Current and Emerging Diagnostic Approaches to Bacterial Diseases of Ruminants

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KEYWORDS

• Bovine • Diagnostics • Bacterial pathogens • MALDI-TOF • Real-time PCR • Bovine respiratory disease • Mastitis • Abortion • Bovine keratoconjunctivitis

KEY POINTS

- Real-time polymerase chain reaction (PCR) methods have enabled highly sensitive diagnostic tests for numerous bacterial pathogens of ruminants.
- Multiplexing of real-time PCR tests has enabled development of syndromic PCR panels that can screen for several pathogens in one test.
- Real-time PCR cycle threshold values can be used to estimate relative abundance of pathogens in clinical samples.
- Comparison of molecular diagnostics with culture-based approaches is disease- and sample-dependent.
- Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry has allowed for highly efficient identification and typing of bacterial pathogens associated with ruminant diseases.

INTRODUCTION

Basics of Polymerase Chain Reaction

The polymerase chain reaction (PCR) was discovered in the mid-1980s and remains one of the most important developments to molecular biology.[1] Specifically, the adaptation of this technique to use with fluorescent dyes and labeled probes, allowing measurement of nucleic acid amplification in real time, the real-time PCR (rtPCR) assay

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has had the greatest impact in veterinary diagnostic laboratories (VDLs).[2] The acronym rtPCR will be used throughout this article to describe this approach as has been used in a recent review on this method specific to VDLs.[2] Other nomenclature or acronyms to describe similar methods for RNA detection include reverse transcription rtPCR (RT-rtPCR). The acronyms qPCR and RT-qPCR are recommended for use by the *M*inimum *I*nformation for Publication of *Q*uantitative Real-Time PCR *E*xperiments guidelines, so these are frequently used to describe rtPCR and RT-rtPCR in the literature.[3] However, this convention is not applied in this review, as qPCR implies quantification of target, which does not routinely occur when used for diagnostic testing. rtPCR applied to diagnostic testing of veterinary samples is complex and includes collection and transport of samples, sample preparation, nucleic acid extraction, amplification, analysis, and reporting. One advantage of rtPCR is there is no additional step required to visualize amplification, which decreases analysis time and reduces risk for cross-contamination, as amplified targets do not need additional processing or handling. The extreme sensitivity of the method, which can detect a few nucleic acid copies, requires strict quality control to ensure accuracy.

The rtPCR methods have been developed for detection of numerous pathogens, many of which are multiplexed assays that allow for simultaneous testing for multiple targets in the same sample.[4] Multiplexing typically relies on the use of sequence specific oligonucleotides, called probes, that have a reporter dye and quencher dye attached to the 5′ and 3′ end, and are combined in an assay with flanking oligonucleotide primers. During nucleic acid amplification the reporter and quencher dyes are cleaved from the probe by the polymerase enzyme, resulting in fluorescence of the reporter dye when excited by the light source from the instrument. Numerous reporter dyes are available that can be multiplexed as they differ in their emission spectrum following excitation. This has allowed for the development of syndrome specific panels (syndromic PCR panels) that allow for rapid screening of numerous agents associated with a disease syndrome. The real-time nature of the assay allows for fluorescence to be measured following each amplification cycle (typically 40 total cycles; **Figs. 1** and **2**). The number of cycles required to generate sufficient signal over background levels is called a cycle threshold (Ct) value, which is inversely proportional to the amount of target in the sample. This relationship allows for quantification of target in the sample to be conducted by comparison with a standard curve of a known and quantified target, also called a cycle quantification (Cq) level (see **Fig. 1**). However, the use of a standard curve for quantification is cost and time prohibitive and typically not

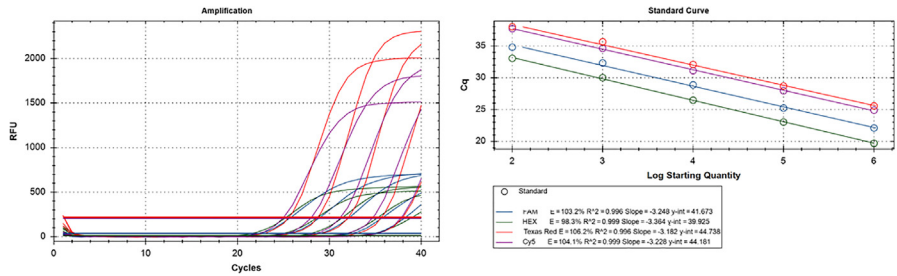

Fig. 1. Real-time PCR amplification plots demonstrating use of four targets in a serial dilution. X-axis is cycles and y-axis is relative fluorescence (*left panel*) and a generation of a standard curve from these data, x-axis is cycle threshold (Ct) value and y-axis is log-starting quantity of target (*right panel*). The increase in Ct value for each serial dilution can be observed, along with relative differences in values for each target.

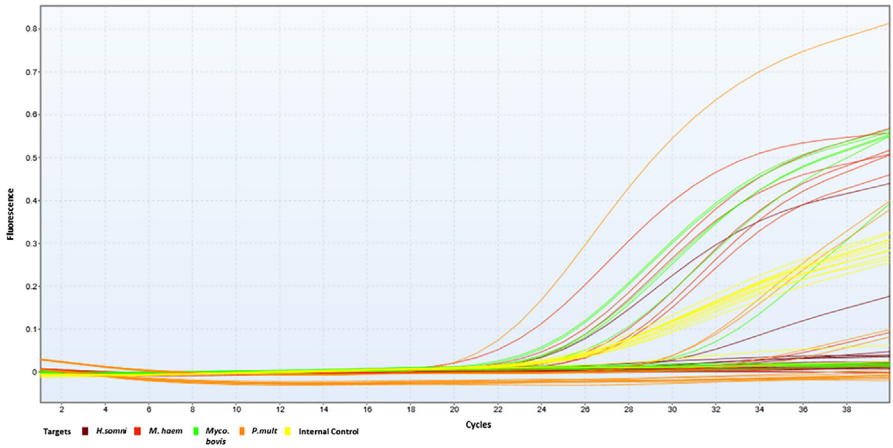

Fig. 2. Real-time PCR data output showing baseline corrected fluorescence in a multiplexed rtPCR panel for bovine respiratory disease run on clinical samples. Each colored line on the curve represents a single target (*H somni*, *M haemolytica, P multocida*, *M bovis*, and Internal Control) within the multiplexed reaction run on nucleic acid extracted from a sample.

performed for routine diagnostics (see note above about rtPCR and qPCR). Instead, the Ct value is used and reported based on the relative abundance of target in comparison to a well characterized positive control. VDLs typically report this on diagnostic reports as a Ct value and have established cutoffs to determine if a sample is considered detected (positive) for a target, or not detected (negative) based on their validation studies. These components are necessary for Ct values to be meaningful in the absence of a standard curve.[2] Therefore, in this article, Ct value will be used instead of Cq or other metrics, as this is the most commonly reported on diagnostic results.

Diagnostic applications of rtPCR using multiplexing have expanded the range of agents available for testing, simplified reporting, and reduced testing costs. There are some limitations, however, because robust optimization must be undertaken to ensure no preferential target amplification is occurring. This can cause false-negative results for some low-copy targets when amplified in the presence of a strong positive, a frequent occurrence for some disease syndromes such as bacteria associated with bovine respiratory disease (BRD).[4,5] To ensure consistent assay performance, VDLs will typically include a low-copy internal positive control (IPC) which is an exogenous source of nucleic acid that can be measured to ensure no inhibitors are present in the sample and that the nucleic acid extraction performed as expected.[6] IPCs are also useful to evaluate the presence of preferential amplification in multiplexed assays, as amplification of the low-copy control can be evaluated to ensure this is not occurring.

Expanded development, validation, and accessibility of rtPCR have made numerous tests for ruminant diagnostics available in VDLs. Research continues to provide meaningful data in associating these test results to both classical tests and field-level investigations. Specific discussion of these tests is provided later in this article using frequently encountered clinical disease syndromes of ruminants.

Matrix-Assisted Laser Desorption Ionization–Time of Flight and Diagnostics

Matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry is an emerging technology that has revolutionized bacterial identification in

human clinical microbiology laboratories and has been widely adopted in VDLs.[7] MALDI-TOF is a robust and highly reproducible method for microbial identification and for many organisms is equivalent to sequencing ribosomal or other housekeeping genes for identification.[8] The MALDI-TOF procedure has been simplified to use on a colony taken from solid media, which is then smeared on a stainless steel target in a thin film.[9] The cells are then treated with a matrix, usually α-cyano-4-hydroxycinnamic acid, which crystallizes on the smear film. When excited by a ultraviolet (UV) laser, the reactive matrix disrupts the film of microbial cells into ionized protein fragments (**Fig. 3**). As the process occurs in a vacuum contained in a flight tube, the application of a timed electromagnetic charge enables an accurate measurement of flight time as the ions transverse the tube and collide with a detector, allowing calculation of mass-to-charge ratio (m/z) of the ions. Each bacterial species generates a unique composition of ion fragments, which are consistently generated by the MALDI-TOF process. The consistency of this fragmentation allows for unique fingerprints (mass spectrum profiles) to be saved and compared with future isolates and strains.

