THESIS

DETECTION OF BOVINE RESPIRATORY PATHOGENS USING REAL-TIME PCR AND BEAD-BASED TECHNOLOGIES

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ABSTRACT

DETECTION OF BOVINE RESPIRATORY PATHOGENS USING REAL-TIME PCR AND BEAD-BASED TECHNOLOGIES

The global cattle industry suffers financial losses of $900 million USD annually from infections caused by respiratory pathogens in the bovine respiratory disease complex (BRD). Accurate and timely detection of BRD pathogens provides cattle producers with a diagnosis so they can institute patient care and prevent pathogen spread. We sought to implement Luminex xTAG technology to detect four pathogens that cause BRD - bovine respiratory syncytial virus (BRSV), bovine viral diarrhea virus (BVDV), bovine herpes virus-1 (BHV-1), and *Mycoplasma bovis* (*M. bovis*). We compared singleplex real-time polymerase chain reaction (real-time PCR) to a newly developed xTAG testing protocol. Nucleic acids were extracted from 28 bovine lung samples that previously tested positive on PCR for each of the viral pathogens: BRSV (5), BVDV (5), BHV-1 (5), and *M. bovis* (5). All samples for BRSV and BHV-1 were detected on xTAG with a mean fluorescent index (MFI) well above 10,000 while detection of BVDV is limited to an MFI of 10,000 and *M. bovis* is detected inconsistently by xTAG. Lungs from six co-infected animals that tested positive for two BRD pathogens were tested on xTAG and real-time PCR side-by-side, revealing similar findings to the single positive lungs where BHV-1 and BRSV targets are more detectable than BVDV and *M. bovis*. Spiked pools of all pathogens resulted in MFI decreases as the number of

pathogens per sample increases. With proper optimization, Luminex xTAG may be utilized in the veterinary diagnostic setting to circumvent issues with multiplex real-time PCR while maintaining high standards of diagnostic testing.

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Chapter 1: Literature Review and Background

Bovine respiratory disease (BRD) affects cattle herds globally, resulting in substantial economic and production losses. BRD can account for up to 70% of mortalities in feed lots and annual global financial losses up to $900 million when accounting for cost of treatment and loss of production (Blakebrough-Hall et al., 2020). Both bacterial and viral pathogens cause BRD and among them, bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), bovine herpesvirus-1 (BHV-1), and *Mycoplasma bovis (M. bovis)* are the most common. Cattle can be co-infected with one or more of these pathogens, including latently infected cattle in the case of BHV-1, and persistently infected (PI) calves in the case of BVDV, increasing severity of disease caused by BRD (Blakebrough-Hall et al., 2020), (Taylor et al., 2010), (Thiry et al., 2006). Young calves are the most susceptible population to BRD due to high stocking density and high stress environments where respiratory disease is more likely to spread, such as during calf shipment (Taylor et al., 2010), giving BRD the alternative name of “shipping fever”. Viral and bacterial pathogens may coincide with environmental stimuli to cause more severe respiratory disease in cattle with a variety of clinical signs, making it difficult to detect BRD in cattle populations. Availability of accurate and rapid diagnostic testing is essential to detecting BRD-associated pathogens and preventing pathogen transmission, ensuring that the loss of production and economic assets are minimized.

Early signs of BRD outbreaks primarily include clinical signs such as appetite loss, depression, and self-isolation (Gaudino et al., 2022). As the disease develops, cattle may show severe signs including abnormal breathing and coughing, as well as

discharge from the eyes and respiratory system. Less common clinical signs of BRD include spontaneous abortions, mastitis, and lesions on infected tissues (de Oliveira et al., 2022), (Rajamanickam et al., 2019), (Griffin, 2014). Disease can be exasperated by environmental factors and stress factors, making good husbandry measures imperative (Pratelli et al., 2021), (Snowder et al., 2006). As respiratory illness can result in a variety of clinical presentations, each pathogen that causes BRD has unique identifiers that can aid veterinarians in diagnosis.

Mycoplasma bovis (*M. bovis*) is a primary pathogen responsible for BRD, with additional possible clinical signs of otitis media, mastitis, and arthritis (Dudek et al., 2020). Due to its low infectious dose for mucosal surfaces, *M. bovis* is prevalent in cattle herds, often resulting in chronic illness and promoting co-infection with other BRD pathogens (Maunsell et al., 2011). Sick cattle will not demonstrate unique signs of infection, especially in co-infection cases, and laboratory testing is essential to identify this pathogen (Ridley & Hateley, 2018). *M. bovis* is transmitted via respiratory secretions sometimes causing pneumonia (Kanci et al., 2017), (Phillips et al., 2003) and can be introduced to herds via breeding due to semen transfer to female cows, and by milk to young calves (Haapala et al., 2018). Viable bacteria have been demonstrated to survive up to 4 months in environmental feces during colder months, making contaminated pastures a significant risk factor in its eradication (Williams & Hoy., 1930). While respiratory lesions are common, *M. bovis* has been demonstrated to disseminate through the hematogenous route causing damage to organs separate from where the initial infection took place (Maunsell & Chase., 2019). Arthritic cases of *M. bovis* have been reported to originate from bronchopneumonia infections which can spread to the

liver, kidney, or to the nervous system (Maeda et al., 2003). *M. bovis* is one of 13 different *Mycoplasma* species detected in cattle and some of these species will result in clinical illness whereas some will exist as normal flora in cattle (Dudek et al., 2020). This can complicate the *M. bovis* diagnosis in subclinical animals and animals infected with more than one *Mycoplasma* species. Accurate testing is required for diagnosis and diagnostic assays that differentiate between *Mycoplasma* species aid in appropriate case and herd management.

Primary detection methods for *M. bovis* include PCR, and bacterial culture. These assays can be performed on milk, respiratory swabs, or tissues of the clinically ill animal (Parkinson, 2019). Routinely testing batches of semen and milk will ensure that dairy production and reproductive management are optimized. Serology is used to identify seroconversion for *M. bovis* in large herds of animals but does not differentiate current shedders. Bacterial culture is regarded as the main confirmatory test for *M. bovis* diagnosis, requiring careful sterile techniques and longer turnaround times compared to PCR (Pfyffer & Wittwer, 2012). Acid fast staining and microscopy are quick and simple diagnostic methods to identify Mycoplasma although they lack the high molecular specificity that PCR has, and the unique growth conditions exhibited in culture (Gormley et al., 2014).

Control of *M. bovis* within a herd is primarily achieved by isolating or culling infected animals within the herd. Mastitic cows can be identified by red or swollen utters and are often isolated from the herd for further treatment and testing (Haapala et al., 2021). Isolation of pneumonic and arthritic cattle gives additional time for diagnosis and keeps the sick cattle from interacting with healthy cattle. Preventing nose-to-nose

contact in calves for the first three months of life has been demonstrated to significantly decrease *M. bovis* presence in eight out of ten herds (Haapala et al., 2021). Six months of limited contact is preferred; however, this is a relatively inexpensive method to implement that may prevent future infections, especially if an infected cow gives birth to a healthy calf. Long term surveillance and control methods are common, and when combined with physical isolation strategies, dairy producers avoid the common risk factors for *M. bovis* transmission within their herd. Long term monitoring of milk yield will indicate if the herd is healthy and may suggest *M. bovis* infection if yield is low (Haapala et al., 2021), meaning if milk yield is high then it is unlikely that the herd is infected with *M. bovis*. Treatment of *M. bovis* has proven difficult due to its innate and acquired immune resistances, as well as its ability to chronically infect cattle. For example, beta-lactam-based antibiotics are not effective against *M. bovis* because the bacterium lacks a cell wall (Loria et al., 2008), (Nicholas et al., 2016). These antibiotics will bind to penicillin-binding proteins to prevent bacterial cell wall synthesis, thus making these methods ineffective for *M. bovis*. Macrolides are less effective at inhibiting growth of recent *M. bovis* isolates (Sulyok et al., 2014) likely due to a genetic mutation changing the bacterial ribosome structure. Fluoroquinolones remain highly effective at inhibiting *M. bovis* growth *in vitro*, recommending that they be effective in treatment.

M. bovis can evade the host immune system through a variety of methods, leading to chronic infection of cattle. The innate immune system is bypassed by *M. bovis* as the organism can inhibit protein kinase C in neutrophils typically located in the respiratory tract. These neutrophils are then unable to produce chemiluminescent trails that activate adaptive immune processes (Askar et al., 2021). This will prevent the

immune system from mounting a reliable response to *M. bovis,* allowing the organism to replicate more readily in other physical locations. In cases of mastitis, *M. bovis* has been demonstrated to promote immune response exhaustion by repressing ligands in T-cells. Immunosuppressive factors including T-lymphocyte-associated protein, programmed cell death 1, and lymphocyte activation gene 3 are utilized by *M. bovis* to prevent the immune system from carrying out cytotoxic functions and further proliferation of immune cells (Gondaira et al., 2020). *M. bovis* is one of the primary disease agents causing BRD and exhibits unique molecular qualities that will exaggerate the clinical disease caused by other BRD pathogens.

Bovine viral diarrhea virus (BVDV) is a single stranded RNA virus belonging to the Pestivirus genus of Flaviviruses. Tropic for immune cells such as lymphocytes, macrophages, and monocytes, BVDV is commonly detected in blood and where resident immune cells thrive (Bolin, 2002) and is shed in milk and other bodily fluids. Infections with BVDV often result in decreased milk production, fertility rates, and growth rates in newborn calves (Evans et al., 2018). This results in financial losses accounting for production and veterinary costs up to nearly $40 million per million calves raised (Houe, 2003).

Animals that are infected transiently through the environment or by fomites typically present with mild clinical illness but can also experience decreased fertility, decreased milk production and spontaneous abortions (Grooms, 2004), (Thurmond, 2005). The virus is often endemic within herds where control methods are minimal (Evans et al., 2018), often aggravated by persistently infected (PI) cattle which shed large amounts of virus through bodily fluids and remain infected through the remainder

of their shorter lifespan (Bauermann, 2014). PI cattle become the primary source of BVDV dissemination in the herd through nose-to-nose contact and bodily fluid contact. Immunotolerant PI cattle may have physical deformities such as smaller overall body-size and oddly shaped heads. These cattle may not display obvious clinical signs of illness but through virus surveillance and careful monitoring, PI cattle can be identified. Persistent infections are caused by the introduction of BVDV to a naïve pregnant cow at any point before roughly 150 days into the pregnancy. BVDV is then vertically transmitted to the fetus before its adaptive immune system has developed, resulting in BVDV replication in the fetus without control via an immune response (Peterhans et al., 2010). If the PI calf nurses from its dam early in life, maternal antibodies acquired from feeding have been demonstrated to hinder detection capabilities for BVDV through antigen capture ELISA on ear notch samples (Fux & Wolf, 2012). PI calves are more prevalent in herds that intermingle multiple cattle populations. Wild cattle introduced to feedlot cattle and even feedlot cattle introduced to alike populations have been demonstrated to have more PI infections compared to herds that control new animal introduction (Evans et al., 2018). In bovids that are acutely infected after birth, infection is cleared naturally and shedding of the virus continues until roughly three weeks post infection.

Two biotypes of BVDV occur in cattle, non-cytopathic (ncp) and cytopathic (cp) strains (Peterhans & Schweizer, 2010). Non-cytopathic strains are responsible for all BVDV infections acquired from outside sources while cp strains mutate from ncp strains in an individual animal either by gene duplication, translocation, or recombination with the host causing mucosal disease (Peterhans et al., 2010). Cytopathic strains mutate

exclusively from ncp strains in PI calves due to their weak immune system which is immunotolerant to BVDV. Both ncp and cp strains utilize N^{pro}, a protease that cleaves transcription factors such as IRF-3, preventing the production of type-1 interferons (Gil et al., 2006). Type-1 interferons are essential in anti-viral immune responses and with their activators cleaved, BVDV is free to infect the host. Nonstructural protein NS3 in cp strains is found alongside NS2-3 whereas ncp strains only contain NS2-3 (Meyers & Thiel, 2008). NS3 is responsible for activating viral replication machinery and activating downstream protease activity (Tamura et al., 1993). Achieving the extra level of viral replicability and protease activity may suggest why cp strains are distinct from ncp strains in causing mucosal disease. A study by Meyers, *et al*. investigated the genomic aspects of cp and ncp strains of BVDV, revealing that two common cp strains, Osloss and NADL, have 228 and 270 nucleotide long insertions that show remarkably similar homology to bovine mRNA sequences (Meyers et al., 1991). These large genomic insertions appear to have similar effects on the infected host, suggesting that ncp strains of BVDV have machinery that selects genes to enhance its cytopathogenicity.

Diagnosis of BVDV can be achieved through a variety of reliable diagnostic methods using samples from live or dead animals. Virus isolation is historically regarded as the most confirmatory diagnostic method for the cytopathic strains of the virus using a buffy coat extracted from whole blood, although it is not as widely used due slow turnaround time and availability of high sensitivity methods such qPCR. For virus isolation testing, bovine cell lines are inoculated with the buffy coat and incubated for 4-5 days, observing for cytopathic effect (CPE) caused by the virus (Saliki & Dubovi, 2004). If no CPE is found, a PCR assay can be performed to confirm BVDV growth in

cells for ncp strains. Nasal swabs or lung tissue can be used to detect transient BVDV infection via PCR, acquired through respiratory routes. Pooled RT-PCR or antigen-capture ELISA testing can be performed a variety of sample types, including milk (Drew et al., 1999) and serum samples (Boulard & Villejoubert, 1991) and ear notches test for the presence of PI cattle since the virus replicates systemically in PI cattle. Serology can be used to detect antibodies to BVDV; however, issues arise with serology when the animal is vaccinated as a positive serology test does not differentiate vaccinated from infected animals.

To prevent the introduction of PI calves into dairy herds and to detect BVDV before it spreads, producers utilize biosecurity interventions and surveillance testing to decrease risk. Beginning in 2008, Switzerland aimed to eradicate BVDV from their cattle population of roughly 1.5 million animals (Stalder et al., 2018). Their plan consisted of three phases, beginning with testing ear notches through PCR, of all cattle in the country followed by the second phase of antigen-capture ELISA testing all calves. The final phase of BVDV eradication is ongoing and involves testing milk from dairy herds twice per year and blood from beef herds every three years. Any BVDV positive animals are culled to prevent viral spread. At the beginning of the first phase, 20% of herds contained a BVDV positive case. By 2020, 99.5% of herds are free of BVDV. Of the positive cases, PI calf prevalence decreased to 0.02% from 1.4% (Stalder et al., 2018). Due to the rigorous planning and commitment to eradicating BVDV from their herds, Swiss dairy producers can rely on control systems in place to ensure that BVDV will cause minimal financial loss for their business. The control programs methods used could be utilized in other countries to control BVDV.

