity of targets, and enhanced sensitivity in some matrices. dPCR is currently being evaluated for use in many veterinary diagnostic labs, and has been shown to be a potentially superior method for detection of bovine leukemia proviral load in infected animals.¹⁶ It has also shown promise for identification sub-clinical animals with only focal bovine paratuberculosis lesions, where it was the most sensitive assay evaluated in a comparison study.¹⁷ Future applications of this technology to bovine diagnostics will certainly continue, and practitioners may begin to see these types of tests and results on diagnostic reports. One notable difference is the absence of a Ct value, where instead results are reported in absolute quantities, typically in copies/ μ L or total copies of pathogen target in a sample.

Next generation sequencing (NGS)

The application of next generation sequencing (NGS) methods to bovine diagnostics is primarily in the research phase of development, where it is extremely useful to answer specific

research questions, but is not yet found its potential as a routine and cost-effective diagnostic tool. The cost and amount of data typically generated by these types of methods is typically not readily translatable to routine diagnostics and requires significant analysis and bioinformatics capacity, and the time for testing and analysis typically takes several days. However, there remains high levels of potential for different tools in the NGS toolbox that can be used for diagnostic purposes. NGS methods that are commercially available include both short read technologies or long read technologies that have different strengths and weaknesses. Short read sequencing can generate millions of small fragments of sequence that must be pieced together or assembled or analyzed by bioinformatics software. Additional frequently used methods include metagenomics using 16S or other common microbial genes that are pre-amplified prior to sequencing, or shotgun approaches where many sequences are generated and ran through databases of sequence for analysis. Short read technologies also have market advantages in that laboratories and researchers have more experience in utilizing these systems and the downstream analysis of the data generated from them. Long read sequencing approaches can be used in similar ways but are also advantageous in generating long reads required for whole genome assembly or other types of sequencing but often have more limited sequence generating capacity, resulting in a higher overall sequencing cost.

Typically, in diagnostic samples, host nucleic acids or other non-pathogenic sequences overwhelm the ability of detection through shotgun sequencing samples. Target pre-enrichment, either through preamplification of target sequences through PCR with pools of target primers, or enrichment through target baits, is often done to enhance the ability to find rare sequences in these complex samples which often have large amounts of off target and host nucleic acid. Tests have been developed for cattle that can test for dozens or hundreds of different pathogens using a pre-amplification step followed by sequencing approach.¹⁸ Others have used long read sequencing to look for respiratory pathogens and antimicrobial resistance in chronic BRD cases, but such an application would be versatile for many diagnostic situations.¹⁹ Shotgun approaches using long read sequences have also been very useful to find pathogens in unknown/unknown samples (those which diagnosticians do not know what they are looking for and may not have tests for them) and additionally may have utility to find high consequence, unexpected or foreign animal diseases in veterinary samples.²⁰

MALDI-TOF MS

Matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry is a method developed to assess complex macromolecules in chemistry laboratories, that has gained rapid adoption in veterinary diagnostic laboratories. The method relies on soft ionization of proteins and separation of them in a flight tube, resulting in a mass spectrum profile. For bacterial pathogens, these profiles are easily generated on colony growth from a culture plate, and are unique and reproducible for most organisms at the species level, and some at the sub-species level.²¹ This allows for rapid identification of organisms, and in some cases further characterization into classifications such as genotypes, toxin types, or serotypes. This technology has enabled veterinary labs to streamline microbial identification workflows, and can save days of testing time when compared to classical phenotypic tests. Discoveries in genomic differences using NGS across opportunistic pathogens, such as *Mannheimia haemolytica*, *Moraxella bovis* and

Moraxella bovoculi, have allowed for these genomic differences to be translated into rapid and accurate MALDI-TOF tests.²² Additional capabilities of MALDI-TOF instrumentation include the ability to conduct strain level comparisons or “biotyping” on clinical isolates to track changes and other variation among circulating strains.²³

Clinical applications

The application of diagnostics varies greatly depending on the disease in question, therefore the interpretation of diagnostic test results varies depending on the disease, clinical history and findings, and other information collected. One important note to consider which is outside the scope of this paper is the use of pre-test probability along with assay performance metrics such as diagnostic sensitivity and specificity (see Buczinski, et al for an excellent review of this application) when interpreting diagnostic testing results.²⁴ When analysis of PCR testing results is performed, it is important to know if the types of infectious disease would be expected to be detected such as in the case of opportunistic pathogens or those where there would be the potential for environmental contamination. One common challenging scenario is the case of Johne’s disease diagnostics in herds with unknown status. Frequently, the presence of several heavy organism shedders in commingled groups can cause false positive PCR testing results in animals, frequently with high, but non-negative Ct values. Additionally, disease complexes such as bovine respiratory disease and bovine infectious keratoconjunctivitis involve opportunistic pathogens that may always be present at some level in normal animals, and certainly be present at the herd level. Use of Ct values, culture and clinical findings is helpful to interpret PCR based testing results.

Additionally, syndromic PCR panels are now available at many laboratories that detect many causes of infectious abortion. Although these panels are useful as adjunct to histopathology to detect organisms, infectious abortion workups should heavily use histopathology to determine if lesions such as placentitis are present, or collect samples that would be less likely to be environmentally contaminated to also include testing such as culture. A recent *Veterinary Clinics of North America* edition on diagnostics, and a chapter on bacterial diagnostics, provides further information on many of these topics.^{6,25}

Conclusions

There are an increasing number of tests available to bovine practitioners to assess animals for the presence or absence of pathogens or prior exposure to them. However, with new technological advancements and increasing sensitivity and complexity of testing, interpretation has never been more challenging. New data from these tests such as Ct values/copy numbers, presence or absence of antimicrobial resistance genes, or pathogen genotype, can help clinicians and practitioners maximize the utility of diagnostic information when taken in the context of clinical findings and herd level information.