MALDI-TOF has become the standard in many laboratories as it is fast, reproducible, cost-efficient, accurate, and eliminates the need for specialized biochemical testing. Commercially available MALDI-TOF libraries have tens of thousands of database entries; some of which include yeasts and molds in addition to human, veterinary, plant, and environmental bacterial pathogens and organisms. Studies have been conducted examining this technology for many veterinary and bovine pathogens including those associated with mastitis, respiratory disease, and ocular disease.[10–13] Laboratories can also curate their own database of profiles for isolates or strains that may be more regional, disease, or host-specific.[11] In addition, methods have been developed that enable discrimination of specific peaks contained in the mass spectrum profile that can be used to characterize isolates to the subspecies level, which may be useful when culturing opportunistic pathogens from sites with normal flora.[14–16] Many instruments also possesses the ability for biotyping, or looking at relatedness dendrograms based on peak profiles, which may be useful for outbreak investigations or evaluating strain diversity in herds or regions for some pathogens.[17]

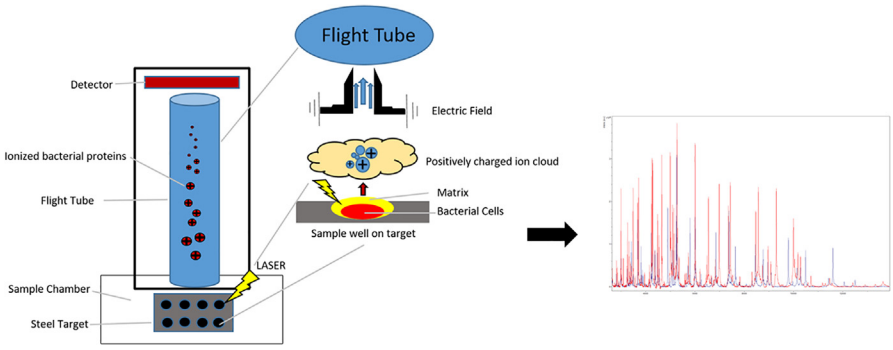

Fig. 3. Diagram of MALDI-TOF process for microbial identification. A thin film of microbial cells is prepared with a matrix solution on a steel target. The sample is vaporized into positively charged ions by a laser, which are propelled through a vacuum flight tube by an electrical field. Ions hitting the detector are measured and mass to charge ratio (m/z) is calculated. Mass spectrum output is shown next to the black arrow. Blue is *M bovis* and red is *M bovoculi*. The x-axis is m/z in Daltons. The y-axis is peak intensity in arbitrary units (arbitrary units). The mass spectrum profiles generated can then be used for downstream identification or strain typing.

Basics of Sequencing for Identification

Identification of bacterial pathogens can be challenging, even with robust tools such as MALDI-TOF, PCR, and gene sequencing. Bacterial genomes typically consist of a circular chromosome, and in some cases, plasmids or phages can be fluid.[18–20] Bacteria can acquire exogenous deoxyribonucleic acid (DNA) through plasmid- or integrative conjugative element (ICE)- induced conjugation, viral-induced transduction, and transformation processes, which collectively result in plasmid acquisitions, and the assimilation of ICEs and other newly acquired DNA segments on to their chromosomes.[21] In addition, insertion sequences and larger transposons can move about bacterial DNA,[21,22] so too can integrons, which are assembly and expression platforms of exogenous gene cassettes that can reside on plasmids and transposons where they have mobility.[23] Bacterial chromosomes and plasmids can also change their tandem repeat numbers due to strand slippage and recombination, and they can undergo inversions.[24–26] Bacteria also have mutation rates with their reproduction that can vary within a community and result in the spontaneous generation of single nucleotide polymorphisms and/or insertion deletion alleles.[27,28] All of these mechanisms can introduce or modulate gene function in bacteria, and thus are essential to their evolution and adaptability to new niches, including ones involving pathogenesis.[21,22,29] Consequently, decoding their genomic playbooks through sequencing provides a means for their identification and a foundation for understanding their biological functions and pathogenic potential.

DNA sequencing technology has advanced tremendously through the years and is categorized into three generations. The first is represented by Maxam–Gilbert and Sanger techniques that provide relatively small-scale sample and targeted region coverage.[30,31] The second consists of massive, short-length sequence production from clonally amplified DNA molecules on platforms such as Ion Torrent and Illumina.[30] The short-length sequences produced with second-generation technologies translate to challenges in generating compete, closed whole-genome assemblies, primarily because of repetitive sequence as well as genomic rearrangements and inversions.[30,32,33] Third-generation sequencing technologies involve moving a strand of DNA along a stationary read out system, such as an immobilized DNA polymerase complex (PacBio platform), or through a pore on a membrane, where ion flow changes through the membrane coincide with the nucleotides passing through it (Oxford Nanopore platform).[30] DNA sequence reads generated from third-generation technologies tend to be much longer than those from the second generation, which means that they assemble better; however, the sequence reads can have higher error rates.[30] Consequently, hybrid assemblies using combinations of second- and third-generation sequences have been used to generate highly accurate, closed bacterial chromosome sequences.[34] Third-generation sequencing continues to improve, and combined with enhanced assembly techniques, shows promise in constructing closed, circular genomes from mixed samples containing DNA of multiple microbes, such as microbiome samples.[35] Thus, the ability to sequence and assemble full bacterial chromosomes, even from preparations of mixed DNA, should only continue to increase in the future.

Nucleic acid sequence is a powerful identifier of bacteria. Historically, 16S ribosomal gene sequence has been used for bacterial identification to the species level.[36] More recently, its taxonomic utility has been recognized more at the level of genus or higher with only segments of the gene used for identification.[37,38] Multilocus sequence analysis, which involves analysis of multiple (4 or more) housekeeping genes, is commonly used for species and subspecies identification.[39] However, with whole-genome sequencing (WGS) of bacteria becoming cheaper, faster, and easier to do,

average nucleotide identity (ANI) has become a preferred metric of choice for species identification.[40] The ANI method was developed in 2005 and is an alignment-based pairwise similarity between two genomes.[40–42] Bacterial genomes do not have to be completely assembled into single chromosomes for ANI comparisons, and simple cut-offs are used to determine if bacteria are members of the same species.[43,44] Thus, it has become somewhat straightforward to identify bacterial species. Whole genomic sequences can also be used to identify bacterial subspecies, usually by phylogenetic or cladistic analyses that show genetic substructure at that level.[34,45,46] As sequencing technologies continue to improve and become cheaper and more accessible, we anticipate that WGS will become increasingly used by VDLs for a variety of applications.

APPLICATION IN DIAGNOSING SPECIFIC CLINICAL SYNDROMES

Bovine Respiratory Disease

BRD is a multifactorial disease complex associated with both viruses and bacteria and is one of the most frequently diagnosed and economically costly diseases of cattle.[47] Bacterial pathogens associated with BRD include *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, *Mycoplasma bovis*, and organisms such as *Trueperella pyogenes*.[47] Culture and downstream identification methods, such as biochemical testing, are the gold standard for diagnosis of bacterial pathogens associated with BRD. However, numerous molecular-based PCR assays are available for detection and identification of bacterial pathogens associated with BRD. The most efficient use highly multiplexed assays that can detect several pathogens in a single test and can be used on nasal swabs, bronchoalveolar lavage fluid, and lung tissues.[5,48–51] Similar approaches have been developed and implemented for viral BRD pathogens for more than a decade.[52] rtPCR for BRD pathogens has significant advantages in testing time and sensitivity; however, interpretation varies depending on the sample type and amount of potential contaminating flora, even in robustly validated assays as many of these pathogens are opportunists that can reside in the nasopharynx of normal animals (see a previous edition for a recent review of BRD clinical diagnostics and sampling).[53] Generally, PCR has been established as more sensitive at BRD pathogen detection than culture, by as much as 20% in one study.[54] Another advantage to syndromic rtPCR panel-based approaches over conventional methods is the enhanced ability to identify co-detections. In one study, co-detection of BRD pathogens were not observed when relying on culture alone but a five-fold increase in co-detections was observed using rtPCR.[5] The rtPCR also has advantages for detecting fastidious and slow growing agents like *Mycoplasma bovis*, where culture can take weeks and rtPCR is rapid and sensitive.[55] Many laboratories also include a culture-based diagnostic as part of the PCR panel to supplement recovery of isolates for downstream testing like antimicrobial susceptibility. Relating PCR results to culture and other traditional diagnostics is complex as the detection of target pathogen nucleic acid is not the same as isolation of a viable pathogen. The limitations for rtPCR-based diagnostics for BRD have been well discussed previously.[56] However, the use of Ct which is associated with relative pathogen abundance can be useful to estimate pathogen burden. For example, in BRD assays that were compared with culture, limits of detection for PCR were quite low (1.2–12 colony forming units [CFU]/mL), with Ct values ranging from ~36 to 37 for this level. Each subsequent decrease of 2 to 3 Ct corresponded to a 10-fold increase in copy number, thus providing some data for clinicians to use to support association of the relative amount of pathogen with the observed disease.[5]