In addition to physical control methods and surveillance, modified live and killed vaccines are effective. Vaccinated bovids mount a strong B cell response to E2 and NS2/3 proteins and are effective at the herd level to prevent BVDV from becoming endemic (Ridpath, 2013). Vaccine programs to prevent PI calves is challenging due to the route of transmission for PI infections to occur. Preventing infection of the fetus from a pregnant cow relies on effective immunization of the pregnant cow prior to BVDV crossing the placenta. Vaccination provides moderate protection to the fetus but is not completely reliable as a PI control method (Larson et al., 2004).

Bovine herpesvirus-1 (BHV-1) is an enveloped double stranded DNA virus known for infecting mucosal surfaces primarily in the respiratory tract and reproductive tracts of cattle (Biswas et al., 2013). BHV-1 causes mild respiratory disease and is the etiologic agent of infectious bovine rhinotracheitis (IBR) and infection may establish latency in the host. If an infection progresses to latency, affected cattle will become reservoirs for BHV-1, increasing the likelihood of pathogen transmission within the herd. Three subtypes of BHV-1 are associated with separate infections where BHV-1.1 results in respiratory disease, BHV-1.2 results in genital infections and BHV-1.3 results in neurological disease (Biswas et al., 2013). These subtypes are differentiated by their envelope proteins allowing them to have differing cell tropism. Following acute infection, BHV-1 can disseminate through the blood from mucosal sites and spread to secondary sites of infection (Mechor et al., 1987), (Nandi et al., 2009). Virus particles can remain stable in the environment and cattle feed for up to 30 days despite being an enveloped virus which is typically less stable outside of the host environment (Biswas et al., 2013). BHV-1 is shed in respiratory droplets (Mars et al., 2000) in acutely infected bovids for up

to 14 days when clinical signs of infection are most noticeable (Straub, 1991) making this a common mode of transmission for the virus.

Infected cattle will experience decreases in growth and milk production, along with a susceptibility to secondary infections, making BHV-1 a primary cause of BRD (Biswas et al., 2013). Clinical signs of IBR include conjunctival discharge, respiratory illness, and occasionally abortions within the first five months of pregnancy. Lesions in the respiratory tract or genitals are common signs of BHV-1 (Yates, 1982).

Once virions contact a mucosal surface, glycoprotein C will utilize heparan sulfate proteoglycans to adhere to host cells (Karger et al., 1995) and glycoprotein D to fuse the viral envelope with the cell membrane with the help of the human polio receptor (Connolly et al., 2001). MHC antigen-processing by macrophages is hindered by BHV-1 infection allowing the pathogen to avoid both innate and adaptive immune responses (Forman et al., 1982). Macrophages and lymphocytes are further impaired by infection leaving the host immunocompromised, leading to increased susceptibility to BRD (Tikoo et al., 1995). Within eight hours of exposure, host cells release productive BHV-1 virions causing further virus distribution and initiating the latency pathways of the virus (Babiuk et al., 1996). Establishment of BHV-1 in the trigeminal ganglia nerves can result in latent infection wherein the virus can remain in the host indefinitely. Latency related genes are present in these nerves nearly one week after the initial BHV-1 exposure (Inman er al., 2002) and reactivation of the virus results in viral shedding and transmission to other cattle typically by respiratory droplets (Mars et al., 2000). Latency is first established by the upregulation of the latency related (LR) gene which inhibits infected cells from undergoing programmed cell death (Ciacci-Zanella et al., 1999) ensuring that infected

cells can be hijacked to produce BHV-1 virions. Productive virions utilize two transcription units, immediate early transcription unit 1 (IEtu1) and IEtu2 that activate early and late phase transcription factors for infection (Jones et al., 2011). These transcription factors enable proteins such as ICP22 to modify RNA polymerase II from the infected cell to help with viral replication (Rice et al., 1995). The LR gene contains ORFs 1 and 2 that help with inhibiting apoptosis as well as aiding the cycle of reactivating infection and returning to latency. Both ORFs lack a leading methionine and are spliced together by a polyA RNA from the LR gene, allowing for translation of ORF2 proteins which inhibit apoptosis (Shen & Jones, 2008). ORF2 proteins interact with the Notch pathway to assist with the cycle of reactivation and returning to latency. Notch signaling proteins regulate a variety of physiological pathways in cells and are not typically specific to one function. Notch 1 and Notch 3 inhibit apoptosis and have been demonstrated to interface with ORF2 proteins meaning that the apoptosis signaling is now under control of viral gene products (Workman et al., 2011). Stress hormone DEX is known to reactivate BHV-1 infection from latency by promoting activity of bICP0, a transcriptional regulator that resides in the IEtu1 gene (Workman et al., 2009). Utilizing DEX and regulating the Notch pathway, BHV-1 has multiple levels in which it can control the latency-reactivation cycle in infected nerves.

BHV-1 virus infection can be confirmed through cell culture, PCR, and histopathology. Tissues that are appropriate for diagnosing BHV-1 include lung, spleen, lymph nodes, and kidney along with swabs from mucosal surfaces (Sandturde et al., 1996). CPE within bovine cell lines can be detected around three days post inoculation, presenting as large plaques and syncytia on occasion (Sandturde et al., 1996). BHV-1

will cause intranuclear inclusion bodies (Biswas et al., 2013) which can be visually detected by a pathologist on histopathology slides of infected tissues. Serology will detect antibodies for BHV-1 typically IgM and IgG (Ungar-Waron & Abraham, 1991). Enzyme-linked immunosorbent assays (ELISAs) are used to detect BHV-1 using antibodies that bind surface glycoproteins used for viral entry (Wellenberg et al., 2001). However, if the cattle are vaccinated for BHV-1, serology is ineffective as the assay may give false positive results. PCR is routinely used for BHV-1 diagnosis due to its high sensitivity and rapid turnaround time.

Treatment and control of BHV-1 has proven difficult due to latent infections where disease is not consistently presenting (Fuchs et al., 1999). The test-and-slaughter method has been effective for affected herds although in some cases, cattle have had latent BHV-1 infections while remaining seronegative (Hage et al., 1998), making this method difficult to implement in an effective way. Dairy producers are not likely to cull animals due to the large financial burden it puts on the farm. Despite this, a variety of producers in Europe have seen success with this method (Nylin et al., 2000), (Ackermann & Engels, 2006). Vaccines are effective as a prophylactic measure for cattle especially modified live vaccines where they stimulate an immune response quickly and mount a robust memory to the virus (Biswas et al., 2013). If administered intranasally, protection from BHV-1 can be acquired in as little as three days. Modified live vaccines may cause abortion in pregnant cattle and in this case, an inactivated vaccine is recommended. Vaccines are a reliable control method for BHV-1 and eradication of the pathogen in herds is assisted by routine diagnostic testing and removal of sick animals.

Bovine respiratory syncytial virus (BRSV) is an enveloped Paramyxovirus containing a segmented single-stranded RNA genome (Makoshey & Berge, 2021). Causing acute respiratory illness and high rates of seropositivity in dairy herds, BRSV infection results in low mortality rates in infected animals (Fulton et al., 2000), (MacGregor & Wray, 2004). Herd morbidity often exceeds 50% seropositivity due to rapid dissemination within a population and the relative ease in which the bovine immune system can clear the virus. After being transmitted by aerosol, BRSV virions will infect the respiratory epithelial lining and cattle will begin shedding virus roughly three days post infection until the tenth day (Gershwin, 2008). Acute infection of BRSV can result in decreased milk production up to 0.9kg less per cow per day, making BRSV a significant financial burden for dairy producers (Beaudeau et al., 2010). Necrosis of the epithelial lining is a common outcome of BRSV as the host cells undergo programmed cell death to limit the infection. Following apoptosis, more damage to the respiratory tract can occur when the host immune response attacks the infected area (Gershwin, 2008). Enzymes released by neutrophils are known to have negative effects on BRSV infected tissue when present in large numbers, commonly associated with high levels of IL-8 (Geerdink et al., 2015). The low specificity of neutrophils' response to an infection often has collateral effects on the host.

Infection is dependent on large glycoproteins (G) and fusion proteins (F) present on the BRSV envelope (Hendricks et al., 1988), (Sarmiento-Silva et al., 2012). Glycoprotein G exists as a heavily glycosylated membrane bound protein as well as a secreted protein which is known to bind neutralizing antibodies (Hendricks et al., 1988). The majority of glycoprotein G exists as the secreted form and is translated from the

same gene as the membrane-bound form although with a different initiation site (Roberts et al., 1994). Heparin binding domains on protein G facilitate envelope attachment to cell membranes to initiate viral entry. F proteins mediate viral envelope and cell membrane fusion allowing the BRSV nucleocapsid to safely enter the cell. Existing as two subunits (F1 and F2), the F protein is activated by cleavage of furin endoproteases not only allowing for cell membrane to virus fusion but also syncytium formation by fusing host cells together (Valarcher & Taylor, 2007). BRSV and human RSV differ from other paramyxoviruses in that they utilize nonstructural (NS) proteins NS1 and NS2 to aid in the infection of the host and evasion of their immune system. Genes for the NS proteins are located on the 3' end of the genome, which may suggest a recombinant event leading to these genes being conserved to the end of the negative-sense RNA and persisting through evolutionary pressures (Bossert et al., 2003). NS proteins have been found only in infected cells and not live virions, suggesting their role in suppressing cellular pathways (Atreya et al., 1998).

Interferon-β (IFN-β) and its pathways are essential for host cells to mount an antiviral response and the NS1/NS2 proteins are specialized in antagonizing this process for viral replication. IFN-stimulated genes (ISGs) are activated in the presence of IFNs and their IFN regulatory factors (IRFs). ISGs code for proteins that prevent viral activity in the cell and include an array of protein kinases and commonly MxA. A study by Conzelman et al. outlines the process in which IFN-β production is impaired by keeping the activated IRF-3 localized to the cell's nucleus instead of being exported to the cytoplasm where protein production will occur (Bossert et al., 2003). *IFN-β* expression is dependent on activation of IRF-3, NF-kB and ATF-2/c-Jun and by using a

luciferase reporter assay, researchers found that IRF-3 activity was dramatically reduced in wild type BRSV-infected cells. All IRF-3 was kept in the nucleus and prevented from nuclear export. Mutant BRSV strains that did not produce NS1/NS2 did not have the same effect on IRF-3 localization. In a healthy cell, IRF-3 is phosphorylated on its C-terminus and processed for export to the cytoplasm by VAK. Cells infected with the mutant BRSV strain, absent of the genes coding for NS1 and NS2, resumed IRF-3 activity and its downstream effects of producing MxA and IFN-β were observed. This proved that NS1 and NS2 proteins are not essential for BRSV infection but heavily impact the association of VAK and IRF-3 making NS1/NS2 prime vaccine targets for future developments.

The presence of BRSV can be confirmed through cell culture and PCR (Makoshey & Berge, 2021). While cell culture is regarded as the gold standard for diagnosis (Larsen, 2000), PCR testing of BRSV is reliable to detect viral RNA in respiratory tissue homogenates and bronchoalveolar lavage (BAL) (Boxus et al., 2005). Where cell culture may fail is if the sample has not been handled correctly resulting in the sample having inactive BRSV virions. In this case, BRSV will not be detected by cell culture but will be detected by PCR. Antibody tests such as ELISAs are used commercially to detect antibodies in serum or milk against BRSV. The caveat to using ELISAs is that milk is typically more readily available than serum while antibodies appear in serum much quicker post-infection than in milk (Näslund et al., 2000). It is difficult to detect BRSV using ELISAs since antibodies are acquired from vaccination and the subsequent immune response and is not readily differentiated from antibodies produced in response to infection.

As BRSV is responsible for high rates of seroconversion in clinical respiratory illness cases in dairy herds, vaccine development is essential to the success of the dairy industry (Elvander, 1996). Despite the unique surface antigens proteins G and F and the high seropositivity of cattle, vaccines have not been fully effective in their current state. Modified live and formalin-inactivated vaccines against BRSV have shown to cause unwanted immune reactions and even fatality in vaccinated cattle (Schreiber et al., 2001), (Kimman et al., 2011). This suggests that vaccine acquired immunity either does not protect against BRSV or the acquired immunity is aggravating the host's immune response. Similar complications were seen in a modified live vaccine where cattle remained healthy if unvaccinated (Kimman et al., 2011). Antibodies acquired from these vaccines may cause complications when combined with viral epitopes, stimulating the incorrect immune response in the host. Disease exacerbation from BRSV vaccines has been traced back to Th2 immune reactivity. IgE associated with Th2 responses was found to be the primary neutralizing antibody against BRSV fusion proteins and glycoproteins in vaccinated cattle with severe respiratory illness (Kalina et al., 2004). Formalin-inactivated vaccines have been demonstrated to elicit this response in the past for Human RSV (Kim et al., 1969) and will be a large roadblock in developing a successful prophylactic for these viruses.