References

1. Mullis K, Faloona F, Scharf S, et al. Specific Enzymatic Amplification of DNA In Vitro: The Polymerase Chain Reaction. *Cold Spring Harbor Symposia on Quantitative Biology* 1986;51:263-273.
2. Toohey-Kurth KL, Mulrooney DM, Hinkley S, et al. Best practices for performance of real-time PCR assays in veterinary diagnostic laboratories. *J Vet Diagn Invest* 2020;32:815-825.

3. Reinbold JB, Coetzee JF, Sirigireddy KR, et al. Detection of *Anaplasma marginale* and *A. phagocytophilum* in bovine peripheral blood samples by duplex real-time reverse transcriptase PCR assay. *J Clin Micro* 2010;48:2424-2432.
4. Ginter Summarell CC, Hairgrove TB, Schroeder ME, et al. Improvements in *Tritrichomonas foetus* molecular testing. *J Vet Diagn Invest* 2018;30:603-608.
5. Walz PH, Newcomer BW, Riddell KP, et al. Virus detection by PCR following vaccination of naive calves with intranasal or injectable multivalent modified-live viral vaccines. *J Vet Diagn Invest* 2017;29:628-635.
6. Loy JD, Clawson ML, Adkins PRF, et al. Current and emerging diagnostic approaches to bacterial diseases of ruminants. *Vet Clin North Am Food Anim Pract* 2023;39:93-114.
7. Loy JD, Leger L, Workman AM, et al. 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 Invest* 2018;30:837-847.
8. Cho Y-I, Kim W-I, Liu S, et al. Development of a panel of multiplex real-time polymerase chain reaction assays for simultaneous detection of major agents causing calf diarrhea in feces. *J Vet Diagn Invest* 2010;22:509-517.
9. Zheng W, Porter E, Noll L, et al. A multiplex real-time PCR assay for the detection and differentiation of five bovine pinkeye pathogens. *J Microbiol Methods* 2019;160:87-92.
10. Casey TA, Bosworth BT. Design and evaluation of a multiplex polymerase chain reaction assay for the simultaneous identification of genes for nine different virulence factors associated with *Escherichia Coli* that cause diarrhea and edema disease in swine. *J Vet Diagn Invest* 2009;21:25-30.
11. Meer RR, Songer JG. Multiplex polymerase chain reaction assay for genotyping *Clostridium perfringens*. *Am J Vet Res* 1997;58:702-705.
12. Dutta E, Loy JD, Deal CA, et al. Development of a multiplex real-time PCR assay for predicting macrolide and tetracycline resistance associated with bacterial pathogens of bovine respiratory disease. *Pathogens* 2021;10.
13. Beker M, Rose S, Lykkebo CA, et al. Integrative and conjugative elements (ICEs) in *Pasteurellaceae* Species and their detection by multiplex PCR. *Front Micro* 2018;9.
14. Clawson ML, Murray RW, Sweeney MT, et al. Genomic signatures of *Mannheimia haemolytica* that associate with the lungs of cattle with respiratory disease, an integrative conjugative element, and antibiotic resistance genes. *BMC Genom* 2016;17:982.
15. Quan P-L, Sauzade M, Brouzes E. dPCR: A Technology Review. *Sensors*, 2018.
16. De Brun ML, Cosme B, Petersen M, et al. Development of a droplet digital PCR assay for quantification of the proviral load of bovine leukemia virus. *J Vet Diagn Invest* 2022;34:439-447.
17. Badia-Bringué G, Canive M, Casais R, et al. Evaluation of a droplet digital PCR assay for quantification of *Mycobacterium avium* subsp. paratuberculosis DNA in whole-blood and fecal samples from MAP-infected Holstein cattle. *Front Vet Sci* 2022;9.
18. Anis E, Hawkins Ian K, Ilha Marcia RS, et al. Evaluation of targeted next-generation sequencing for detection of bovine pathogens in clinical samples. *J Clin Microbiol* 2018;56:e00399-00318.
19. Freeman CN, Herman EK, Abi Younes J, et al. Evaluating the potential of third generation metagenomic sequencing for the detection of BRD pathogens and genetic determinants of antimicrobial resistance in chronically ill feedlot cattle. *BMC Vet Res* 2022;18:211.

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20. Neujahr AC, Loy DS, Loy JD, et al. Rapid detection of high consequence and emerging viral pathogens in pigs. *Front Vet Sci* 2024;11:1341783.
 21. Khot PD, Couturier MR, Wilson A, et al. Optimization of matrix-assisted laser desorption ionization–time of flight mass spectrometry analysis for bacterial identification. *J Clin Microbiol* 2012;50:3845-3852.
 22. Loy JD, Clawson ML. From Genomics to MALDI-TOF MS. *Microbiological Identification using MALDI-TOF and Tandem Mass Spectrometry*, 2023;283-302.
 23. Berrazeg M, Diene SM, Drissi M, et al. Biotyping of multidrug-resistant *Klebsiella pneumoniae* clinical isolates from France and Algeria using MALDI-TOF MS. *PLoS ONE* 2013;8:e61428.
 24. Buczinski S, Dufour S, Arango-Sabogal JC. Interpretation and analysis of individual diagnostic tests and performance. *Vet Clin North Am Food Anim Pract* 2023;39:1-19.
 25. Loy JD, Monday JD, Smith DR. Ruminant diagnostics and interpretation. *Vet Clin North Am Food Anim Pract* 2023;39:i.