Gaps still exist in objectively relating Ct values to clinical disease, especially with antemortem samples like nasal swabs. However, a recent study looked at pathogen Ct values detected in nasal swabs in relationship to BRD clinical scores to estimate clinically relevant Ct cutoffs. Although the cutoffs calculated are not directly comparable with other assays, because the method uses a pre-amplification step, it means that there are potentially clinically useful relationships between nasal swab pathogen shedding as detected by PCR and clinical BRD for some pathogens.[57]

Other applications for these tools are also being realized, such as examining the relationships between pathogen circulation and BRD outbreaks.[58,59] The rtPCR technology is also being applied to rapidly determine the presence of antimicrobial resistance genes and thus be able to assist therapy selection in near real time. One study showed high to moderate levels of agreement between isolation of resistant bacterial BRD pathogens and detection of macrolide or tetracycline resistance genes in clinical samples.[60] Third-generation sequencing tools are also being applied to look at both antimicrobial resistance and pathogen detection.[61]

Other emerging tools to rapidly identify and characterize pathogens from samples include the use of MALDI-TOF. Recent advantages allow for rapid genotyping of *M haemolytica* isolates to discern those types more likely to be associated with BRD, which is especially helpful when culturing samples like nasal swabs that may have mixed populations.[14,62] Other new methods include the use of MALDI-TOF to characterize tetracycline resistance and directly detect BRD pathogens from enriched clinical samples.[63,64]

Recent research highlights the advantages of multimodal BRD diagnostics, where PCR or molecular-based testing can be combined with classical culture-based approaches and even direct application of mass spectrometry to clinical samples to rapidly determine the presence or absence of agents associated with disease, estimate the relative pathogen burdens in complex samples, and provide some rudimentary assessment of the presence or absence of antimicrobial resistance before pathogen isolation. Given that pathogenicity within a bacterial species such as *M haemolytica* can vary at the strain level, isolates can be characterized by MALDI-TOF to determine if they are more or less likely to be a pathogen, before being subjected to MIC testing for antimicrobial resistance (AMR) phenotypes.

Infectious Bovine Keratoconjunctivitis

Like BRD, infectious bovine keratoconjunctivitis (IBK) is a disease complex, with numerous pathogens associated with the disease, each with varying degrees of support for causality. The IBK complex remains one of the most frequently reported diseases of cow–calf producers.[65] This subject was recently reviewed in a previous edition of Veterinary Clinics of North America (VCNA) dedicated to ruminant opthamology.[66,67] Typically, diagnostic testing includes detection and/or isolation of *Moraxella bovis*, *Moraxella bovoculi*, bovine herpesvirus-1 (Bo-HV1), and *Mycoplasma* spp. The use of flocked swabs in a liquid transport media is used to facilitate testing both by PCR panels and culture as many of these pathogens are intimately associated with epithelial cells.

Specific testing approaches for IBK are similar to BRD, where syndromic panels have been developed that can screen for relevant pathogens using rtPCR, and Ct values may be useful to estimate pathogen shedding.[68] Newer panels include repeats-in-toxin (RTX) gene targets for *M bovis* and *M bovoculi,* which may help support clinical relevance of detection. Culture is performed as a supplement to collect isolates for downstream testing such as autogenous vaccine manufacture and/or antimicrobial susceptibility testing.[69] However, in the authors' experience, the high rates of

detection by rtPCR of some pathogens, especially *M bovis* and *Mycoplasma bovoculi*, do not associate with high rates of isolation by culture. This may be due to fastidiousness of the organisms, contaminating flora, or the rapid penetration of some strains into the cornea following infection.[70] Culture is also beneficial because *in vitro* multidrug resistance to the approved IBK therapies (tulathromycin and oxytetracycline) has been observed in some strains of *M bovoculi,* although not frequently in *M bovis*.[45,71]

MALDI-TOF has been used to develop rapid typing tools to discern genotypes of *M bovoculi* at the subspecies level. Thus, it can rapidly discriminate between one genotype associated with IBK and another more likely to be found in normal animals.[15] MALDI-TOF-based typing tools have also been developed to rapidly screen for isolates that carry hemolytic RTXs using supplemented agar media.[72]

Infectious Abortion

Infectious abortion in cattle can be caused by pathogenic bacteria, fungi, parasites, and viruses.[73] Although diagnosis of infectious abortion should rely heavily on histopathology (see Matthew M. Hille and colleagues' article, "The Role of Histopathology in Ruminant Diagnostics," in this issue), it can benefit from supplemental diagnostics such as culture, rtPCR, syndromic rtPCR panels, and even WGS of isolates. Infectious abortions in one study accounted for 58% of abortion cases submitted to one US diagnostic laboratory, so detection and/or identification of these agents is important.[74] It is also critical to determine if the cause of abortion may provide risk to other herdmates or is the result of an opportunistic infection. The most frequently encountered bacterial pathogens include *T pyogenes*, *Bacillus* spp, *Listeria* spp, *Leptospira* spp, and enterobacteria such as *Salmonella* and *Escherichia coli.*[73,75] Other US studies have also found *Campylobacter* spp in some cases.[74] Traditionally, classical diagnostics focused on culture-based approaches; however, PCR has advantages in speed and sensitivity. New technologies including fluorescent *in situ* hybridization and 16S ribosomal deoxyribonucleic acid (rDNA) sequencing have also shown improvement in detection of infectious agents, especially for fungi.[76] Tissues available and lesion types may guide testing. For example, placenta with histological evidence of placentitis benefits from PCR, because cultures are frequently contaminated and may have false-negative or false-positive culture results.[77] Placenta seems to be infrequently submitted, potentially due to placentophagia or scavenging (one study had inclusion in only 12.5% of submissions); however, it would be important for detection of *Chlamydia* and *Coxiella burnetii* if these agents are suspected.[74,78] Other tissues are generally less susceptible to contamination, so detections and/or recovery of infectious agents with consistent lesions may be more meaningful. One study showed fetal abomasum had the greatest likelihood of pathogen detection, so this would be useful to include in submissions for both culture and PCR testing.[74]

To supplement other diagnostics, multiplexed rtPCR assays have been developed that target a broad range of agents associated with bovine infectious abortion.[79] One challenge over other disease syndromes is the agents associated with bovine abortions vary greatly with management strategies and geographical and international boundaries and includes zoonotic pathogens. Many agents, such as *Brucella* spp are infrequent in the United States due to control programs, and therefore, the need for inclusion in routine diagnostic panels is limited. Another example is the foothill abortion agents, or epizootic bovine abortion, caused by *Pajaroellobacter abortibovis*, which is predominant in cases occurring where the agent is endemic.[74,80] Other laboratories in Europe report *Neospora* and *Bacillus licheniformis* as leading causes of infectious abortions, which may not be observed with the same frequency elsewhere.[77]

One study from Switzerland detected *C burnetii* in 20% of placentomes tested.[78] Currently, author Loy's laboratory uses a multiplex rtPCR panel that contains viral, bacterial, and parasitic causes of infectious abortion to supplement histopathology. These include bovine viral diarrhea virus, Bo-HV1, *Neospora caninum*, and *Leptospira* spp.[52,81–83] Even though it is not a bacterium, *Neospora* PCR is a helpful target to include in syndromic panels, as it remains a widespread issue in numerous herds and may not always induce lesions. A review of control and diagnosis of *Neospora* can be found in the earlier VCNA editions.[84]

WGS of isolates may also be a useful tool to investigate infectious abortion. A recent study examined a large abortion outbreak caused by *Listeria monocytogenes* using WGS to compare strains isolated from clinical cases and their environment, which identified two distinct strains isolated from the abortions, with one also found in water and silage sources. The origin of other strain was not idenitifed.[85] This highlights the potential impact of WGS to help identify and eliminate sources of pathogens associated with abortion in the environment.