The presence of one or more bovine respiratory pathogens often increases susceptibility to other pathogens in an individual or in a population of bovids, making multiplexed detection methods essential for diagnosis and disease control. PCR is a commonplace diagnostic technique for BRD pathogens due to high sensitivity and short test time. Singleplex PCRs are easier to optimize due to fewer assay components and

genetic targets whereas multiplexed PCRs are more complex and are more likely to have cross-reactivity between primers/probes or higher amplification efficiencies for certain genetic targets than others due to varying cycling preferences (Polz & Cavanaugh, 1998), (Brownie et al., 1997). With enough optimization and analysis, multiplexed PCRs can be accurate and efficient. However, while optimizing the assay to bolster the efficiency of one target, the changes may hinder the efficiency of detecting other targets (Zangenberg et al., 1999). Optimizing an assay to detect multiple pathogens can allow for increased speed of detection as well as decreased cost (Mahony et al., 2009). Multiplexed assays are widely used as a detection method for respiratory pathogens in the veterinary and human medicine fields (Pang et al., 2002), (Gadsby et al., 2015). Multiplex assay development for respiratory panels is important for detection and differentiation of multiple pathogens since most respiratory pathogens present very similarly (Ferraro et al., 2021) and cannot be diagnosed without accurate testing. Practitioners can give an informed diagnosis over a shorter timeline and ultimately, the laboratory will decide if a multiplex or singleplex assay will be performed based on the demands of the client base. For example, if there are multiple pathogens that exhibit the same clinical signs like groups of respiratory pathogens do, they may be included in a multiplexed panel to quickly rule out other organisms (García-Arroyo et al., 2015). This has aided BRD diagnostics due to the high number of pathogens that could be causing disease and their similar clinical presentations in cattle. Due to their robust molecular qualities and general familiarity within most laboratories, PCR and bead-based technologies are often multiplexed into panels (Elshal & McCoy, 2006), allowing laboratory teams to streamline their molecular detection capabilities.

xTAG is a molecular diagnostic assay developed by Luminex Corporation and utilizes fluorescent beads and machine fluidics to detect a desired molecular target (Luminex Corporation, 2006-2024). Detection of DNA targets using xTAG depends on specific modifications to the 5’ end of PCR primers. The forward primer contains a specific TAG sequence, consisting of only T, A and G bases, roughly 20 nucleotides long with a three-carbon spacer between the TAG sequence and the 5’ end of the forward primer (Hrdy et al., 2021). This spacer increases primer stability by blocking unwanted enzymes from interfering with polymerase binding (Cradic et al., 2004). Reverse primers contain a biotin bead on their 5’ end, utilized for co-reporter binding. After conducting conventional PCR, all 5’ primer modifications are present on either strand of the double stranded product of the DNA target. Anti-TAG sequences are provided pre-conjugated to fluorescent microspheres and are hybridized to the TAG sequence attached to newly generated PCR amplicons. During this hybridization step, streptavidin phycoerythrin (SAPE) is added as a co-reporter molecule and binds to the biotin bead on the 5’ end of the reverse primer (Cong et al., 2018). Once hybridization is complete, each double stranded product will have one strand bound to the fluorescent microsphere, attached by the TAG and anti-TAG sequences, and one strand with SAPE bound to biotin. The MagPix instrument uses fluidics to uptake samples, reading each positive event as they are magnetized by their magnetic beads to the imaging surface. Two lasers are used to confirm the presence of hybridized products. A 635nm red laser identifies which fluorescent microspheres are present while a 532nm yttrium aluminum garnet laser detects SAPE (Pang et al., 2002). Other Luminex instruments do not utilize the magnetic technology in the MagPix instrument but rather use flow cytometry fluidics.

The lasers gather forward scatter data to determine if a single microsphere or a clump of microspheres is passing through the fluidics system, ensuring that only single microsphere events are used to calculate the sample's fluorescence. Using the MagPix instrument and xPONENT analysis software, each sample is given a mean fluorescence index (MFI) representing the fluorescent intensity of each microsphere color.

Multiplexing xTAG begins at the PCR step where forward and reverse primers are designed for conserved regions of the desired genomic target. The TAG sequence must be different from the TAG sequences for other targets in the multiplex assay. If four DNA targets are included in the multiplexed assay, four different fluorescent microspheres (referred to as bead regions) with anti-TAG sequences complementary to their TAG sequences must be present. The MagPix instrument will differentiate between each bead region color. Background fluorescence is detected by a control well containing all bead regions. This MFI is subtracted from the total MFI of each well to give a net MFI total.

Hybridization of the TAG and anti-TAG sequences is essential to the efficacy of the xTAG assay. The hybridization buffer's contents and their working concentrations play a key role in reaction efficiency, while also making the buffer an essential reagent for optimizing the assay. The hybridization buffer is comprised of a mixture of NaCl, Tris-HCl, Triton x-100 and bovine serum albumin (BSA). Salt in the hybridization buffer acts to balance the charges of the solution due to DNA being a negatively charged molecule. Balancing the charges will decrease the electrostatic repulsion between DNA molecules and mitigates non-specific binding (Gadsby et al., 2015). Tris-HCl is used to buffer the pH during hybridization, ensuring that the pH remains at 8.0 for an efficient reaction. If

the buffer is too acidic, hybridization will be less efficient due to surface tension forces caused by the addition of electrostatic ions. On the other hand, hybridization buffer that is too basic will inhibit hybridization by decreasing steric interactions between DNA molecules and deprotonating the phosphate backbone (Polz & Cavanaugh, 1998). Triton x-100 acts as a detergent and lyses all cell membrane artifacts leftover from extraction, allowing DNA to hybridize without the interference of phospholipid aggregates (Brownie et al., 1997). Bovine serum albumin (BSA) is used as a blocking agent during hybridization to minimize non-specific binding. It will bind single stranded DNA and lower its affinity for other DNA molecules, allowing the complementary single stranded DNA molecule to hybridize (Zangenberg et al., 1999). Adding BSA in moderation will allow for less non-specific DNA hybridization events while still promoting accurate hybridization; too much BSA may inhibit TAG and anti-TAG interactions.

A primary difference between real-time PCR and xTAG is the results they output and how they may be interpreted. Real-time PCR results in a cycle threshold (Ct) value representing the PCR cycle where sufficient DNA product was present to cross a threshold of detection (Mackay, 2004). xTAG provides an MFI value which is not based on a threshold value like Ct values are, but still provides relative quantification of the sample (Shi et al., 2021). The assays differ in that Ct values involve data collection through the duration of the PCR protocol where MFI is only measuring the fluorescence after PCR amplification. This means that real-time PCR has an additional factor to interpret in its results by incorporating a time aspect. xTAG was developed by Luminex to multiplex large amounts of genetic targets with ease. xTAG's protocol and instruments allow for development of multiplexed assays that are uniquely different from

multiplex PCR assays. Both assays may accomplish the same goal of multiplexing although providing different output values that may not allow for the same interpretation. This gives diagnosticians the ability to choose how they wish to collect results and how those results fulfill the needs of the client while still being cost effective and accurate.

In this study, lung samples from 28 bovids were tested using a multiplexed xTAG assay for four common bovine respiratory pathogens, *M. bovis,* BVDV, BRSV, and BHV-1. These pathogens are part of the bovine respiratory disease complex and commonly present as clinical respiratory illness. Differentiating between pathogens requires laboratory diagnostic techniques. Using xTAG, we aimed to detect all four pathogens and compare the MFIs from xTAG to Ct values obtained from singleplex PCR. This comparison will give insight into how the two assays compare as far as their ability to accurately quantify genetic targets and the relative ease of performing the assays.

Chapter 2: xTAG and Real-Time PCR Comparison

Introduction:

The bovine respiratory disease (BRD) complex is a collection of roughly ten pathogens (Griffin et al., 2010), (Klima et al., 2014) both bacterial and viral, that cause clinical respiratory illness in cattle. BRD is commonly referred to as “shipping fever” due to its high prevalence in calves (Campbell, 2022) who are infected during shipping in high stocking density environments to cattle owners. BRD results in an overall morbidity of up to 50%, and mortality reaching 10% of the herd depending on co-infection rates and disease management (Campbell, 2022). General clinical signs of BRD include fever, nasal discharge, and coughing, along with altered respiration and mentation (Ferraro et al., 2021). Due to the relatively high rate of co-infections in individual animals and the variety of pathogens that contribute to BRD, diagnostic testing should be utilized in herds showing clinical signs to confirm the pathogens spreading within the population. Rapid diagnosis supports disease management and pathogen reduction within a herd. PCR is primarily used to diagnose BRD pathogens and has proven to be a robust and reliable way to detect respiratory pathogens. However, when multiple pathogens are to be tested for, especially in BRD cases, the inability to develop multiplexed assay increases the financial cost and time to produce a diagnosis.

The xTAG assay was developed to detect BRD pathogens due to its high potential for multiplexing genetic targets and its modular design, allowing for targets to

be added or removed from the assay with ease. With all targets being detected simultaneously, multiplex xTAG is a more time-efficient assay compared to singleplex PCR. Real-time PCR and xTAG are both semiquantitative assays; however, xTAG utilizes fluorescent beads to bind the genetic target and real-time PCR utilizes fluorescent oligonucleotide probes. Comparing these two methods will give insight into how the differences between the two assays benefit or hinder diagnostic output.

We selected four BRD pathogens for this multiplexed assay because of their prevalence in cattle as well as their interconnecting roles in causing disease. This study compares PCR and xTAG as diagnostic assays for detection of BRD pathogens on bovine lung samples that were submitted from BRD positive cattle. We aim to determine if real-time PCR or xTAG is more suitable for diagnosing the four BRD pathogens previously mentioned. Considering the cost of multiplexing assays, this study outlines scenarios where either assay is suitable to use. By analyzing results from both assays, we will begin to understand how MFI changes as Ct values are higher or lower depending on the sample and how this may affect the interpretation of the results. In comparing the two assays, we aimed to define a threshold of detection that will provide a diagnostic point of comparison between both methodologies.

Materials and Methods:

Clinical Samples Selection:

The twenty-eight bovine lung samples used in this project were submitted to the Colorado State University Veterinary Diagnostic Laboratories and tested by singleplex real-time PCR for BVD, BRSV, and BHV-1 and conventional PCR for *Mycoplasma spp*.

The samples were frozen at -80°C for up to 5 years prior to use in this study. Five positive samples were selected for each of the four pathogens and six samples were selected for the co-infection sample analysis. For each pathogen tested, the five samples were assigned a letter A through E. The six co-infected samples were assigned a letter A through F. Each letter was randomly assigned. Eight samples were chosen for the pooled sample experiment, two for each pathogen. *M. bovis* samples were bacterial isolates confirmed by MALDI-TOF while BVDV, BRSV, and BHV-1 samples were bovine lung samples. These eight samples were assigned to either group 1 or group 2 ensuring that each group contains a positive sample for each pathogen.

Sample Preparation:

Homogenized suspensions of bovine lung samples were created by making a 10% homogenate with 1ml of sterile PCR-grade H_2O and 100mg of lung tissue. Nucleic acids were extracted using either the Qiagen RNeasy Mini Kit or the QIAamp DNA Mini Kit on Qiagen Qiacube instruments according to the manufacturer's protocols (Qiagen, Hilden, Germany).

Pooled Sample Testing:

Positive samples for each pathogen were pooled after conventional PCR in combinations of two, three, and four pathogens. 5µl of PCR product from each pathogen was pooled immediately prior to the testing.

Real-Time PCR:

The BVD real-time PCR assay used in this project was conducted using the Applied Biosystems VetMAX-Gold BVDV PI Detection Kit (Applied Biosystems,

Waltham, USA) following manufacturer guidelines and using the following PCR protocol: a reverse transcription step 45°C for 10 minutes, an activation step at 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds with 60°C for 45 seconds. The primer and probe sequences associated with this kit are proprietary and not disclosed by Applied Biosystems. *M. bovis* real-time PCR was performed using the AgPath-ID One-Step PCR Kit (Applied Biosystems, Waltham, USA). The total reaction volume was 23µl, using 12.5µl of 2x buffer, 1µl of 25x buffer, 0.6µl of forward and reverse primers (10µM), and 0.2µl of probe (10µM) and 8µl of extracted DNA per reaction. The *M. bovis* cycling protocol consists of a two-part precycling stage 45°C for 10 minutes and 94°C for 10 minutes and a cycling stage of 40 cycles at 95°C for 10 seconds, 58°C for 15 seconds and 72°C for 30 seconds. BRSV real-time PCR was performed using the QIAGEN QuantiTect Probe RT-PCR Kit (Qiagen, Hilden, Germany) at a total volume of 25µl containing 12.5µl probe RT-PCR Master Mix, 0.25µl RT Mix, 0.25µl of forward and reverse primers (10µM), 0.50µl of probe (10µM), 10.25µl H_2O and 1µl of extracted RNA. The following cycling protocol was used for BRSV detection; precycling stage at 50°C for 2 minutes followed by 95°C for 10 minutes. The cycling stage included 40 cycles of 95°C for 15 seconds and 59°C for 60 seconds. BHV-1 real-time PCR was performed using the QuantiTect Multiplex PCR NoROX Kit. With a total reaction volume of 25µl, each reaction contains 12.5µl of QuantiTect Multiplex PCR NoROX Master Mix, 5.50µl of H2O, 2µl of primer/probe mix (equal volumes of working stocks, 10µM for primers, 1.25µM for probe), and 5µl of extracted DNA. The cycling protocol begins at 95°C for 15 minutes followed by 43 cycles of 95°C for 60 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. The primers included in real-time PCR assays are included in Table 1.

All real-time PCRs were conducted using the Applied Biosystems 7500 Fast thermocycler instrument. The Ct value represents the PCR cycle at which there was sufficient fluorescence from the amplified gene target to cross the positive threshold. Thresholds were set at 10% of the plateau of the positive amplification control. If a sample does not produce enough of the genetic target to cross the threshold, its Ct result is recorded as zero and the sample is considered negative.

Table 1: Real-Time PCR Primer Design

Pathogen	Forward Primer Sequence	Reverse Primer Sequence	Probe Sequence	Gene Target	Fluorescent Marker
M. bovis	TCAAGGAACCCCACCAGAT	AGGCAAAGTCATTTCTAGGTGCAA	TGGCAAACTTACCTATCGGTGACCCT	*oppD*	Cy5
BRSV	GCAATGCTGCAGGACTAGGTATAAT	ACACTGTAATTGATGACCCCATTCT	ACCAAGACTTGTATGATGCTGCCAAAGCA	Nucleocapsid Protein	FAM/TAMRA
BHV-1	CCGCCGTATTTTGAGGAGTCG	TCGGTCTCCCCTTCRTCCTC	TACGAGCCGCCGCCTGCCGC	glycoprotein D	CAL Fluor Red 610/BHQ-2

xTAG Primer Design:To detect the presence of the four desired targets, forward and reverse primers were modified from various publications for genomic xTAG (Letellier and Kerkhofs, 2003), (Behara et al., 2018), (Boxus et al., 2005), (Wernike et al., 2011), targeting BRSV, BVDV, BHV-1, and *M. bovis* in conserved regions of coding genes (Table 2). Additionally, each forward primer contained a unique TAG sequence provided by Luminex (Table 3). A biotin molecule is conjugated to the reverse primer, allowing for the fluorescent molecule SAPE to adhere to generated amplicons containing a copy of the reverse primer.