Enteric Infections: Calf Diarrhea

Calf diarrhea is one of the most economically significant diseases in beef and dairy cattle and is estimated to cause more than half of calf mortality on dairy farms.[86] The most frequently identified pathogens associated with calf diarrhea (calves less than 30 days) include *Cryptosporidium*, rotavirus, bovine coronavirus, *Salmonella* spp, and pathogenic *E coli.*[87] Classically, these pathogens were diagnosed with a combination of methods including culture, histopathology (at postmortem), electron microscopy, and other methods such as an enzyme-linked immunosorbent assay (ELISA).[87] However, syndromic PCR panels have been developed which enable rapid screening for many of them using multiplexed rtPCR.[48,88] Other non-PCR-based rapid tests (antigen-capture ELISA dipsticks) are also available that screen for antigens of a similar array of pathogens; however, sensitivity, especially for viral agents may be decreased.[89] Additional information on detection and diagnosis of viral agents, including those associated with diarrhea is found in another article of this edition and in reviews including pathophysiology and treatments in earlier VCNA editions.[90]

For bacterial infections, such as *E coli*, the vast majority of organisms in the intestinal tract are commensals, and only a small percentage are pathogenic. Therefore, determining the causality of isolates recovered from clinical cases often requires the determination of the presence or absence of virulence factors. *E coli* is primarily associated with two enteric diseases in cattle, one being neonatal diarrhea primarily caused by enterotoxigenic *E coli* (ETEC) and another associated with Shiga toxin-producing *E coli*/attaching and effacing *E coli*.[91] For a comprehensive review of *E coli* associated with both attaching and effacing disease and calf diarrhea please see additional chapters in previous VCNA editions.[87,92] ETEC is the primary cause of neonatal diarrhea in the first 4 days of life.[86] However, determination of the presence of virulence factors is routinely conducted on isolates from clinical cases and can include PCR panels to determine the presence or absence of both toxins and adherence factors in bacterial isolates.[93] Direct detection of the *E coli* K99/F5 gene, which encodes a fimbrial antigen involved with adhesion, is usually included in syndromic panels run on fecal samples due to overlap in age and clinical presentation with other pathogens.[88] In some of the authors' experience, ETEC K99/F5 is readily detected by rtPCR when present, with a corresponding low Ct value and heavy growth of a mucoid isolate on selective agar when cultured.

Salmonellosis in calves 2 to 6 weeks of age can cause diarrhea and severe enteric disease with variable severity.[94] In adult cattle, acute forms of disease can be

characterized by fever followed by diarrhea and abortions in pregnant animals.[94] Most clinical infections with *Salmonella* are caused by the host-adapted serotype Dublin in addition to Typhimurium.[94] However, a recent study reported more than 143 serotypes found in normal cattle, with the most frequent being Montevideo, Typhimurium, Kentucky, Meleagridis, Anatum, Cerro, Mbandaka, Muenster, Newport, and Senftenberg.[95] This indicates that multiple serotypes are circulating in cattle populations worldwide. *Salmonella* spp are routinely detectable by culture and samples from animals with fecal shedding levels of 100 CFU/gram or greater are readily detected, which is a level routinely found in clinical cases.[96] However, culture for *Salmonella* spp typically includes several enrichment and/or selection steps that take several days. Therefore, *Salmonella* is an ideal target for PCR panels and any detection should be considered significant. In the VDL of author Loy, laboratory culture testing is routinely performed on any nonnegative *Salmonella* PCR test from feces or tissues, and positive culture results are typically yielded in cases where Ct values are $\leq$ 35. Isolation of *Salmonella* following a culture-independent diagnostic test like PCR is an important reflex test to perform, as strains vary significantly in virulence depending on serotype and antimicrobial susceptibility testing may be requested. One diagnostic challenge is *Salmonella* Dublin, which can be shed at low levels and/or is intermittently shed and does not grow well on routine selective media. Thus, sensitivity in subclinical shedding animals is estimated to be 20%.[97] New methods of detection of very low levels of *Salmonella* are useful for environmental and testing of subclinical shedders that combine enrichment followed by PCR.[97] Serological tests using an indirect ELISA are available for *Salmonella* Dublin and may be helpful to determine previous or current infection or herd status.[98] Recent reviews of *Salmonella* in dairy cattle and calves can be found in previous VCNA editions.[99,100]

Enteric Infections: Johne's Disease

Johne's disease, caused by *Mycobacterium avium* ssp *paratuberculosis* (MAP), is an economically significant and widespread disease of ruminants worldwide. An excellent review of MAP diagnostics is provided in a previous VCNA edition, which states "There is a suitable diagnostic test for virtually every paratuberculosis need."[101] The gold standard diagnostic test is fecal culture; however, this is time-consuming, laborious, and very few VDLs currently offer it due to cost and time requirements. Most laboratories rely on rtPCR (typically insertion sequence 900) for MAP diagnostics in addition to serological testing for MAP antibodies. New testing methods using mycobacteriophage D29 have been developed that may enable enhanced diagnostic testing, as they only infect and lyse viable MAP cells; however, they need further validation to become commercially viable.[102] Consensus recommendations on MAP diagnostics have been developed by experts based on different animal production purposes, species, and systems.[103] Tests and test recommendations are frequently designed with control programs in mind and are extremely useful for determining herd-level status and risk. In addition to control programs, frequently diagnosticians and veterinarians are tasked with applying MAP diagnostics to individual valuable or seedstock animals, where the recommended test is typically fecal PCR. Interpretation of PCR results for MAP is usually straightforward, as the specificity of the test is greater than 95%, thus animals that are PCR positive are likely infected.[103,104] However, this interpretation must be evaluated at the population level, for example, as tested sample size increases so does the risk of a detection being a false-positive result. Also, due to the analytical sensitivity of the test, most rtPCR assays classify animals with Ct values greater than 36 to 37 as suspect or inconclusive, as the higher Ct values do not correlate well with gold standard fecal culture results. This is especially

true in MAP-infected herds where there is commingling with heavy shedding animals, which can increase the likelihood of ingested organisms from the environment or cross-contamination during sampling. This may cause false-positive testing results, especially for those individuals in the high Ct suspect range. The authors typically recommend resampling and retesting individuals that test in the suspect range after their removal from exposure to or commingling with heavy shedders. These individuals could also be cultured for MAP to determine disease status in high value animals. Interpretation of negative or not detected results also depends on the herd-level status (prevalence) and risk status, as the clinical sensitivity of the assay is ~60%, because animals only shed MAP intermittently in the early stages of disease.[104] Generally, MAP diagnostics and testing programs heavily depend on multiple factors and vary greatly on producer goals, production systems, and herd prevalence, see Sébastien Buczinski and colleagues' article, "Interpretation and Analysis of Individual Diagnostic Tests and Performance," in this issue of Veterinary Clinics for additional information.

Anaerobic Infections

Anaerobic infections in ruminants tend to fall into two major categories: enteric and tissue infections with toxin producing members of the genus *Clostridium* and soft tissue infections caused or associated with Gram-negative, nonspore forming organisms, by members of the genera *Fusobacterium, Bacteroides*, *Dichelobacter*, *Porphyromona*s, and *Prevotella*.[105,106] The most significant of the Gram-negative pathogens is *Fusobacterium necrophorum*, which is associated with bovine foot rot and liver abscesses, whereas *Dichelobacter nodosus* is associated with ovine foot rot. Classically, anaerobes provided a significant diagnostic challenge, as they are highly susceptible to oxygen and require specialized sampling, transport, handling, and culture and isolation procedures. In addition, identification based on biochemical testing can be challenging, especially to establish species-level identification of veterinary pathogens. However, robust databases of anaerobic pathogens have been developed for MALDI-TOF, which have proven extremely accurate for identification.[107,108] These advances have greatly improved identification of anaerobes, especially the Gram-negative pathogens described above. If infection with one of these pathogens is suspected, please contact your VDL for specific transport, collection, and submission instructions.

Clostridium perfringens is the primary pathogenic species that causes enterotoxic infections in ruminants, which includes enteritis and abomasitis. Several recent clinical reviews of these diseases in ruminants are available.[109,110] *C perfringens* is classified based on the presence or absence of different toxins genes (Type A–Type G), which make identification alone insufficient for diagnosis. This typing scheme has recently been expanded to reflect two new toxin types, F and G.[111] In VDLs, after isolation and identification of *C perfringens*, isolates are usually subjected to a multiplex toxin typing PCR assay to determine the presence or absence of toxin genes.[111] This is important as *C perfringens* can be found in normal, healthy animals, and isolation from fecal samples may not be clinically relevant. Findings should be interpreted in the context of histopathology and other clinical findings, especially for *C perfringens* Type A.

Clostridium is also responsible for numerous histotoxic and neurotoxic diseases. Histotoxic diseases include clostridial myositis (blackleg) caused by *Clostridium chauvoei* and gas gangrene usually caused by *Clostridium septicum* but also *Clostridium novyi, Clostridium sordellii,* and *C perfringens.*[112] Diagnosis and detection of these agents typically involves identification of organisms associated with lesions. In VDLs, this is performed by fluorescent antibody testing of fixed tissues or tissue

smears from affected areas or immunohistochemistry staining of tissue sections.[113,114] Culture and identification of these organisms may be helpful, but interpretation is challenging as they may represent postmortem growth. Neurotoxic diseases associated with *Clostridium* include botulism (*C botulinum*) and tetanus (*C tetani*). Diagnosis of neurotoxic *Clostridium* is usually based on clinical findings and exclusion of other causes; however, culture and identification of the pathogens in lesions may be supportive of the diagnosis. In addition, for botulism, PCR testing for toxins is available for use on clinical samples.[112,115] Methods for detection of preformed botulinum toxin using MALDI-TOF have been developed and used on rumen contents; however, these tests may not be as widely available.[116]

Mastitis

Mastitis, defined as inflammation of the mammary gland, is the most common bacterial disease of adult dairy cows. Mastitis also affects prepubertal and gestational dairy heifers, beef cattle, goats, and sheep. Although usually a disease of females, occasional cases of mastitis are seen in male animals. Mastitis is diagnosed based either on overt clinical signs of inflammation, for example, changes in the appearance of the milk, redness, heat, pain and swelling of the mammary gland, and/or signs of systemic illness, or, for subclinical disease, detection of inflammatory cells (somatic cell count [SCC]) in milk. Mastitis is most frequently caused by a bacterial intramammary infection (IMI). Generally speaking, mastitis-causing bacterial pathogens are grouped into categories based on their clinical behavior (clinical vs subclinical) and/or mode of acquisition. With regard to the latter, pathogens have been classically grouped as contagious, those that spread from animal-to-animal usually during the milking process, and environmental, those that are acquired from the animal's environment between milkings. Among the ruminants commonly encountered in clinical practice, the prevalent bacterial pathogens that cause IMI are generally similar with a few exceptions.

The most commonly isolated bacteria from ruminant milk are the staphylococci, with the so-called non-aureus staphylococci (NAS) being most prevalent. Among the NAS, *Staphylococcus chromogenes* tends to predominate.[117] Some of the NAS species tend to be primarily associated with mammary sources, whereas others tend to be associated with extra-mammary sites, for example, the animal's environment.[118] Common contagious pathogens include *Staphylococcus aureus*, *Streptococcus agalactiae*, *Mycoplasma* spp, *Corynebacterium bovis*, and *T pyogenes*. Common environmental pathogens include Gram-negative bacteria including fecal coliforms and Gram-positive bacteria including streptococcal and streptococcal-like organisms other than *S agalactiae,* among which *Streptococcus uberis* and *Streptococcus dysgalactiae* are most frequent. Sheep and goats may also get abscesses in their udder caused by *Corynebacterium pseudotuberculosis*, the causative agent of caseous lymphadenitis, and can have alterations in milk SCC associated with small ruminant lentiviruses.[119]

Diagnosis of the etiology of an IMI is performed to select appropriate treatment protocols and/or initiate preventive measures such as milking time hygiene improvements for contagious bacteria or improvements in bedding management for environmental pathogens. Hence, determining broad groups of pathogens present in milk may be sufficient to implement changes in management or initiate treatment. For example, in the case of clinical mastitis, intramammary treatment is usually limited to Gram-positive infections, whereas diagnosis of subclinical IMI during lactation is usually performed to help guide prevention measures rather than guide treatment as subclinical mastitis is not usually treated during lactation. Currently, bacterial culture and PCR are the most commonly used methods to diagnose IMI.

Bacterial culture of milk is relatively simple to perform and inexpensive.[120] Many mastitis pathogens grow under aerobic conditions on blood agar-based media. There are exceptions, such as *Mycoplasma* spp, which usually require specific growth media and conditions. False-negative results can thus occur when the cow has an IMI with a pathogen that does not grow on routine screening media under aerobic conditions. Although most culture techniques are easily performed, consistent and standardized protocols coupled with established definitions for what constitutes an IMI are necessary to establish a diagnosis. Accurate diagnosis begins with aseptic sample collection.[120] Briefly, teats should be clean and dry and scrubbed with 70% isopropyl alcohol before sample collection. Teats are then stripped of a few streams of milk to reduce the potential for streak canal contaminants in the sample before collecting milk in a sterile vial while wearing disposable gloves. Following collection, samples should be chilled or frozen for transportation to the laboratory. In the laboratory, any frozen samples should be thawed before culture. Using aseptic techniques, a known volume of milk is plated onto culture media and incubated under conditions and times suitable for growth of the potential pathogen(s) of interest.[120] Standardized CFU/mL thresholds are used to define an IMI with a particular pathogen as well as define when a sample is considered contaminated.[121] Generally, a sample is considered contaminated when greater than 2 different colony types are identified in any given sample. Sampling handling is as an important factor to consider when interpreting culture results. Although freezing samples for transportation and/or storage can help reduce bacterial overgrowth in the sample, the impact of storage and freezing on culture results can vary. Some organisms do not always survive freezing or storage for extended periods of time; this includes *E coli*, *T pyogenes*, *Nocardia* spp, and *Mycoplasma* spp. On the other hand, long-term storage does not generally affect the viability of Gram-positive cocci and freezing of milk samples can increase the likelihood of detecting some staphylococcal species and *S aureus*.[122]

Although conventional culture methods are still frequently used to diagnose an IMI, molecular techniques have also become commonplace. The two most common molecular techniques used to diagnose IMI are MALDI-TOF and PCR. Although PCR can be used as a culture-independent method to detect DNA from mastitis-causing organisms directly from the milk sample, MALDI-TOF still requires bacterial culture to isolate the organism of interest. Hence, MALDI-TOF, in its current form, is used to identify isolated organisms to the genus and/or species level. In many laboratories, MALDI-TOF has thus largely replaced traditional biochemistry-based phenotypic methods for identifying bacteria. The method has been validated against traditional phenotypic methods and genotypic methods for bacterial identification and has been found to correctly classify genus and species most of the time, whereas misclassification errors can occur with traditional phenotypic speciation methods for some mastitis pathogens, for example, some species of staphylococci.[123,124] One limitation of MALDI-TOF is that the database used to identify the organism based on its mass spectra must contain the organism of interest. Hence, many mastitis laboratories have developed custom libraries of spectra for mastitis pathogens that are used in concert with the manufacturer's library of organisms to make a diagnosis.[123]

The use of commercially available quantitative rtPCR-based tests for detecting bacterial DNA in milk samples has become quite common in some regions of the world. Advantages of PCR-based techniques include faster turnaround time, user-independent identification of bacteria, and ability to identify organisms that are difficult to culture in a timely fashion, for example, *Mycoplasma* spp. Further, commercially available PCR assays can be directly applied to milk samples containing preservatives, allowing storage and shipment at room temperature and obviating the need

for chilled or frozen storage and shipping conditions required for samples undergoing conventional culture. Much like CFU/mL thresholds are used to define an IMI with conventional culture, diagnosis of an IMI with rtPCR is based on the Ct-value needed to detect DNA from a suspected pathogen. With commercial PCR-based tests, the manufacturer determines the Ct-value linked to a diagnosis. Also, like the culture methods, detection of DNA from greater than 2 bacterial genera and/or species indicates contamination. Because PCR detects bacterial DNA, it is possible to detect DNA from organisms that are no longer viable and thus no longer causing an infection. One study comparing conventional culture to PCR longitudinally throughout the course of an infection showed that milk samples remain positive by PCR after conventional cultures have become negative, which could lead to overtreatment of cases where the cow has resolved the infection.[125] Another limitation of PCR-based tests is that the commercial test kit must include PCR primers to detect the organism of interest. Hence, false-negative results can occur when the cow has an infection with an organism for which primers are not included in the test kit.

Although not applied in the routine diagnosis of IMI, a number of molecular methods can be applied to strain-type specific organisms to better understand the epidemiology of mastitis pathogen(s) on farms, for example, to understand contagiousness or better understand reservoir(s) of infection. These methods include, among others, WGS, RAPD-PCR, and pulse-field gel electrophoresis. Metagenomic approaches have also been applied in an effort to understand the milk microbiome.[126] Our understanding of the latter is still in its infancy.