Table 2: xTAG Conventional PCR Primers

Pathogen	Forward Primer Sequence	Reverse Primer Sequence	Amplicon Size (bp)	Gene Target	Source
BVD	CTCGAGATGCCATGTGGAC	CTCCATGTGCCATGTACAGCA	149	5'UTR (NADL)	(Letellier and Kerkhofs, 2003)
M. bovis	AAGTTGAAGTTGACCGGTTTG	TCCATATTTGGACCTAGTCCTTT	106	uvrC	(Behara et al., 2018)
BRSV	GCAATGCTGCAGGACTAGGTATAAT	ACACTGTAATTGATGACCCCATTCT	75	Nucleoprotein gene	(Boxus et al., 2005)
BHV-1	CCGCCGTATTTTGAGGAGTCG	TCGGTCTCCCCTTCRTCCTC	70	glycoprotein G	(Wernike et al., 2011)

Table 3: Bead Regions and TAG Sequences

Pathogen	Bead Region	TAG Sequence 5'-3'	anti-TAG Sequence 5'-3'
BVD	12	CATAATCAATTTCAACTTTCTACT	AGTAGAAAGTTGAAATTGATTATG
M. bovis	18	ACACTTATCTTTCAATTCAATTAC	GTAATTGAATTGAAAGATAAGTGT
BRSV	29	TACTACTTCTATAACTCACTTAAA	TTTAAGTGAGTTATAGAAGTAGTA
BHV-1	54	TTAATACAATTCTCTCTTTCTCTA	TAGAGAAAGAGAGAATTGTATTAA

Conventional PCR for xTAG Preparation:

Prior to conventional PCR, extracted RNA was reverse-transcribed to cDNA using the Quantitect Reverse Transcriptase Kit (Qiagen, Hilden, Germany). The cDNA protocol was carried out on the Applied Biosystems GeneAmp PCR System 9700 thermocycler using the following conditions: a gDNA wipeout step for 2 minutes at 42°C followed by the RT step at 42°C for 12 minutes and 95°C for three minutes. Using Qiagen HotStarTaq PCR Master Mix Kit (Qiagen, Hilden, Germany), cDNA and extracted DNA were subject to multiplex conventional PCR using xTAG primers for BVD, BRSV, BHV-1 and *M. bovis.* For multiplex PCR, 5µl of extracted DNA and 5µl of cDNA from each lung was added to the PCR reaction for co-infected samples with 25µl HotStarTaq Master Mix, 1µl of each forward and reverse primer (10µM), and 12µl PCR-

grade H_2O. For *M. bovis* and BHV-1 positives, 5µl of extracted DNA was added to PCR. For BVD and BRSV positives, 5µl of cDNA was added to the PCR reaction. The singleplex reaction consists of 5µl of DNA extract or cDNA, 25µl HotStarTaq Master Mix, 1µl of the respective forward and reverse primers (10µM), and 18µl of PCR-grade H_2O. The following cycling protocol was used for PCR, 95°C for 15 minutes, 35 cycles of 94°C for 30 seconds with 60°C for 30 seconds and 72°C for 30 seconds, and a final step at 72°C for 5 minutes.

Luminex Data Acquisition and Analysis:

For analyte detection through Luminex xTAG, hybridization of the anti-TAG and the genomic targets was performed at 45°C for 30 minutes on a plate heater. Each reaction contained 16µg/ml of the co-reporter molecule, SAPE, in a filter sterilized hybridization buffer containing 0.2M NaCl, 0.1M Tris-HCl, 0.08% Triton x100, and 1% BSA at pH 8.0. The bead-mix contained four Luminex bead regions (12, 18, 29, 54) at a concentration of 1250 beads/reaction for each region diluted in hybridization buffer. 70µl of hybridization buffer + SAPE, 5µl of PCR template, and 20µl of bead-mix were mixed thoroughly before incubating. The Luminex MagPix instrument was set to read 70µl of sample and to record 100 events for each of the four bead regions. All data is represented as net MFI which is calculated by subtracting the MFI the background well from each sample's raw MFI value.

Optimization of the xTAG Assay:

After initial data were collected, the xTAG assay was improved at various steps of the protocol. SAPE was utilized at two concentrations, 8µg/µl and 16µg/µl to determine if SAPE concentration affects the final MFI. Samples pooled with all four targets were tested in duplicate using both SAPE concentrations. To determine the most optimal SAPE product for this study, Moss Bio SAPE and Thermo Fisher SAPE were compared using BVD and *M. bovis* positive samples tested in duplicate. The microsphere concentration of 1,250 beads/ml was decreased from the recommended 2,500 beads/ml. The conventional PCR annealing temperature was tested within a range of 55°C-63°C using band patterns on gel electrophoresis to determine the efficacy of each annealing temperature.

After collecting the data included below, more optimization techniques were explored to further improve the assay. The xTAG hybridization temperature was set to at 40°C, 45°C and 50°C on all positive controls and one positive sample for each pathogen in duplicate. The volume of extracted DNA or cDNA was increased to 10µl, and the resulting MFI was compared to when 10µl of PCR product was added to xTAG hybridization. Genetic product volume was investigated using BVDV positive samples.

Results:

Five bovine lung samples positive for each of the four pathogens were tested by xTAG and real-time PCR; MFI from xTAG and Ct values from RT-PCR are represented in Figure 1. BVDV is detected in 60% of the samples on xTAG with sample A nearly reaching the positive threshold of 1000 MFI (Figure 1A), providing a limit of detection at a Ct of 31. Using xTAG, *M. bovis* was detected in 20% of the lung samples that had previously tested positive on real-time PCR, revealing challenges with xTAG detection

of *M. bovis* (Figure 1B). There was a 100% detection rate for BRSV positive lung samples with MFI's above 14,000 in each sample and replicate (Figure 1C). Similarly, as seen in Figure 1D, BHV-1 is detected in 100% of the replicates with MFIs consistently crossing the positive threshold at high levels (Figure 1D).

Figure 1

BVDV Results

Sample ID	BVDV Ct	MFI
A	30.8	800
B	30.14	1,500
C	35.79	100
D	28.87	11,000
E	31.31	1,900

M. bovis Results

Sample ID	*M. bovis* Ct	MFI
A	37.43	-100
B	25.13	0
C	32.9	-100
D	33.19	4,000
E	22.86	-100

BRSV Results

Sample ID	BRSV Ct	MFI
A	33.98	17,500
B	32.9	18,200
C	36.4	14,300
D	28.36	18,500
E	30.37	18,800

BHV-1 Results

Sample ID	BHV-1 Ct	MFI
A	31.01	11,600
B	28.89	15,800
C	25.42	18,100
D	26.78	18,100
E	22.16	18,600

Figure 1: Twenty bovine lung samples tested side-by-side on real-time PCR and xTAG, in duplicate. Ct values measured by real-time PCR represent one replicate per sample and MFIs represent the average of two replicates rounded to the nearest hundred. Positive and negative controls were included with each sample set (data not shown).

A side-by-side comparison for six co-infected bovine lung samples demonstrates how xTAG detection may be affected when two pathogens are present (Figure 2). Co-infected BVDV samples demonstrate similar detection ability as in the single positive experiment, with detection dropping off at a Ct of 31 as obtained when comparing samples A and D. *M. bovis* is detected in 80% of the replicates with no clear trend of

MFI to Ct value and when compared to more detectable pathogens in this assay, the MFIs seen for *M. bovis* are low for the corresponding Ct value. BRSV was detected in 100% of the replicates that were also positive on real-time PCR, the outlier being sample B which had tested positive when initial diagnostic testing was conducted and appeared negative in this study. BHV-1 is detected in 100% of its replicates (samples E and F) with MFIs surpassing the positive threshold at high levels similar to the single positive lungs (Figure 1).

Figure 2: Co-infected Comparison

Sample ID	Target	xTAG Result	Real-Time Ct
A	BVDV	14,200	22.97
	M. bovis	3,700	21.46
B	BRSV	0*	0*
	M. bovis	5,200	23.25
C	BRSV	13,700	39.92
	M. bovis	3,200	25.05
D	BRSV	3,300	34.27
	BVDV	1,000	31.43
E	BHV-1	19,000	27.31
	M. bovis	300	34.65
F	BHV-1	19,500	26.15
	M. bovis	0	23.93

Figure 2: Bovine lung samples that previously tested positive for two BRD pathogens were tested side-by-side on real-time PCR and xTAG in duplicate. Ct values gathered from RT-PCR represent one replicate per sample and MFIs represent the average of two replicates rounded to the nearest hundred. *This sample previously tested positive for BRSV prior to this study and upon testing for this study tested negative on both real-time PCR and xTAG.

Figure 3 represents two groups of samples positive for one of the four pathogens, pooled in all combinations of two, three, and four pathogens. The y-axis indicates the MFI obtained from xTAG and the x-axis contains the groups of pathogens that were

pooled for testing. Each MFI is represented by a colored bar in the pathogen's respective pool. Error bars represent the standard deviation between two replicates run simultaneously on xTAG. For each of the single positive pools, all MFIs are well above the positive threshold of 1000 MFI; although, as pathogens are added to each pool, the MFI for each respective pathogen decreases. This trend is seen in group 1 (Figure 3a) and group 2 (Figure 3b).

Figure 3a: Pooled Sample Comparison (Group 1)

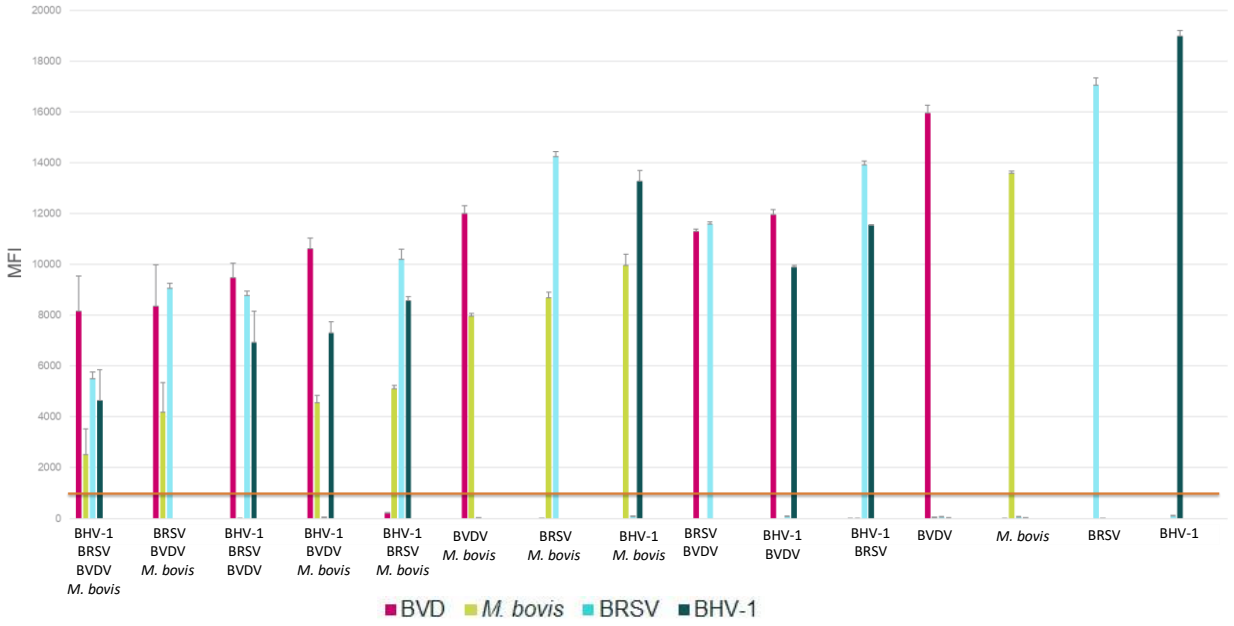

Figure 3b: Pooled Sample Comparison (Group 2)

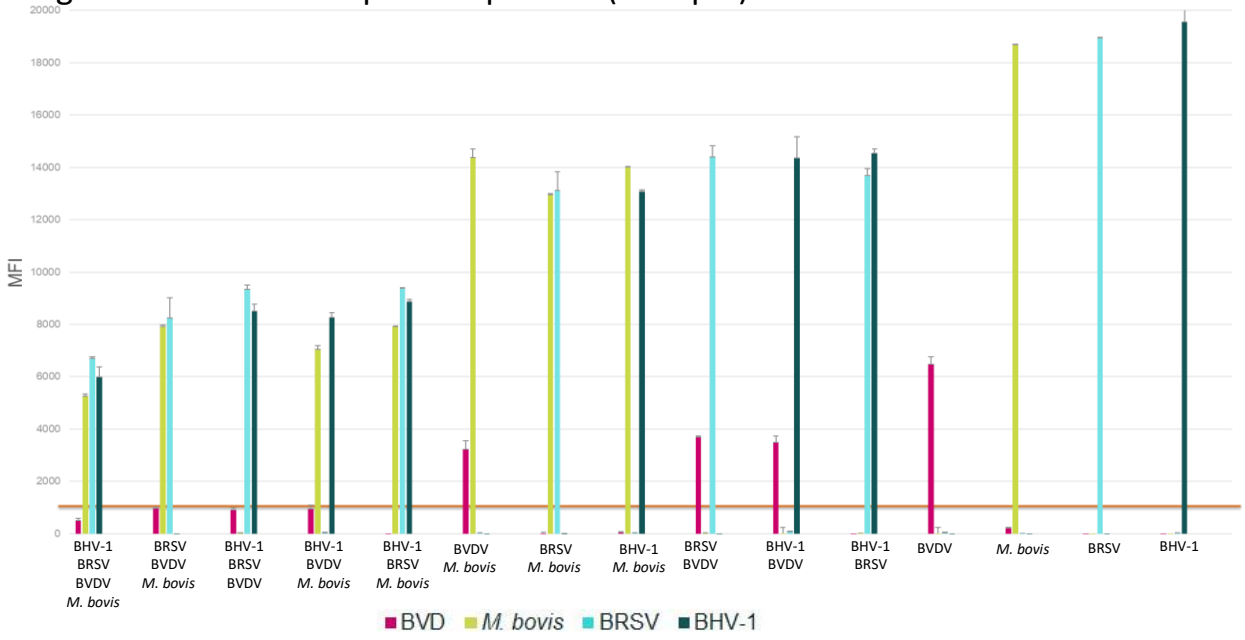

Figure 3: Positive samples for each pathogen were tested in duplicate using the multiplex xTAG assay and pooled in each combination of two, three and four pathogens. Two distinct groups of four samples are shown. The data shown represents the average of both replicates and error bars are set as the standard deviation between the two replicates. The positive threshold of 1000 MFI is shown as an orange line. 3a: Sample Group 1. 3b: Sample Group 2.

Through most of the assay's development, SAPE from Moss Bio was utilized. According to data provided on Moss Bio's website, ThermoFisher SAPE was found to produce notably lower MFIs which was also seen in preliminary tests in this study. On BVD positive lung samples, Moss Bio SAPE yields an MFI of 16,253, while ThermoFisher SAPE yields an MFI of 4,617. This trend was detected in *M. bovis* samples as well as the positive amplification controls (PAC) used in a previous run with ThermoFisher SAPE.