A more comprehensive overview of mastitis diagnostics can be found in the November 2018 edition of VCNA, Food Animal Practice.[127]

SUMMARY

Emerging diagnostic approaches such as rtPCR, MALDI-TOF, and gene sequencing have enhanced diagnostic testing for a variety of infectious diseases in ruminants. rtPCR has enhanced sensitivity, reduced testing time, and minimizes the need for culture. However, interpretation of result requires additional considerations. For MALDI-TOF and WGS, isolation of the pathogen in pure culture is still required. Sequence-based approaches applied directly to clinical samples hold promise for diagnostic testing for these pathogens, but advances in technology, reduction in cost, and ease of analysis need to be comparable with existing methods to be practical.

CLINICS CARE POINTS

- Real-time polymerase chain reaction (PCR) has had a tremendous impact on the ability of VDLs to rapidly detect pathogens in diagnostic samples.
- Multiplexed real-time PCR (rtPCR) syndromic panels have the ability to screen for numerous pathogens simultaneously.
- Interpretation of rtPCR results varies by the clinical disease, pathogen, and sample type. Results may not always directly correlate to culture results or clinical disease, especially with opportunistic pathogens in sites with normal flora.
- Genomic sequencing continues to enhance the ability to identify and characterize bacterial pathogens. When combined with additional technologies such as Matrix -assisted laser desorption ionization–time of flight (MALDI-TOF) and rtPCR, genomic information can be rapidly translated into diagnostic results.

- MALDI-TOF has revolutionized veterinary microbiology and has provided a rapid means to identify most pathogenic bacteria.
- New applications of MALDI-TOF hold promise for strain typing, rapid AMR detection, and use directly on clinical samples.
- Classic culture-based approaches for pathogen isolation continue to be vitally important for bacterial diagnostics, which enable reflex testing like antimicrobial susceptibility, MALDI-TOF typing, and whole -genome sequencing.

STATEMENT

The use of product and company names is necessary to accurately report the methods and results; however, the US Department of Agriculture (USDA) neither guarantees nor warrants the standard of the products, and the use of names by the USDA implies no approval of the product to the exclusion of others that may also be suitable. The USDA is an equal opportunity employer.

DISCLOSURE

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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 Quantitative Biol 1986;51:263–73.
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(6):815–25.
3. Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009;55(4):611–22.
4. Gunson RN, Bennett S, Maclean A, et al. Using multiplex real time PCR in order to streamline a routine diagnostic service. J Clin Virol 2008;43(4):372–5.
5. 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(6):837–47.
6. Hoffmann B, Depner K, Schirrmeier H, et al. A universal heterologous internal control system for duplex real-time RT-PCR assays used in a detection system for pestiviruses. J Virol Methods 2006;136(1–2):200–9.
7. Randall LP, Lemma F, Koylass M, et al. Evaluation of MALDI-ToF as a method for the identification of bacteria in the veterinary diagnostic laboratory. Res Vet Sci 2015;101:42–9.
8. Wragg P, Randall L, Whatmore AM. Comparison of Biolog GEN III MicroStation semi-automated bacterial identification system with matrix-assisted laser desorption ionization-time of flight mass spectrometry and 16S ribosomal RNA gene sequencing for the identification of bacteria of veterinary interest. J Microbiol Methods 2014;105:16–21.

9. 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(12):3845–52.
10. Wilson David J, Middleton John R, Adkins Pamela RF, et al. Test agreement among biochemical methods, matrix-assisted laser desorption ionization–time of flight mass spectrometry, and 16S rRNA sequencing for identification of microorganisms isolated from bovine milk. J Clin Microbiol 2019;57(3):013811-18.
11. Robbins K, Dickey AM, Clawson ML, et al. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry identification of Moraxella bovoculi and Moraxella bovis isolates from cattle. J Vet Diagn Invest 2018;30(5):739–42.
12. Kuhnert P, Bisgaard M, Korczak BM, et al. Identification of animal Pasteurellaceae by MALDI-TOF mass spectrometry. J Microbiol Methods 2012;89(1):1–7.
13. Spergser J, Hess C, Loncaric I, et al. Matrix-Assisted laser desorption ionization–time of flight mass spectrometry is a superior diagnostic tool for the identification and differentiation of mycoplasmas isolated from animals. J Clin Microbiol 2019;57(9):e00316–9.
14. Loy JD, Clawson ML. Rapid typing of Mannheimia haemolytica major genotypes 1 and 2 using MALDI-TOF mass spectrometry. J Microbiol Methods 2017;136:30–3.
15. Hille M, Dickey A, Robbins K, et al. Rapid differentiation of Moraxella bovoculi genotypes 1 and 2 using MALDI-TOF mass spectrometry profiles. J Microbiol Methods 2020;173:105942.
16. Mani RJ, Thachil AJ, Ramachandran A. Discrimination of Streptococcus equi subsp. equi and Streptococcus equi subsp. zooepidemicus using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J Vet Diagn Invest 2017;29(5):622–7.
17. 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(4):e61428.
18. Bobay LM, Ochman H. The evolution of bacterial genome architecture. Front Genet 2017;8:72.
19. Pfeifer E, de Sousa JAM, Touchon M, et al. Bacteria have numerous distinctive groups of phage-plasmids with conserved phage and variable plasmid gene repertoires. Nucleic Acids Res 2021;49(5):2655–73.
20. Andreani NA, Hesse E, Vos M. Prokaryote genome fluidity is dependent on effective population size. ISME J 2017;11(7):1719–21.
21. Partridge SR, Kwong SM, Firth N, et al. Mobile genetic elements associated with antimicrobial resistance. Clin Microbiol Rev 2018;31(4). https://doi.org/10.1128/CMR.00088-17.
22. Siguier P, Gourbeyre E, Chandler M. Bacterial insertion sequences: their genomic impact and diversity. FEMS Microbiol Rev 2014;38(5):865–91.
23. Mazel D. Integrons: agents of bacterial evolution. Nat Rev Microbiol 2006;4(8):608–20.
24. Zhou K, Aertsen A, Michiels CW. The role of variable DNA tandem repeats in bacterial adaptation. FEMS Microbiol Rev 2014;38(1):119–41.
25. Chen J, Leblanc DJ, Galli DM. DNA inversion on conjugative plasmid pVT745. J Bacteriol 2002;184(21):5926–34.
26. Merrikh CN, Merrikh H. Gene inversion potentiates bacterial evolvability and virulence. Nat Commun 2018;9(1):4662.
27. Ramiro RS, Durao P, Bank C, et al. Low mutational load and high mutation rate variation in gut commensal bacteria. Plos Biol 2020;18(3):e3000617.

28. Weinroth MD, Clawson ML, Arthur TM, et al. Rates of evolutionary change of resident Escherichia coli O157:H7 differ within the same ecological niche. BMC Genomics 2022;23(1):275.
29. Ehrlich GD, Hiller NL, Hu FZ. What makes pathogens pathogenic. Genome Biol 2008;9(6):225.
30. Hu T, Chitnis N, Monos D, et al. Next-generation sequencing technologies: an overview. Hum Immunol 2021. https://doi.org/10.1016/j.humimm.2021.02.012.
31. Slatko BE, Gardner AF, Ausubel FM. Overview of Next-Generation Sequencing Technologies. Curr Protoc Mol Biol 2018;122(1):e59.
32. Phillippy AM, Schatz MC, Pop M. Genome assembly forensics: finding the elusive mis-assembly. Genome Biol 2008;9(3):R55.
33. Koren S, Harhay GP, Smith TP, et al. Reducing assembly complexity of microbial genomes with single-molecule sequencing. Genome Biol 2013;14(9):R101.
34. Clawson ML, Schuller G, Dickey AM, et al. Differences between predicted outer membrane proteins of genotype 1 and 2 Mannheimia haemolytica. BMC Microbiol 2020;20(1):250.
35. Bickhart DM, Kolmogorov M, Tseng E, et al. Generating lineage-resolved, complete metagenome-assembled genomes from complex microbial communities. Nat Biotechnol 2022. https://doi.org/10.1038/s41587-021-01130-z.
36. Franco-Duarte R, Cernakova L, Kadam S, et al. Advances in chemical and biological methods to identify microorganisms-from past to present. Microorganisms 2019;7(5). https://doi.org/10.3390/microorganisms7050130.
37. Kamble A, Sawant S, Singh H. 16S ribosomal RNA gene-based metagenomics: a review. Biomed Res J 2020;7:5–11.
38. Yang B, Wang Y, Qian PY. Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. BMC Bioinformatics 2016;17:135.
39. Glaeser SP, Kampfer P. Multilocus sequence analysis (MLSA) in prokaryotic taxonomy. Syst Appl Microbiol 2015;38(4):237–45.
40. Sant'Anna FH, Bach E, Porto RZ, et al. Genomic metrics made easy: what to do and where to go in the new era of bacterial taxonomy. Crit Rev Microbiol 2019;45(2):182–200.
41. Yoon SH, Ha SM, Lim J, et al. A large-scale evaluation of algorithms to calculate average nucleotide identity. Antonie Van Leeuwenhoek 2017;110(10):1281–6.
42. Parks DH, Chuvochina M, Chaumeil PA, et al. A complete domain-to-species taxonomy for Bacteria and Archaea. Nat Biotechnol 2020;38(9):1079–86.
43. Richter M, Rossello-Mora R, Oliver Glockner F, et al. JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. Bioinformatics 2016;32(6):929–31.
44. Ciufo S, Kannan S, Sharma S, et al. Using average nucleotide identity to improve taxonomic assignments in prokaryotic genomes at the NCBI. Int J Syst Evol Microbiol 2018;68(7):2386–92.
45. Dickey AM, Loy JD, Bono JL, et al. Large genomic differences between Moraxella bovoculi isolates acquired from the eyes of cattle with infectious bovine keratoconjunctivitis versus the deep nasopharynx of asymptomatic cattle. Vet Res 2016;47:31.
46. Wynn EL, Hille MM, Loy JD, et al. Whole genome sequencing of diverse Moraxella bovis strains reveals two genotypes with different genetic determinants. BMC Microbiol 2022;22:258. https://doi.org/10.1186/s12866-022-02670-3.
47. Griffin D, Chengappa MM, Kuszak J, et al. Bacterial pathogens of the bovine respiratory disease complex. Vet Clin North Am Food Anim Pract 2010;26(2):381–94.