Discussion:

We compared commonly used real-time PCR assays to a newly developed multiplex Luminex xTAG assay, testing for BRSV, BVDV, BHV-1, and *M. bovis.* We analyzed Ct value results from the real-time PCR and compared to MFI from the xTAG assay. In the diagnostic setting, Luminex xTAG technology may improve upon PCR when detecting multiple pathogens through multiplexing the assay, due to its high-throughput and modular qualities. By adding or removing primers and Luminex microspheres for certain pathogens in the protocol, xTAG can detect up to 80 analytes per sample demonstrating that Luminex xTAG has a unique flexibility that is uncommon in other multiplex assays. This flexibility ensures that the panel can evolve if certain pathogens are no longer needed in the panel, or a new pathogen needs to be added to the panel. The bovine respiratory disease panel developed in this study demonstrates that multiplex Luminex xTAG results are comparable to real-time PCR when testing BRSV positive and BHV-1 positive samples and are less comparable when testing

BVDV positive and *M. bovis* positive samples. Additionally, this assay was able to detect multiple BRD pathogens in co-infected animals as well as in artificially spiked replicates.

We wanted to determine the ability of each assay to detect four BRD pathogens and, where possible, assess the thresholds of detection between both assays. A positive threshold of 1000 MFI is applied to all samples regardless of which pathogen is being detected. BRSV positive lung samples were compared on both assays revealing high sensitivity for the virus. With Ct values ranging from 28 to 36, xTAG maintained MFIs above 14,000 through all replicates providing an MFI of 13,655 on a sample with a corresponding Ct of 39.92. However, a sample in the co-infected group has a BRSV Ct of 34.27 and an MFI of 3,298 which is an outlier to the trend seen in single-positive samples. With MFI values so high for high Ct samples, the BRSV portion of the xTAG assay may provide better detection of the pathogen than PCR. Comparing the behavior of MFI and Ct values reveals that MFI may not always provide qualitative information that Ct values provide, which can be used to assess how much of the genetic target is present relative to other samples.

BVDV samples were tested by both assays side-by-side, revealing that xTAG provides accurate detection of BVDV in samples that are positive on PCR with Ct values below 31, detecting BVDV in three out of five samples. This suggests that the BVDV portion of the xTAG assay is not as effective at detecting BVDV compared to the real-time PCR assay currently in-use. This trend is observed in bovine lungs co-infected with BVDV and other BRD pathogens where an MFI of 1045 corresponds to a Ct of 31.43 and an MFI of 14,000 corresponds to a Ct of 22.97. Results for *M. bovis* positives reveal poor detection of all but one sample through xTAG. Comparing Ct values to MFI,

it is evident that the real-time PCR assay is noticeably more effective only detecting *M. bovis* in a sample with a Ct of 33. Poor detection of *M.* bovis was also found in co-infected samples E and F where real-time PCR detected *M. bovis*.

BHV-1 positive lung samples were tested on xTAG and real-time PCR. BHV-1 detection through xTAG shows high levels of detection throughout the Ct values detected by real-time PCR. With Ct values ranging from 22.16 to 31.01 and corresponding MFIs ranging from 18,600 and 11,600, xTAG detected BHV-1 consistently at a range of Ct values; however, it is unclear where the assay's ability to detect this pathogen drops off. Both assays perform similarly in BHV-1 detection and without weakly positive (high Ct) samples we are unable to assess which assay out-performs the other for weaker positive samples. Co-infected bovine lungs exhibit similar levels of detection as single-positive lungs, providing high MFIs of 19,000 for Ct values of 26 and 27.

It does not appear that MFI has a similar linear relationship to Ct values as samples are more positive or less positive. For example, samples D and E tested for BHV-1 have Ct values of 22.16 and 26.78 and MFIs of 18,100 and 18,600 respectively. The difference in Ct values represents roughly a 10-fold increase in BHV-1 nucleic acid. This increase is not represented in a change in MFI, especially since the higher Ct sample yields a slightly higher MFI in this case. It was common for MFI values to decrease dramatically as they approached the threshold of detection for the assay unlike real-time PCR where Ct values will increase linearly until the end of the cycling stage. Based on these findings with lung samples from BRD positive animals, real-time

PCR exhibits reliable detection capabilities and consistent relative quantification when compared to xTAG.

As more pathogens are present in a sample, MFI decreases consistently for all pathogens. This phenomenon further explains that MFI is not recommended as a quantification metric. The decrease in MFI is attributed to competition for SAPE in the hybridization step. Increasing or decreasing the SAPE concentration during hybridization corresponds with the resulting MFI, and further suggests that if more SAPE is present, MFI for all targets will increase. After finding the appropriate concentration of 16µg/µl, positive samples for all pathogens result above the threshold of detection through two different sample sets, aside from the *M. bovis* positive lung in Group 2. This competition for SAPE poses a concern for co-infected samples where one pathogen is much less present than another, resulting in decreased detection of all pathogens. This phenomenon was not observed in the co-infected animals; however, the trend of decreasing MFIs may be seen if additional pathogens were present in the co-infected sample group. It is important that the assay should detect suspect pathogens in co-infected samples.

In a publication by Xu *et al,* (Xu *et al.,* 2020) the authors mention that when designing primers for their xTAG assay, they ensured that all amplicons were close to 200 base pairs long to ensure that DNA amplification and hybridization were consistent through all targets. After pooling positive samples through two sample sets (Figure 3), there does not appear to be a preference for longer or shorter amplicons, at least during the hybridization step. However, the two smallest amplicons in this study, BRSV and BHV-1, coincidently have the most efficient xTAG reactions when compared to BVDV

and *M. bovis* amplicons which are larger. There may be aspects of these amplicons affecting the assay although it remains unclear if these differences in assay performance could be fixed through optimization or due to inconsistent amplicon size.

This protocol applies 100 events per bead region to the final MFI. Upwards of 1500 events are collected and 100 are applied to the mean fluorescence given by the software. It is possible that if the event number per bead is increased, the following MFI may be more representative of a quantitative result. xTAG panels provided by Luminex provide qualitative results and it is reasonable to conclude that the parameters set in the xPONENT software are optimized for qualitative output. This means that if the minimum event count is increased, each positive event represents a more accurate ratio of the genetic target in the sample. Overall, if Luminex xTAG were to be used in-place of RT-PCR, the information that Ct values provide would be sacrificed for the ease of multiplexing using xTAG.

Optimization:

Through developing an xTAG assay, optimizing temperatures and concentrations of reagents at various steps provided an in depth understanding of the assay. The major steps in the assay are the conventional PCR and the hybridization step. The hybridization step is simpler to optimize than PCR due to the reaction occurring at one temperature, 45°C. Unlike PCR, hybridization does not produce new genomic products which may affect reaction kinetics later in the protocol. To optimize the hybridization step, total sample volume, temperature, and bead concentration were adjusted without yielding significant changes to MFI. Increasing SAPE concentration from 8µg/µl to 16µg/µl yields higher MFIs for all targets without increasing the background

fluorescence. Higher SAPE concentration affected the co-infected samples' MFI in a similar manner which only partially mitigated the MFI inhibition detected when multiple pathogens are present. Hybridization temperature was determined to be most effective at 45°C when compared to 40°C and 50°C. At 45°C, MFIs for all samples were higher with the lowest MFIs resulting from hybridization at 50°C. TAG sequences contain primarily A/T base pairing which explains why the hybridization temperatures are lower than what is often used for other DNA assays. If more G/C base pairs were present this would increase the efficiency of the hybridization at higher temperatures.

Moss Bio (SAPE-001) and ThermoFisher (S866) SAPE were compared using BVD and *M. bovis* positive samples. We determined that the maximum MFI that can be achieved depends on the SAPE being used. The ThermoFisher SAPE peaks around 4,000-5,000 MFI whereas Moss Bio SAPE peaks near 16,000-18,000 MFI, showing no change in background MFI levels through both SAPE products. Five BVDV positive samples were tested in duplicate using ThermoFisher SAPE did not cross the positive threshold of 1000 MFI, demonstrating that this reagent is outperformed by the Moss Bio SAPE. The SAPE chosen for an xTAG experiment is extremely important to the output of the assay.

The Luminex Participant Guide (Luminex Corporation, 2014-2020) provided by Luminex recommends using 2,500 beads of each region per reaction. Through assay development, this was halved to 1,250 beads per region per reaction without a loss of MFI. The MagPix instrument is set to calculate only 100 events per bead region and having 1,250 beads per region in each sample, the instrument was consistently giving "total event" readings of 1,500 and as high as 2,200 in some cases. Decreasing the

number of beads in the assay does not optimize the assay for better results but rather saves money on reagents.

Doubling the extracted DNA or cDNA used in the PCR reaction had a greater effect on MFI than doubling the PCR product used in hybridization. This was conducted using the single positive BVDV lungs. BVDV lungs were chosen since only three out of five were detected on xTAG previously. The goal was to investigate if the conventional PCR step or hybridization step was responsible for increasing the final MFI value. All MFIs for these samples increased when 10µl of sample was added to PCR compared to when 10µl was added at the hybridization step. One sample is the exception where it appeared negative on both experiments. This suggests that if the PCR is optimized correctly, additional DNA extracts or cDNA can be a main point of optimizing the xTAG assay.

Conventional PCR became another point of optimization for this assay due to it being the first stage of the assay. The issues observed in early assay development were attributed to complications caused by multiplexing four primer sets with varying annealing temperatures. The annealing temperature was tested from 55°C to 63°C with 60°C being the most optimal. This annealing temperature resulted in distinct bands for each pathogen through gel electrophoresis while other temperatures resulted in weak bands or multiple banding patterns, indicating non-specific binding. Should certain primers interact poorly with other primers, the conventional PCR reactions can be run separately, and the separate reactions can be pooled during the hybridization step. This adds to xTAG's flexibility because if primers for two pathogens interact poorly, the assay can still be a reliable method of detection. In real-time PCR, poor primer design may

invalidate the entire assay with little to no workaround aside from optimization, which may decrease the assay's specificity for other targets.

Cost Analysis:

Practical implementation of xTAG depends on assay cost compared to the assay currently in use. These factors include: 1. accounting for only the reagents used in the xTAG protocol; and 2., each diagnostic sample from extraction to xPONENT analysis will cost $23.27 USD. This includes the optimization steps stated earlier in this manuscript regarding SAPE and bead region concentrations. However, each run of xTAG requires four positive PCR controls, one negative PCR control, and an xTAG background control increasing the cost per run at $60.21 USD. Depending on the laboratory, this may be significantly less expensive than PCR, particularly if the pathogens in the desired panel are currently being detected through singleplex RT-PCR. Calibration kits for the MagPix instrument cost roughly $1,136 USD annually if they are being used each week as directed by Luminex and add to the overall reagent cost to perform this assay.

Final Assessment and Future Directions:

Throughout the course of this study, the benefits and drawbacks of each assay became apparent. Both assays can achieve high quality diagnostic results where multiplex xTAG shows promise as an effective molecular assay due to its cost efficiency and its ease to multiplex targets while real-time PCR remains to be a robust and reliable method for molecular detection. Depending on a laboratory's financial budget,

standards for how results are quantified, and specific goals for their multiplex panel, either xTAG or real-time PCR may suit their needs. Real-time PCR detected all samples whether they were positive for one or multiple pathogens, where xTAG did not perform as well in the cases of *M. bovis* and BVDV. Using the optimization steps outlined previously, it is apparent that these detection issues with xTAG are a result of poor conventional PCR efficiency prior to hybridization. With ample PCR optimization, xTAG can achieve similar detection levels as real-time PCR.

Real-time PCR is a reliable choice for singleplex assays due to xTAG's design as a multiplex technique. The technician time allotted for a singleplex xTAG run would be greater than for a simple PCR run. Real-time PCR is desirable if the results need to be semi-quantifiable either by the technician or the client. Ct values provide semi quantification of the genetic target where MFI is designed to give a qualitative result. The client may prefer having Ct values to compare to other submissions in the past, gathering insight on the current case or previous cases.

xTAG has the advantage of testing large panels or pathogens are performed. Hospitals may run large, multiplexed panels to rule out common pathogens to ensure prompt treatment. These assays typically provide only qualitative results. In this environment, xTAG can provide the desired results in a reasonable turnaround time. The total cost of an xTAG run is cheaper compared to real-time PCR assays and utilizes less reagents compared to multiple singleplex PCRs where various kits are used. xTAG provides a streamlined method to multiplex genetic targets into one assay and while it may be effective in certain fields of diagnostics, real-time PCR ultimately is more effective and well known throughout the industry.

In the future, the xTAG assay in this study can be adapted and expanded. The turnaround time from sample prep to results is much longer than that of real-time PCR. Utilizing a DNA/RNA extraction kit instead of performing two extractions will save time and optimizing a one-step PCR kit will remove the need to make cDNA for all samples. With further optimization of the BVDV and *M. bovis* conventional PCRs, their detection using xTAG may be on par with the current real-time PCR. This will include changing primer concentrations or separating these two pathogens into their own duplex PCR to ensure their reaction kinetics are not interfered with by other components of the assay. To gain a better understanding of how MFI changes as the target DNA increases or decreases, a limit of detection assay with DNA concentrations should be performed.

Chapter 3: Discussion

Project Summary

We tested 28 bovine lung samples by real-time PCR and Luminex xTAG for four common bovine respiratory pathogens: BVDV, *M. bovis,* BRSV, and BHV-1. This study compares commonly used real-time PCR methodology to a multiplex Luminex xTAG assay. The bovine respiratory disease panel developed in this study shows, using diagnostic samples, that this multiplex Luminex xTAG assay gives comparable results to real-time PCR when testing BRSV positive and BHV-1 positive samples, and less comparable results when testing BVDV positive and *M. bovis* positive samples. Additionally, this assay was able to detect multiple BRD pathogens in co-infected animals as well as in artificially spiked replicates. Standardizing Ct values that result from real-time PCR as compared to MFI results from xTAG will provide diagnosticians with a deeper understanding of the newly developed assay. In the diagnostic setting, Luminex xTAG technology may be a great choice when testing for pathogens in panels due to the high-throughput and modular nature of this assay. By adding or removing primers and Luminex beads for specific pathogen genomic targets, Luminex xTAG has a unique flexibility that is uncommon in other multiplex assays. This flexibility ensures that the panel can evolve if certain pathogens are no longer needed in the panel, or a new pathogen target needs to be added to the panel.