48. Goecke NB, Nielsen BH, Petersen MB, et al. Design of a high-throughput real-time PCR system for detection of bovine respiratory and enteric pathogens. original research. Front Vet Sci 2021. https://doi.org/10.3389/fvets.2021.677993.
49. Pansri P, Katholm J, Krogh KM, et al. Evaluation of novel multiplex qPCR assays for diagnosis of pathogens associated with the bovine respiratory disease complex. Vet J 2020;256:105425.
50. Wisselink HJ, Cornelissen JBWJ, van der Wal FJ, et al. Evaluation of a multiplex real-time PCR for detection of four bacterial agents commonly associated with bovine respiratory disease in bronchoalveolar lavage fluid. BMC Vet Res 2017;13(1):221.
51. Goto Y, Yaegashi G, Fukunari K, et al. Design of a multiplex quantitative reverse transcription-PCR system to simultaneously detect 16 pathogens associated with bovine respiratory and enteric diseases. J Appl Microbiol 2020;129(4):832–47.
52. Horwood PF, Mahony TJ. Multiplex real-time RT-PCR detection of three viruses associated with the bovine respiratory disease complex. J Virol Methods 2011;171(2):360–3.
53. Buczinski S, Pardon B. Bovine respiratory disease diagnosis: what progress has been made in clinical diagnosis? Vet Clin North Am Food Anim Pract 2020;36(2):399–423.
54. Bell CJ, Blackburn P, Elliott M, et al. Investigation of polymerase chain reaction assays to improve detection of bacterial involvement in bovine respiratory disease. J Vet Diagn Invest 2014;26(5):631–4.
55. Sachse K, Salam HSH, Diller R, et al. Use of a novel real-time PCR technique to monitor and quantitate Mycoplasma bovis infection in cattle herds with mastitis and respiratory disease. Vet J 2010;186(3):299–303.
56. Fulton RW, Confer AW. Laboratory test descriptions for bovine respiratory disease diagnosis and their strengths and weaknesses: gold standards for diagnosis, do they exist? Can Vet J 2012;53(7):754–61.
57. Klompmaker AF, Brydensholt M, Michelsen AM, et al. Estimating clinically relevant cut-off values for a high-throughput quantitative real-time PCR detecting bacterial respiratory pathogens in cattle. Brief research report. Front Vet Sci 2021;8:674771. https://doi.org/10.3389/fvets.2021.674771.
58. Workman AM, Kuehn LA, McDaneld TG, et al. Longitudinal study of humoral immunity to bovine coronavirus, virus shedding, and treatment for bovine respiratory disease in pre-weaned beef calves. BMC Vet Res 2019;15(1):161.
59. Workman AM, Kuehn LA, McDaneld TG, et al. 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. Am J Vet Res 2017;78(9):1065–76.
60. 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(1). https://doi.org/10.3390/pathogens10010064.
61. 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(1):211.
62. 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 Genomics 2016;17(1):982.
63. Van Driessche L, Bokma J, Deprez P, et al. Rapid identification of respiratory bacterial pathogens from bronchoalveolar lavage fluid in cattle by MALDI-TOF MS. Scientific Rep 2019;9(1):18381.
64. Van Driessche L, Bokma J, Gille L, et al. Rapid detection of tetracycline resistance in bovine Pasteurella multocida isolates by MALDI Biotyper antibiotic susceptibility test rapid assay (MBT-ASTRA). Scientific Rep 2018;8(1). https://doi.org/10.1038/s41598-018-31562-8.
65. Martin MS, Grau SA, Rutherford BW, et al. Survey of cow-calf producer perspectives on management strategies and industry challenges. Part 1: handling practices, and health and industry challenges. Translational Anim Sci 2018;3(1):195–203.
66. Loy JD, Hille M, Maier G, et al. Component causes of infectious bovine keratoconjunctivitis - the role of moraxella species in the epidemiology of infectious bovine keratoconjunctivitis. Vet Clin North Am Food Anim Pract 2021/07/01/2021;37(2):279–93.
67. Loy JD, Clothier KA, Maier G. Component causes of infectious bovine keratoconjunctivitis—non-moraxella organisms in the epidemiology of infectious bovine keratoconjunctivitis. Vet Clin North Am Food Anim Pract 2021;37(2):295–308.
68. 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.
69. Schmellik-Sandage CS, Hill RE Jr. Regulation of autogenous biologicals in the United States. Dev biologicals 2004;117:9–13.
70. Rogers DG, Cheville NF, Pugh GW Jr. Pathogenesis of corneal lesions caused by Moraxella bovis in gnotobiotic calves. Vet Pathol 1987;24(4):287–95.
71. Loy JD, Brodersen BW. Moraxella spp. isolated from field outbreaks of infectious bovine keratoconjunctivitis: a retrospective study of case submissions from 2010 to 2013. J Vet Diagn Invest 2014;26(6):761–8.
72. Hille MM, Clawson ML, Dickey AM, et al. MALDI-TOF MS Biomarker Detection Models to Distinguish RTX toxin phenotypes of moraxella bovoculi strains are enhanced using calcium chloride supplemented agar. Original Research. Front Cell Infect Microbiol 2021;11(202). https://doi.org/10.3389/fcimb.2021.632647.
73. Mee JF. Investigation of bovine abortion and stillbirth/perinatal mortality - similar diagnostic challenges, different approaches. Irish Vet J 2020;73(1). https://doi.org/10.1186/s13620-020-00172-0.
74. Clothier K, Anderson M. Evaluation of bovine abortion cases and tissue suitability for identification of infectious agents in California diagnostic laboratory cases from 2007 to 2012. Theriogenology 2016;85(5):933–8.
75. Kirkbride CA. Bacterial agents detected in a lo-year study of bovine abortions and stillbirths. J Vet Diagn Invest 1993;5(1):64–8.
76. Wolf-Jäckel GA, Strube ML, Schou KK, et al. Bovine Abortions Revisited—Enhancing Abortion Diagnostics by 16S rDNA Amplicon Sequencing and Fluorescence in situ Hybridization. Original Research. Front Vet Sci 2021. https://doi.org/10.3389/fvets.2021.623666.
77. Cabell E. Bovine abortion: aetiology and investigations. Practice 2007;29(8):455–63.
78. Botta C, Pellegrini G, Hässig M, et al. Bovine fetal placenta during pregnancy and the postpartum period. Vet Pathol 2018;56(2):248–58.