Concerns with Assay Optimization

As the xTAG assay was developed, it became apparent which aspects could be improved upon. Notably, BVDV and *M. bovis* detection using xTAG was poor when compared to BHV-1 and BRSV. Likewise, the results of MFI decreasing as additional pathogens were added is concerning to the validity of the assay. To address the poor detection of BVDV, the volume of BVDV product was doubled in preparation for xTAG hybridization or the conventional PCR reaction. In samples where the volume was doubled for xTAG hybridization, MFI either increased or decreased depending on the samples (Figure 1). None of these changes were significant enough to increase MFIs above the threshold of detection. Doubling the volume of BVDV cDNA prior to the conventional PCR reaction increased MFIs for each sample by varying degrees, resulting in a previously negative sample above the threshold of detection. This sample's MFI increased from 800 to 2,500 (Figure 4) while other samples had similar results. The BVDV portion of this assay was improved by increasing the volume of BVDV cDNA prior to the PCR reaction, bringing the limit of detection from a Ct of 31 to nearly 35. This suggests that limit of detection issues are associated with PCR efficiency instead of an issue within the xTAG protocol. Increasing the cDNA volume put into the PCR reaction was more effective because PCR amplifies the target exponentially whereas the hybridization step is simply detecting the product that is already there. Furthermore, increasing cDNA volume for PCR will more than double the amplified target when compared to taking twice the volume of your PCR product.

Figure 4

	MFI		
Sample ID	Original	10uL into xTAG	10uL into PCR
A	800	900	2,500
B	1,500	4,000	8,200
C	100	0	600
D	11,000	8,100	11,400
E	1,900	3,500	4,800

Figure 4: Five BVDV positive lungs tested on three different xTAG protocols. Original: Uses the xTAG protocol outlines in the material and methods section. 10µL into xTAG: Doubles the PCR product volume from 5µL to 10µL going into the xTAG hybridization reaction. 10µL into PCR: Doubles the cDNA volume from 5µL to 10µL going into the conventional PCR reaction. Each sample was run in duplicate and MFI is the average of each replicate rounded to the nearest hundred.

MFI was measured for four samples, one for each pathogen, while adjusting the hybridization temperature to 40°C, 45°C, or 50°C (Figure 5). The duration of the hybridization step was kept at 30 minutes to observe how hybridization temperature affects MFI. The diagnostic samples exhibited the highest MFI when hybridized at 45°C, with minimal variation between the 40°C and 50°C groups. Regarding the positive controls for each pathogen, a maximum difference of 2,000 MFI was detected for BHV-1 and BRSV samples between the 45°C and 50°C runs. Positive controls for each pathogen resulted in similar variation in some cases (BVDV and BHV-1) although their

Figure 5

		MFI		
Pathogen	Sample ID	40C	45C	50C
BVD	A	5,200	5,500	4,500
M. bovis	B	0	0	0
BRSV	C	6,200	6,500	4,400
BHV-1	D	14,000	15,100	13,100
BVD	BVD PAC	17,500	17,800	16,000
M. bovis	*M. bovis* PAC	13,100	14,400	14,100
BRSV	BRSV PAC	18,500	19,400	18,500
BHV-1	BHV-1 PAC	19,000	20,000	18,000

Figure 5: Positive lungs for each pathogen were tested using the xTAG protocol with modifications to the hybridization temperature. 45°C is representative of the control group as this is the temperature recommended by Luminex. Each sample was run in duplicate and MFI is the average of each replicate rounded to the nearest hundred.

MFI was consistently at the upper limit and a 2,000 MFI increase or decrease is common intraassay variation that may be seen if the hybridization temperature was kept consistent. While the hybridization temperature seems to play a role in the reaction's efficiency and the resulting MFI, optimizing this step for each target will be difficult given that all samples are present during this step. If different hybridization temperatures are determined to be optimal for each target, multiple 30-minute incubations would have to

be performed, lengthening the overall turnaround time of the assay. Keeping the hybridization temperature at 45°C as Luminex recommends will be optimal for xTAG efficiency.

To further optimize the assay, the hybridization buffer pH was altered to increase MFI for suspect samples. The xTAG Participant Guide suggests that the hybridization buffer has a pH of 8.0 and a more acidic pH will promote a stronger hybridization between nucleic acid strands. HCl was added to the hybridization buffer until it reached a pH of roughly 6. Upon xTAG analysis, all samples and positive controls resulted with an approximate MFI of 4000. This is surprising not only because MFI was expected to increase, but also because each sample had varying MFIs before the hybridization. All samples resulting in an MFI of roughly 4000 suggest that a more acidic hybridization buffer caps the amount of hybridization that can occur during the incubation period. Ensuring that the hybridization buffer has a pH of 8.0 is essential for this assay to output reliable results between runs and batches of reagents.

Luminex instructions (Luminex Corporation, 2006-2024) direct that 35 events are required to achieve reliable MFIs and that anything above 35 events will not change MFI; however, the xPONENT software has a default of 50 events. This was increased to 100 events after consultation with the Luminex company for optimal parameters for this study. It is possible that if the event number per bead is increased, the resulting MFI may be more representative of a quantitative result. This indicates that as the minimum event count is increased, each positive event represents a more accurate ratio of the genetic target in the sample. Nevertheless, xTAG panels provided by Luminex provide qualitative results and it is reasonable to conclude that the parameters set in the

xPONENT software are optimized for qualitative output. It is imperative to note that a variety of publications using xTAG technology are obtaining their samples from plasmid replication. In those cases, the samples will be much cleaner and free of potential PCR inhibitors when compared to the tissue samples used in this study. While real-time PCR and xTAG were conducted using the same tissue preparation, variation within the samples may arise especially when compared to nearly pure samples gathered from plasmid amplification.

Future Directions:

A limit of detection assay involving serial dilutions would provide a more detailed comparison of the two assays, observing how MFI and Ct values change with varying organism copy numbers. This will allow a diagnostician an in-depth analysis of target detection as it reaches the threshold, particularly if certain targets have less robust detection capabilities than others. The assay could then be modified, referencing the optimization techniques previously outlined. Including the DNA/cDNA copy number of samples before each assay would allow for points of comparison for other assays. Through identifying the limit of detection for each assay, it will become clear if certain pathogens are detected more readily than others as the dilution factor increases. Achieving reproducibility of high Ct/low MFI samples will demonstrate that this xTAG assay has low intra-assay variation. Currently, the xTAG workflow outlined here is more time consuming given that two nucleic acid extractions and a cDNA step are required for each sample prior to conventional PCR, which is typically longer than the time to conduct a real-time PCR assay. Ideally, xTAG preparation time will be similar to or faster than preparation time for real-time PCR. This can be achieved by using a DNA/RNA

extraction kit as well as a one-step PCR master mix kit to avoid the cDNA step prior to conventional PCR. Due to the underperforming *M. bovis* portion of the assay, reevaluating primers and the samples used in the study would be essential in further development of the panel. Utilizing the same primer sequence for both *M. bovis* RT-PCR and conventional xTAG PCR would provide consistencies between the assays, ensuring that each primer sequence is detecting the same target. There are dozens of *Mycoplasma* species and even though the *M. bovis* primers were designed to detect *M. bovis*, there is potential for cross reactivity between other *Mycoplasma* species.

Use in Molecular Laboratories:

Dundas et al, at the Children's Medical Center of Dallas, conducted a study comparing their direct fluorescent antibody (DFA) assay and the xTAG Respiratory Viral Panel (RVP) provided by Luminex. The researchers determined that the xTAG RVP assay had a higher detection rate compared to their DFA and was more suited for high sample volume. The RVP assay includes primers and beads for 12 viral respiratory pathogens, making it a more cost-efficient multiplexed assay, considering that hospitals are typically testing samples for a larger number of pathogens. Luminex xTAG was beneficial in its ability to be multiplexed while maintaining accurate detection of certain pathogens. Additionally, the client's needs are met with this assay because human diagnostic panels are commonly used to only observe the presence of a pathogen to inform treatment of the patient. This assay shows promise within the human hospital setting as outlined by Dundas et al. where caseload is high and xTAG was demonstrated to be a more sensitive assay.

xTAG may be useful in diagnostic laboratories depending on the needs of the client. MFI results are not a quantitative output as compared to Ct values, meaning that if a lab utilizes xTAG they would be sacrificing a semi-quantitative assay for a qualitative assay. One benefit of using xTAG in certain cases is the multiplexing capability, which in a molecular lab would save time. Compared to running four separate PCRs, this xTAG assay provides a streamlined way to test for BRD pathogens. However, PCRs for BVDV, BRSV, BHV-1 and *M. bovis*. are not exclusively reserved for bovine respiratory screens. There are a variety of sample types and animals where testing for one or more of these pathogens would be reasonable, meaning that even if this multiplex xTAG assay were to be implemented, all listed singleplex PCRs would still be used at nearly the same frequency depending on the laboratory. The multiplex xTAG would only remove bovine respiratory screen requests from these PCR runs, overall adding a whole new assay to train personnel on and maintain reagents for. Each run using xTAG would require a minimum of seven reactions, a control for each of the four pathogens, negative PCR and xTAG controls with at least one diagnostic sample. Diagnostic caseload for bovine respiratory screens may not justify using this assay given that multiple diagnostic samples would have to be included to offset the cost of all necessary controls. xTAG provides qualitative results and does not take large differences in MFI into account during analysis, meaning that the results are analogous to running an electrophoresis gel to observe the presence or absence of PCR product. Developing a multiplex conventional PCR with four targets is achievable and would provide qualitative results similar to the premade Luminex panels.

Based on this analysis, Luminex xTAG would be better suited for panel testing of more than four targets. As more targets are added to a conventional PCR, the gel will become more difficult to read due to similar band lengths. Real-time PCR may also be difficult to multiplex as more targets are added due to additional probes that may interfere with reaction kinetics. Since the beads with anti-TAGs are added after the PCR reaction, this allows for more targets to be included in PCR. It is important to address the possibility of contamination when regarding newer assays, and this assay poses a higher risk of contamination compared to real-time PCR. The hybridization step requires amplified genetic product be added to the reaction, leaving room for the highly concentrated positive samples to contaminate nearby wells. While this can be mitigated by careful technique, it is still worth noting as an important difference between the two assays' protocols. Future comparisons of xTAG to other assays such as serology tests or ELISAs may demonstrate other uses for xTAG assays in ways that this PCR comparison did not highlight.

Bibliography:

Ackermann, M., & Engels, M. (2006). Pro and contra IBR-eradication. *Veterinary Microbiology*, *113*(3), 293–302. https://doi.org/10.1016/j.vetmic.2005.11.043

Askar, H., Chen, S., Hao, H., Yan, X., Ma, L., Liu, Y., & Chu, Y. (2021). Immune Evasion of Mycoplasma bovis. *Pathogens*, *10*(3), Article 3. https://doi.org/10.3390/pathogens10030297

Atreya, P. L., Peeples, M. E., & Collins, P. L. (1998). The NS1 Protein of Human Respiratory Syncytial Virus Is a Potent Inhibitor of Minigenome Transcription and RNA Replication. *Journal of Virology*, *72*(2), 1452–1461.

Babiuk, L. A., van Drunen Littel-van den Hurk, S., & Tikoo, S. K. (1996). Immunology of bovine herpesvirus 1 infection. *Veterinary Microbiology*, *53*(1), 31–42. https://doi.org/10.1016/S0378-1135(96)01232-1

Bauermann, F. V., Falkenberg, S. M., Vander Ley, B., Decaro, N., Brodersen, B. W., Harmon, A., Hessman, B., Flores, E. F., & Ridpath, J. F. (2020). Generation of Calves Persistently Infected with HoBi-Like Pestivirus and Comparison of Methods for Detection of These Persistent Infections. *Journal of Clinical Microbiology*, *52*(11), 3845–3852. https://doi.org/10.1128/jcm.01563-14

Beaudeau, F., Ohlson, A., & Emanuelson, U. (2010). Associations between bovine coronavirus and bovine respiratory syncytial virus infections and animal performance in Swedish dairy herds. *Journal of Dairy Science*, *93*(4), 1523–1533. https://doi.org/10.3168/jds.2009-2511

Behera, S., Rana, R., Gupta, P. K., Kumar, D., Sonal, Rekha, V., Arun, T. R., & Jena, D. (2018). Development of real-time PCR assay for the detection of Mycoplasma bovis. *Tropical Animal Health and Production*, *50*(4), 875–882. https://doi.org/10.1007/s11250-018-1510-1

Biswas, S., Bandyopadhyay, S., Dimri, U., & H. Patra, P. (2013). Bovine herpesvirus-1 (BHV-1) – a re-emerging concern in livestock: A revisit to its biology, epidemiology, diagnosis, and prophylaxis. *Veterinary Quarterly*, *33*(2), 68–81. https://doi.org/10.1080/01652176.2013.799301

Bolin, S. R. (2002). Bovine Viral Diarrhea Virus in Mixed Infection. In *Polymicrobial Diseases*. ASM Press. https://www.ncbi.nlm.nih.gov/books/NBK2491/

Bossert, B., Marozin, S., & Conzelmann, K.-K. (2003). Nonstructural Proteins NS1 and NS2 of Bovine Respiratory Syncytial Virus Block Activation of Interferon Regulatory Factor 3. *Journal of Virology*, *77*(16), 8661–8668. https://doi.org/10.1128/JVI.77.16.8661-8668.2003

Boulard, C., & Villejoubert, C. (1991). Use of pooled serum or milk samples for the epidemiological surveillance of bovine hypodermosis. *Veterinary Parasitology*, *39*(1), 171–183. https://doi.org/10.1016/0304-4017(91)90072-4

Boxus, M., Letellier, C., & Kerkhofs, P. (2005). Real Time RT-PCR for the detection and quantitation of bovine respiratory syncytial virus. *Journal of Virological Methods*, *125*(2), 125–130. https://doi.org/10.1016/j.jviromet.2005.01.008

Brownie, J., Shawcross, S., Theaker, J., Whitcombe, D., Ferrie, R., Newton, C., & Little, S. (1997). The elimination of primer-dimer accumulation in PCR. *Nucleic Acids Research*, *25*(16), 3235–3241. https://doi.org/10.1093/nar/25.16.3235

Campbell, J. (n.d.). *Overview of Bovine Respiratory Disease Complex—Respiratory System*. Merck Veterinary Manual. Retrieved April 10, 2024, from https://www.merckvetmanual.com/respiratory-system/bovine-respiratory-disease-complex/overview-of-bovine-respiratory-disease-complex

Ciacci-Zanella, J., Stone, M., Henderson, G., & Jones, C. (1999). The Latency-Related Gene of Bovine Herpesvirus 1 Inhibits Programmed Cell Death. *Journal of Virology*, *73*(12), 9734–9740. https://doi.org/10.1128/jvi.73.12.9734-9740.1999

Cong, F., Zhu, Y., Wang, J., Lian, Y., Liu, X., Xiao, L., Huang, R., Zhang, Y., Chen, M., & Guo, P. (2018). A multiplex xTAG assay for the simultaneous detection of five chicken immunosuppressive viruses. *BMC Veterinary Research*, *14*(1), 347. https://doi.org/10.1186/s12917-018-1663-1

Connolly, S. A., Whitbeck, J. C., Rux, A. H., Krummenacher, C., van Drunen Littel-van den Hurk, S., Cohen, G. H., & Eisenberg, R. J. (2001). Glycoprotein D Homologs in Herpes Simplex Virus Type 1, Pseudorabies Virus, and Bovine Herpes Virus Type 1 Bind Directly to Human HveC (Nectin-1) with Different Affinities. *Virology*, *280*(1), 7–18. https://doi.org/10.1006/viro.2000.0747

Cradic, K. W., Wells, J. E., Allen, L., Kruckeberg, K. E., Singh, R. J., & Grebe, S. K. G. (2004). Substitution of 3′-Phosphate Cap with a Carbon-Based Blocker Reduces the Possibility of Fluorescence Resonance Energy Transfer Probe Failure in Real-Time PCR Assays. *Clinical Chemistry*, *50*(6), 1080–1082. https://doi.org/10.1373/clinchem.2004.033183

Drew, T. W., Yapp, F., & Paton, D. J. (1999). The detection of bovine viral diarrhoea virus in bulk milk samples by the use of a single-tube RT-PCR. *Veterinary*

Microbiology, *64*(2), 145–154. https://doi.org/10.1016/S0378-1135(98)00266-1

Dudek, K., Nicholas, R. A. J., Szacawa, E., & Bednarek, D. (2020). Mycoplasma bovis Infections—Occurrence, Diagnosis and Control. *Pathogens*, *9*(8), Article 8. https://doi.org/10.3390/pathogens9080640

Dundas, N. E., Ziadie, M. S., Revell, P. A., Brock, E., Mitui, M., Leos, N. K., & Rogers, B. B. (2011). A Lean Laboratory: Operational Simplicity and Cost Effectiveness of the Luminex xTAG™ Respiratory Viral Panel. *The Journal of Molecular Diagnostics*, *13*(2), 175–179. https://doi.org/10.1016/j.jmoldx.2010.09.003

Elshal, M. F., & McCoy, J. P. (2006). Multiplex bead array assays: Performance evaluation and comparison of sensitivity to ELISA. *Methods*, *38*(4), 317–323. https://doi.org/10.1016/j.ymeth.2005.11.010

Elvander, M. (1996). Severe respiratory disease in dairy cows caused by infection with bovine respiratory syncytial virus. *Veterinary Record*, *138*(5), 101–105. https://doi.org/10.1136/vr.138.5.101

Evans, C. A., Pinior, B., Larska, M., Graham, D., Schweizer, M., Guidarini, C., Decaro, N., Ridpath, J., & Gates, M. C. (2019). Global knowledge gaps in the prevention and control of bovine viral diarrhoea (BVD) virus. *Transboundary and Emerging Diseases*, *66*(2), 640–652. https://doi.org/10.1111/tbed.13068

Ferraro, S., Fecteau, G., Dubuc, J., Francoz, D., Rousseau, M., Roy, J.-P., & Buczinski, S. (2021). Scoping review on clinical definition of bovine respiratory disease complex and related clinical signs in dairy cows. *Journal of Dairy Science*, *104*(6), 7095–7108. https://doi.org/10.3168/jds.2020-19471

Forman, A. J., Babiuk, L. A., Misra, V., & Baldwin, F. (1982). Susceptibility of bovine

macrophages to infectious bovine rhinotracheitis virus infection. *Infection and Immunity*, *35*(3), 1048–1057. https://doi.org/10.1128/iai.35.3.1048-1057.1982

Fuchs, M., Hübert, P., Detterer, J., & Rziha, H.-J. (1999). Detection of Bovine Herpesvirus Type 1 in Blood from Naturally Infected Cattle by Using a Sensitive PCR That Discriminates between Wild-Type Virus and Virus Lacking Glycoprotein E. *Journal of Clinical Microbiology*, *37*(8), 2498–2507. https://doi.org/10.1128/jcm.37.8.2498-2507.1999

Fulton, R. W., Purdy, C. W., Confer, A. W., Saliki, J. T., Loan, R. W., Briggs, R. E., & Burge, L. J. (2000). Bovine viral diarrhea viral infections in feeder calves with respiratory disease: Interactions with Pasteurella spp., parainfluenza-3 virus, and bovine respiratory syncytial virus. *Canadian Journal of Veterinary Research*, *64*(3), 151–159.

Fux, R., & Wolf, G. (2012). Transient elimination of circulating bovine viral diarrhoea virus by colostral antibodies in persistently infected calves: A pitfall for BVDV-eradication programs? *Veterinary Microbiology*, *161*(1), 13–19. https://doi.org/10.1016/j.vetmic.2012.07.001

Gadsby, N. J., McHugh, M. P., Russell, C. D., Mark, H., Conway Morris, A., Laurenson, I. F., Hill, A. T., & Templeton, K. E. (2015). Development of two real-time multiplex PCR assays for the detection and quantification of eight key bacterial pathogens in lower respiratory tract infections. *Clinical Microbiology and Infection*, *21*(8), 788.e1-788.e13. https://doi.org/10.1016/j.cmi.2015.05.004

García-Arroyo, L., Prim, N., Martí, N., Roig, M. C., Navarro, F., & Rabella, N. (2016). Benefits and drawbacks of molecular techniques for diagnosis of viral respiratory

infections. Experience with two multiplex PCR assays. *Journal of Medical Virology*, *88*(1), 45–50. https://doi.org/10.1002/jmv.24298

Geerdink, R. J., Pillay, J., Meyaard, L., & Bont, L. (2015). Neutrophils in respiratory syncytial virus infection: A target for asthma prevention. *Journal of Allergy and Clinical Immunology*, *136*(4), 838–847. https://doi.org/10.1016/j.jaci.2015.06.034

Gershwin, L. J. (2007). Bovine respiratory syncytial virus infection: Immunopathogenic mechanisms. *Animal Health Research Reviews*, *8*(2), 207–213. https://doi.org/10.1017/S1466252307001405

Gil, L. H. V. G., Ansari, I. H., Vassilev, V., Liang, D., Lai, V. C. H., Zhong, W., Hong, Z., Dubovi, E. J., & Donis, R. O. (2006). The Amino-Terminal Domain of Bovine Viral Diarrhea Virus Npro Protein Is Necessary for Alpha/Beta Interferon Antagonism. *Journal of Virology*, *80*(2), 900–911. https://doi.org/10.1128/jvi.80.2.900-911.2006

Gondaira, S., Nishi, K., Tanaka, T., Yamamoto, T., Nebu, T., Watanabe, R., Konnai, S., Hayashi, T., Kiku, Y., Okamoto, M., Matsuda, K., Koiwa, M., Iwano, H., Nagahata, H., & Higuchi, H. (2020). Immunosuppression in Cows following Intramammary Infusion of Mycoplasma bovis. *Infection and Immunity*, *88*(3), e00521-19. https://doi.org/10.1128/IAI.00521-19

Gormley, E., Corner, L. A. L., Costello, E., & Rodriguez-Campos, S. (2014). Bacteriological diagnosis and molecular strain typing of *Mycobacterium bovis* and *Mycobacterium caprae*. *Research in Veterinary Science*, *97*, S30–S43. https://doi.org/10.1016/j.rvsc.2014.04.010

Griffin, D., Chengappa, M. M., Kuszak, J., & McVey, D. S. (2010). Bacterial Pathogens

of the Bovine Respiratory Disease Complex. *Veterinary Clinics of North America: Food Animal Practice*, *26*(2), 381–394. https://doi.org/10.1016/j.cvfa.2010.04.004

Grooms, D. L. (2004). Reproductive consequences of infection with bovine viral diarrhea virus. *Veterinary Clinics: Food Animal Practice*, *20*(1), 5–19. https://doi.org/10.1016/j.cvfa.2003.11.006

Haapala, V., Pohjanvirta, T., Vähänikkilä, N., Halkilahti, J., Simonen, H., Pelkonen, S., Soveri, T., Simojoki, H., & Autio, T. (2018). Semen as a source of Mycoplasma bovis mastitis in dairy herds. *Veterinary Microbiology*, *216*, 60–66. https://doi.org/10.1016/j.vetmic.2018.02.005

Haapala, V., Vähänikkilä, N., Kulkas, L., Tuunainen, E., Pohjanvirta, T., Autio, T., Pelkonen, S., Soveri, T., & Simojoki, H. (2021). *Mycoplasma bovis* infection in dairy herds—Risk factors and effect of control measures. *Journal of Dairy Science*, *104*(2), 2254–2265. https://doi.org/10.3168/jds.2020-18814

Hage, J. J., Glas, R. D., Westra, H. H., Maris-Veldhuis, M. A., Van Oirschot, J. T., & Rijsewijk, F. A. M. (1998). Reactivation of latent bovine herpesvirus 1 in cattle seronegative to glycoproteins gB and gE. *Veterinary Microbiology*, *60*(2), 87–98. https://doi.org/10.1016/S0378-1135(97)00152-1

Hendricks, D. A., McIntosh, K., & Patterson, J. L. (1988). Further characterization of the soluble form of the G glycoprotein of respiratory syncytial virus. *Journal of Virology*, *62*(7), 2228–2233. https://doi.org/10.1128/jvi.62.7.2228-2233.1988

Houe, H. (2003). Economic impact of BVDV infection in dairies. *Biologicals*, *31*(2), 137–143. https://doi.org/10.1016/S1045-1056(03)00030-7

Hrdy, J., Vasickova, P., Nesvadbova, M., Novotny, J., Mati, T., & Kralik, P. (2021).

MOL-PCR and xMAP Technology: A Multiplex System for Fast Detection of Food- and Waterborne Viruses. *The Journal of Molecular Diagnostics*, *23*(6), 765–776. https://doi.org/10.1016/j.jmoldx.2021.03.005

Inman, M., Lovato, L., Doster, A., & Jones, C. (2002). A Mutation in the Latency-Related Gene of Bovine Herpesvirus 1 Disrupts the Latency Reactivation Cycle in Calves. *Journal of Virology*, *76*(13), 6771–6779. https://doi.org/10.1128/jvi.76.13.6771-6779.2002

Jones, C., da Silva, L. F., & Sinani, D. (2011). Regulation of the latency–reactivation cycle by products encoded by the bovine herpesvirus 1 (BHV-1) latency-related gene. *Journal of NeuroVirology*, *17*(6), 535–545. https://doi.org/10.1007/s13365-011-0060-3

Kalina, W. V., Woolums, A. R., Berghaus, R. D., & Gershwin, L. J. (2004). Formalin-inactivated bovine RSV vaccine enhances a Th2 mediated immune response in infected cattle. *Vaccine*, *22*(11), 1465–1474. https://doi.org/10.1016/j.vaccine.2003.10.024

Kanci, A., Wawegama, N. K., Marenda, M. S., Mansell, P. D., Browning, G. F., & Markham, P. F. (2017). Reproduction of respiratory mycoplasmosis in calves by exposure to an aerosolised culture of Mycoplasma bovis. *Veterinary Microbiology*, *210*, 167–173. https://doi.org/10.1016/j.vetmic.2017.09.013

Karger, A., Saalmüller, A., Tufaro, F., Banfield, B. W., & Mettenleiter, T. C. (1995). Cell surface proteoglycans are not essential for infection by pseudorabies virus. *Journal of Virology*, *69*(6), 3482–3489. https://doi.org/10.1128/jvi.69.6.3482-3489.1995

KIM, H. W., CANCHOLA, J. G., BRANDT, C. D., PYLES, G., CHANOCK, R. M., JENSEN, K., & PARROTT, R. H. (1969). RESPIRATORY SYNCYTIAL VIRUS DISEASE IN INFANTS DESPITE PRIOR ADMINISTRATION OF ANTIGENIC INACTIVATED VACCINE12. *American Journal of Epidemiology*, *89*(4), 422–434. https://doi.org/10.1093/oxfordjournals.aje.a120955

Kimman, T. G., Sol, J., Westenbrink, F., & Strayer, P. J. (1989). A severe outbreak of respiratory tract disease associated with bovine respiratory syncytial virus probably enhanced by vaccination with modified live vaccine. *Veterinary Quarterly*, *11*(4), 250–253. https://doi.org/10.1080/01652176.1989.9694231

Klima, C. L., Zaheer, R., Cook, S. R., Booker, C. W., Hendrick, S., Alexander, T. W., & McAllister, T. A. (2014). Pathogens of Bovine Respiratory Disease in North American Feedlots Conferring Multidrug Resistance via Integrative Conjugative Elements. *Journal of Clinical Microbiology*, *52*(2), 438–448. https://doi.org/10.1128/JCM.02485-13

Larsen, L. E. (2000). Bovine Respiratory Syncytial Virus (BRSV): A review. *Acta Veterinaria Scandinavica*, *41*(1), 1–24. https://doi.org/10.1186/BF03549652

Larson, R. L., Grotelueschen, D. M., Brooks, K. V., Hunsaker, B. D., Smith, R. A., Sprowls, R. W., MacGregor, D. S., Loneragan, G. H., & Dargatz, D. A. (n.d.). *Bovine viral diarrhea (BVD): Review for beef cattle veterinarians | The Bovine Practitioner*. The Bovine Practitioner. Retrieved April 9, 2024, from https://bovine-ojs-tamu.tdl.org/bovine/article/view/1705

Letellier, C., & Kerkhofs, P. (2003). Real-time PCR for simultaneous detection and genotyping of bovine viral diarrhea virus. *Journal of Virological Methods*, *114*(1),

21–27. https://doi.org/10.1016/j.jviromet.2003.08.004

Luminex Platform Technology—US. (n.d.). Retrieved April 9, 2024, from https://www.thermofisher.com/us/en/home/life-science/antibodies/immunoassays/procartaplex-assays-luminex/features.html

MacGregor, S., & Wray, M. (n.d.). *The Effect of bovine respiratory syncytial virus vaccination on health, feedlot performance and carcass characteristics of feeder cattle | The Bovine Practitioner*. Retrieved April 9, 2024, from https://bovine-ojs-tamu.tdl.org/bovine/article/view/1717