79. Tramuta C, Lacerenza D, Zoppi S, et al. Development of a set of multiplex standard polymerase chain reaction assays for the identification of infectious agents from aborted bovine clinical samples. J Vet Diagn Invest 2011;23(4):657–64.
80. Brooks RS, Blanchard MT, Clothier KA, et al. Characterization of Pajaroellobacter abortibovis, the etiologic agent of epizootic bovine abortion. Vet Microbiol 2016;192:73–80.
81. Pereira GR, Vogel FSF, Bohrer RC, et al. Neospora caninum DNA detection by TaqMan real-time PCR assay in experimentally infected pregnant heifers. Vet Parasitol 2014;199(3):129–35.
82. Stoddard RA, Gee JE, Wilkins PP, et al. Detection of pathogenic Leptospira spp. through TaqMan polymerase chain reaction targeting the LipL32 gene. Diagn Microbiol Infect Dis 2009;64(3):247–55.
83. Mahlum CE, Haugerud S, Shivers JL, et al. Detection of bovine viral diarrhea virus by TaqMan® reverse transcription polymerase chain reaction. J Vet Diagn Invest 2002;14(2):120–5.
84. McAllister MM. Diagnosis and control of bovine neosporosis. Vet Clin North Am Food Anim Pract 2016/07/01/2016;32(2):443–63.
85. Whitman KJ, Bono JL, Clawson ML, et al. Genomic-based identification of environmental and clinical Listeria monocytogenes strains associated with an abortion outbreak in beef heifers. BMC Vet Res 2020;16(1):70.
86. Foster DM, Smith GW. Pathophysiology of diarrhea in calves. Vet Clin North Am Food Anim Pract 2009;25(1):13–36.
87. Blanchard PC. Diagnostics of dairy and beef cattle diarrhea. Vet Clin North Am Food Anim Pract 2012;28(3):443–64.
88. 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(4):509–17.
89. Cho Y-I, Sun D, Cooper V, et al. Evaluation of a commercial rapid test kit for detecting bovine enteric pathogens in feces. J Vet Diagn Invest 2012;24(3):559–62.
90. Heller MC, Chigerwe M. Diagnosis and treatment of infectious enteritis in neonatal and juvenile ruminants. Vet Clin North Am Food Anim Pract 2018;34(1):101–17.
91. Gyles CL, Fairbrother JM. Escherichia coli. Pathogenesis Bacterial Infections Anim 2010;267–308. https://doi.org/10.1002/9780470958209.ch15. Wiley Online Books.
92. Moxley RA, Smith DR. Attaching-effacing escherichia coli infections in cattle. Vet Clin North Am Food Anim Pract 2010;26(1):29–56.
93. Franck SM, Bosworth BT, Moon HW. Multiplex PCR for Enterotoxigenic, Attaching and Effacing, and Shiga Toxin-Producing Escherichia coli Strains from Calves. J Clin Microbiol 1998;36(6):1795–7.
94. Barrow PA, Jones MA, Thomson N. Salmonella. Pathogenesis Bacterial Infections Anim 2010;231–65. https://doi.org/10.1002/9780470958209.ch14. Wiley Online Books.
95. Gutema FD, Agga GE, Abdi RD, et al. Prevalence and serotype diversity of salmonella in apparently healthy cattle: systematic review and meta-analysis of published studies, 2000–2017. Systematic Review. Front Vet Sci 2019;6:102. https://doi.org/10.3389/fvets.2019.00102.
96. Nielsen LR. Review of pathogenesis and diagnostic methods of immediate relevance for epidemiology and control of Salmonella Dublin in cattle. Vet Microbiol 2013;162(1):1–9.

97. Goodman LB, McDonough PL, Anderson RR, et al. Detection of Salmonella spp. in veterinary samples by combining selective enrichment and real-time PCR. J Vet Diagn Invest 2017;29(6):844–51.
98. Nielsen LR, Ersbøll AK. Age-stratified validation of an indirect salmonella dublin serum enzyme-linked immunosorbent assay for individual diagnosis in cattle. J Vet Diagn Invest 2004;16(3):212–8.
99. Mohler VL, Izzo MM, House JK. Salmonella in calves. Vet Clin North Am Food Anim Pract 2009;25(1):37–54.
100. Holschbach CL, Peek SF. Salmonella in dairy cattle. Vet Clin North Am Food Anim Pract 2018;34(1):133–54.
101. Collins MT. Diagnosis of paratuberculosis. Vet Clin North Am Food Anim Pract 2011;27(3):581–91.
102. Grant IR. Bacteriophage-Based Methods for Detection of Viable Mycobacterium avium subsp. paratuberculosis and Their Potential for Diagnosis of Johne's Disease. Mini Review. Front Vet Sci 2021;8:632498. https://doi.org/10.3389/fvets.2021.632498.
103. Collins MT, Gardner IA, Garry FB, et al. Consensus recommendations on diagnostic testing for the detection of paratuberculosis in cattle in the United States. J Am Vet Med Assoc 2006;229(12):1912–9.
104. Alinovi CA, Ward MP, Lin TL, et al. Real-time PCR, compared to liquid and solid culture media and ELISA, for the detection of Mycobacterium avium ssp. paratuberculosis. Vet Microbiol 2009;136(1–2):177–9.
105. Nagaraja TG. Gram-negative, non-spore-forming anaerobes. Vet Microbiol 2022;294–308.
106. Gohari IM, Prescott JF. Clostridium. Vet Microbiol 2022;309–34.
107. Alcalá L, Marín M, Ruiz A, et al. Identifying Anaerobic Bacteria Using MALDI-TOF mass spectrometry: a four-year experience. Original Research. Front Cell Infect Microbiol 2021;11:521014. https://doi.org/10.3389/fcimb.2021.521014.
108. Justesen Ulrik S, Holm A, Knudsen E, et al. Species identification of clinical isolates of anaerobic bacteria: a comparison of two matrix-assisted laser desorption ionization–time of flight mass spectrometry systems. J Clin Microbiol 2011;49(12):4314–8.
109. Simpson KM, Callan RJ, Van Metre DC. Clostridial abomasitis and enteritis in ruminants. Vet Clin North Am Food Anim Pract 2018;34(1):155–84.
110. Otter A, Uzal FA. Clostridial diseases in farm animals: 1. Enterotoxaemias and other alimentary tract infections. Practice 2020;42(4):219–32. https://doi.org/10.1136/inp.m1462.
111. Rood JI, Adams V, Lacey J, et al. Expansion of the Clostridium perfringens toxin-based typing scheme. Anaerobe 2018;53:5–10.
112. Otter A, Uzal FA. Clostridial diseases in farm animals: 2. Histotoxic and neurotoxic diseases. Practice 2020;42(5):279–88, m1984.
113. Uzal FA, Paramidani M, Assis R, et al. Outbreak of clostridial myocarditis in calves. Vet Rec 2003;152(5):134–6.
114. Sterne M, Batty I. Pathogenic clostridia. London WC2B 6AB.: Butterworth & Co.(Publishers) Ltd, 88 Kingsway; 1975.
115. De Medici D, Anniballi F, Wyatt Gary M, et al. Multiplex PCR for Detection of Botulinum Neurotoxin-Producing Clostridia in Clinical, Food, and Environmental Samples. Appl Environ Microbiol 2009;75(20):6457–61.
116. Frye EA, Egan C, Perry MJ, et al. Outbreak of botulism type A in dairy cows detected by MALDI-TOF mass spectrometry. J Vet Diagn Invest 2020;32(5):722–6.

117. Fry PR, Middleton JR, Dufour S, et al. Association of coagulase-negative staphylococcal species, mammary quarter milk somatic cell count, and persistence of intramammary infection in dairy cattle. J Dairy Sci 2014;97(8):4876–85.
118. De Visscher A, Supre K, Haesebrouck F, et al. Further evidence for the existence of environmental and host-associated species of coagulase-negative staphylococci in dairy cattle. Vet Microbiol 2014;172(3–4):466–74.
119. Sanchez A, Contreras A, Corrales JC, et al. Relationships between infection with caprine arthritis encephalitis virus, intramammary bacterial infection and somatic cell counts in dairy goats. Vet Rec 2001;148(23):711–4.
120. Middleton JR, Fox LK, Pighetti G, et al. Laboratory Handbook on Bovine Mastitis. 3rd. New Prague: National Mastitis Council; 2017.
121. Dohoo IR, Smith J, Andersen S, et al. Diagnosing intramammary infections: evaluation of definitions based on a single milk sample. J Dairy Sci 2011;94(1):250–61.
122. Schukken YH, Grommers FJ, Smit JA, et al. Effect of freezing on bacteriologic culturing of mastitis milk samples. J Dairy Sci 1989;72(7):1900–6.
123. Cameron M, Perry J, Middleton JR, et al. Short communication: Evaluation of MALDI-TOF mass spectrometry and a custom reference spectra expanded database for the identification of bovine-associated coagulase-negative staphylococci. J Dairy Sci 2018;101(1):590–5.
124. Park JY, Fox LK, Seo KS, et al. Comparison of phenotypic and genotypic methods for the species identification of coagulase-negative staphylococcal isolates from bovine intramammary infections. Vet Microbiol 2011;147(1–2):142–8.
125. Hiitio H, Pyorala S, Taponen S, et al. Elimination of experimentally induced bovine intramammary infection assessed by multiplex real-time PCR and bacterial culture. J Dairy Sci 2018;101(6):5267–76.
126. Ruegg PL. The bovine milk microbiome - an evolving science. Domest Anim Endocrinol 2022;79:106708.
127. Adkins PRF, Middleton JR. Methods for diagnosing mastitis. Vet Clin North Am Food Anim Pract 2018;34(3):479–91.