Mackay, I. M. (2004). Real-time PCR in the microbiology laboratory. *Clinical Microbiology and Infection*, *10*(3), 190–212. https://doi.org/10.1111/j.1198-743X.2004.00722.x

Maeda, T., Shibahara, T., Kimura, K., Wada, Y., Sato, K., Imada, Y., Ishikawa, Y., & Kadota, K. (2003). *Mycoplasma bovis*-associated Suppurative Otitis Media and Pneumonia in Bull Calves. *Journal of Comparative Pathology*, *129*(2), 100–110. https://doi.org/10.1016/S0021-9975(03)00009-4

Mahony, J. B., Blackhouse, G., Babwah, J., Smieja, M., Buracond, S., Chong, S., Ciccotelli, W., O'Shea, T., Alnakhli, D., Griffiths-Turner, M., & Goeree, R. (2009). Cost Analysis of Multiplex PCR Testing for Diagnosing Respiratory Virus Infections. *Journal of Clinical Microbiology*, *47*(9), 2812–2817. https://doi.org/10.1128/jcm.00556-09

Makoschey, B., & Berge, A. C. (2021). Review on bovine respiratory syncytial virus and bovine parainfluenza – usual suspects in bovine respiratory disease – a narrative review. *BMC Veterinary Research*, *17*(1), 261. https://doi.org/10.1186/s12917-

021-02935-5

Mars, M. H., de Jong, M. C. M., van Maanen, C., Hage, J. J., & van Oirschot, J. T. (2000). Airborne transmission of bovine herpesvirus 1 infections in calves under field conditions. *Veterinary Microbiology*, *76*(1), 1–13. https://doi.org/10.1016/S0378-1135(00)00218-2

Maunsell, F. p., Woolums, A. r., Francoz, D., Rosenbusch, R. f., Step, D. l., Wilson, D. j., & Janzen, E. d. (2011). Mycoplasma bovis Infections in Cattle. *Journal of Veterinary Internal Medicine*, *25*(4), 772–783. https://doi.org/10.1111/j.1939-1676.2011.0750.x

Mechor, G. D., Rousseaux, C. G., Radostits, O. M., Babiuk, L. A., & Petrie, L. (1987). Protection of newborn calves against fatal multisystemic infectious bovine rhinotracheitis by feeding colostrum from vaccinated cows. *Canadian Journal of Veterinary Research*, *51*(4), 452–459.

Meyers, G., Tautz, N., Dubovi, E. J., & Thiel, H.-J. (1991). Viral cytopathogenicity correlated with integration of ubiquitin-coding sequences. *Virology*, *180*(2), 602–616. https://doi.org/10.1016/0042-6822(91)90074-L

Meyers, G., & Thiel, H.-J. (1996). Molecular Characterization of Pestiviruses. In K. Maramorosch, F. A. Murphy, & A. J. Shatkin (Eds.), *Advances in Virus Research* (Vol. 47, pp. 53–118). Academic Press. https://doi.org/10.1016/S0065-3527(08)60734-4

Nandi, S., Kumar, M., Manohar, M., & Chauhan, R. S. (2009). Bovine herpes virus infections in cattle. *Animal Health Research Reviews*, *10*(1), 85–98. https://doi.org/10.1017/S1466252309990028

Näslund, K., Tråvén, M., Larsson, B., Silván, A., & Linde, N. (2000). Capture ELISA systems for the detection of bovine coronavirus-specific IgA and IgM antibodies in milk and serum. *Veterinary Microbiology*, *72*(3), 183–206. https://doi.org/10.1016/S0378-1135(99)00208-4

Nicholas, R. A. J., Fox, L. K., & Lysnyansky, I. (2016). Mycoplasma mastitis in cattle: To cull or not to cull. *The Veterinary Journal*, *216*, 142–147. https://doi.org/10.1016/j.tvjl.2016.08.001

Nicholas, R., Ayling, R., & McAuliffe, L. (Eds.). (2008). *Mycoplasma diseases of ruminants* (1st ed.). CAB International. https://doi.org/10.1079/9780851990125.0000

Nylin, B., Strøger, U., & Rønsholt, L. (2000). A retrospective evaluation of a Bovine Herpesvirus-1 (BHV-1) antibody ELISA on bulk-tank milk samples for classification of the BHV-1 status of Danish dairy herds. *Preventive Veterinary Medicine*, *47*(1), 91–105. https://doi.org/10.1016/S0167-5877(00)00163-X

Pang, Y., Wang, H., Girshick, T., Xie, Z., & Khan, M. I. (2002). Development and Application of a Multiplex Polymerase Chain Reaction for Avian Respiratory Agents. *Avian Diseases*, *46*(3), 691–699. https://doi.org/10.1637/0005-2086(2002)046[0691:DAAOAM]2.0.CO;2

Parkinson, T. J. (2019). 24—Specific Infectious Diseases Causing Infertility and Subfertility in Cattle. In D. E. Noakes, T. J. Parkinson, & G. C. W. England (Eds.), *Veterinary Reproduction and Obstetrics (Tenth Edition)* (pp. 434–466). W.B. Saunders. https://doi.org/10.1016/B978-0-7020-7233-8.00024-0

Peterhans, E., Bachofen, C., Stalder, H., & Schweizer, M. (2010). Cytopathic bovine

viral diarrhea viruses (BVDV): Emerging pestiviruses doomed to extinction. *Veterinary Research*, *41*(6), 44. https://doi.org/10.1051/vetres/2010016

Peterhans, E., & Schweizer, M. (2010). Pestiviruses: How to outmaneuver your hosts. *Veterinary Microbiology*, *142*(1), 18–25. https://doi.org/10.1016/j.vetmic.2009.09.038

Pfyffer, G. E., & Wittwer, F. (2012). Incubation Time of Mycobacterial Cultures: How Long Is Long Enough To Issue a Final Negative Report to the Clinician? *Journal of Clinical Microbiology*, *50*(12), 4188–4189. https://doi.org/10.1128/JCM.02283-12

Phillips, C. J. C., Foster, C. R. W., Morris, P. A., & Teverson, R. (2003). The transmission of *Mycobacterium bovis* infection to cattle. *Research in Veterinary Science*, *74*(1), 1–15. https://doi.org/10.1016/S0034-5288(02)00145-5

Polz, M. F., & Cavanaugh, C. M. (1998). Bias in Template-to-Product Ratios in Multitemplate PCR. *Applied and Environmental Microbiology*, *64*(10), 3724–3730. https://doi.org/10.1128/AEM.64.10.3724-3730.1998

Rice, S. A., Long, M. C., Lam, V., Schaffer, P. A., & Spencer, C. A. (1995). Herpes simplex virus immediate-early protein ICP22 is required for viral modification of host RNA polymerase II and establishment of the normal viral transcription program. *Journal of Virology*, *69*(9), 5550–5559. https://doi.org/10.1128/jvi.69.9.5550-5559.1995

Ridley, A., & Hateley, G. (2018). Mycoplasma bovis investigations in cattle. *Veterinary Record*, *183*(8), 256–258. https://doi.org/10.1136/vr.k3722

Ridpath, J. F. (2013). Immunology of BVDV vaccines. *Biologicals*, *41*(1), 14–19.

https://doi.org/10.1016/j.biologicals.2012.07.003

Roberts, S. R., Lichtenstein, D., Ball, L. A., & Wertz, G. W. (1994). The membrane-associated and secreted forms of the respiratory syncytial virus attachment glycoprotein G are synthesized from alternative initiation codons. *Journal of Virology*, *68*(7), 4538–4546. https://doi.org/10.1128/JVI.68.7.4538-4546.1994

Saliki, J. T., & Dubovi, E. J. (2004). Laboratory diagnosis of bovine viral diarrhea virus infections. *Veterinary Clinics: Food Animal Practice*, *20*(1), 69–83. https://doi.org/10.1016/j.cvfa.2003.11.005

Santurde, G., Da Silva, N., Villares, R., Tabarés, E., Solana, A., Bautista, J. M., & Castro, J. M. (1996). Rapid and high sensitivity test for direct detection of bovine herpesvirus—1 genome in clinical samples. *Veterinary Microbiology*, *49*(1), 81–92. https://doi.org/10.1016/0378-1135(95)00169-7

Sarmiento-Silva, R. E., Nakamura-Lopez, Y., & Vaughan, G. (2012). Epidemiology, Molecular Epidemiology and Evolution of Bovine Respiratory Syncytial Virus. *Viruses*, *4*(12), Article 12. https://doi.org/10.3390/v4123452

Schreiber, P., Matheise, J. P., Dessy, F., Heimann, M., Letesson, J. J., Coppe, P., & Collard, A. (2000). High Mortality Rate Associated with Bovine Respiratory Syncytial Virus (BRSV) Infection in Belgian White Blue Calves Previously Vaccinated with an Inactivated BRSV Vaccine. *Journal of Veterinary Medicine, Series B*, *47*(7), 535–550. https://doi.org/10.1046/j.1439-0450.2000.00380.x

Shen, W., & Jones, C. (2008). Open Reading Frame 2, Encoded by the Latency-Related Gene of Bovine Herpesvirus 1, Has Antiapoptotic Activity in Transiently Transfected Neuroblastoma Cells. *Journal of Virology*, *82*(21), 10940–10945.

https://doi.org/10.1128/jvi.01289-08

Shi, Y., Li, B., Tao, J., Cheng, J., & Liu, H. (2021). The Complex Co-infections of Multiple Porcine Diarrhea Viruses in Local Area Based on the Luminex xTAG Multiplex Detection Method. *Frontiers in Veterinary Science*, *8*. https://doi.org/10.3389/fvets.2021.602866

Stalder, H., Bachofen, C., Schweizer, M., Zanoni, R., Sauerländer, D., & Peterhans, E. (2018). Traces of history conserved over 600 years in the geographic distribution of genetic variants of an RNA virus: Bovine viral diarrhea virus in Switzerland. *PLOS ONE*, *13*(12), e0207604. https://doi.org/10.1371/journal.pone.0207604

Straub, O. C. (1991). BHV1 infections: Relevance and spread in Europe. *Comparative Immunology, Microbiology and Infectious Diseases*, *14*(2), 175–186. https://doi.org/10.1016/0147-9571(91)90130-6

Sulyok, K. M., Kreizinger, Z., Fekete, L., Hrivnák, V., Magyar, T., Jánosi, S., Schweitzer, N., Turcsányi, I., Makrai, L., Erdélyi, K., & Gyuranecz, M. (2014). Antibiotic susceptibility profiles of Mycoplasma bovis strains isolated from cattle in Hungary, Central Europe. *BMC Veterinary Research*, *10*(1), 256. https://doi.org/10.1186/s12917-014-0256-x

Tamura, J. K., Warrener, P., & Collett, M. S. (1993). RNA-Stimulated NTPase Activity Associated with the p80 Protein of the Pestivirus Bovine Viral Diarrhea Virus. *Virology*, *193*(1), 1–10. https://doi.org/10.1006/viro.1993.1097

Tikoo, S. K., Campos, M., & Babiuk, L. A. (1995). Bovine Herpesvirus 1 (Bhv-1(: Biology, Pathogenesis, and Control. In K. Maramorosch, F. A. Murphy, & A. J. Shatkin (Eds.), *Advances in Virus Research* (Vol. 45, pp. 191–223). Academic

Press. https://doi.org/10.1016/S0065-3527(08)60061-5

Ungar-Waron, H., & Abraham, A. (1991). Immunoglobulin M (IgM) indirect enzyme-linked immunosorbent assay and the involvement of IgM-rheumatoid factor in the serodiagnosis of BHV-1 infection. *Veterinary Microbiology*, *26*(1), 53–63. https://doi.org/10.1016/0378-1135(91)90041-D

Valarcher, J.-F., & Taylor, G. (2007). Bovine respiratory syncytial virus infection. *Veterinary Research*, *38*(2), 153–180. https://doi.org/10.1051/vetres:2006053

Virus Transmission—Bovine Viral Diarrhea Virus—Wiley Online Library. (n.d.). Retrieved April 9, 2024, from https://onlinelibrary.wiley.com/doi/10.1002/9780470344453.ch5

Wellenberg, G. J., Mars, M. H., & Van Oirschot, J. T. (2001). Antibodies against bovine herpesvirus (BHV) 5 may be differentiated from antibodies against BHV1 in a BHV1 glycoprotein E blocking ELISA. *Veterinary Microbiology*, *78*(1), 79–84. https://doi.org/10.1016/S0378-1135(00)00283-2

Wernike, K., Hoffmann, B., Kalthoff, D., König, P., & Beer, M. (2011). Development and validation of a triplex real-time PCR assay for the rapid detection and differentiation of wild-type and glycoprotein E-deleted vaccine strains of Bovine herpesvirus type 1. *Journal of Virological Methods*, *174*(1–2), 77–84. https://doi.org/10.1016/j.jviromet.2011.03.028

Williams, R. S., & Hoy, W. A. (1930). The Viability of B. tuberculosis (Bovinus) on Pasture Land, in Stored Faeces and in Liquid Manure. *Epidemiology & Infection*, *30*(4), 413–419. https://doi.org/10.1017/S0022172400010561

Workman, A., Perez, S., Doster, A., & Jones, C. (2009). Dexamethasone Treatment of

Calves Latently Infected with Bovine Herpesvirus 1 Leads to Activation of the bICP0 Early Promoter, in Part by the Cellular Transcription Factor C/EBP-Alpha. *Journal of Virology*, *83*(17), 8800–8809. https://doi.org/10.1128/jvi.01009-09

Workman, A., Sinani, D., Pittayakhajonwut, D., & Jones, C. (2011). A Protein (ORF2) Encoded by the Latency-Related Gene of Bovine Herpesvirus 1 Interacts with Notch1 and Notch3. *Journal of Virology*, *85*(6), 2536–2546. https://doi.org/10.1128/jvi.01937-10

Xu, F., Min, F., Wang, J., Luo, Y., Huang, S., Chen, M., Wu, R., & Zhang, Y. (2020). Development and evaluation of a Luminex xTAG assay for sulfonamide resistance genes in *Escherichia coli and Salmonella* isolates. *Molecular and Cellular Probes*, *49*, 101476. https://doi.org/10.1016/j.mcp.2019.101476

Yates, W. D. (1982). A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. *Canadian Journal of Comparative Medicine*, *46*(3), 225–263.

Zangenberg, G., Saiki, R. K., & Reynolds, R. (1999). 6 - Multiplex PCR: Optimization guidelines. In M. A. Innis, D. H. Gelfand, & J. J. Sninsky (Eds.), *PCR Applications* (pp. 73–94). Academic Press. https://doi.org/10.1016/B978-012372185-3/50007-9