Application of metagenomic sequencing to characterization of the virome in bovine respiratory disease

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University of Saskatchewan

Saskatoon

By

MAODONG ZHANG

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ABSTRACT

Bovine respiratory disease (BRD) is the most concerning disease in the cattle industry worldwide due to the enormous economic losses it causes, and concerns regarding antimicrobial resistance that is emerging due to use of massive amounts of antimicrobial agents to control the disease. The concept of primary environmental, management or viral factors with ensuing secondary bacterial infections is widely accepted, however, morbidity and mortality of feedlot cattle due to BRD are not reduced despite viral vaccination and antimicrobial usage. This indicates that the etiology and pathogenesis of BRD are not yet fully understood, and that all of the infectious agents and environmental causal factors may not have been identified. The advent of high throughput sequencing (HTS) using Illumina MiSeq and Oxford Nanopore sequencing technologies has revolutionized the genomics field, particularly microorganism identification, antimicrobial resistance prediction and microbiome analysis. The main objective of my study was to use HTS to identify unconventional viruses (those viruses are not included in BRD vaccine and no available diagnostic assays) and characterize their association with BRD.

We first characterized and compared the upper and lower respiratory tract viromes of Canadian feedlot cattle with or without BRD through metagenomic sequencing on the Illumina MiSeq platform. The presence of influenza D virus (IDV), bovine rhinitis A virus (BRAV), bovine rhinitis B virus (BRBV), bovine coronavirus (BCV) and bovine respiratory syncytial virus (BRSV) was associated with BRD. Agreement between identification of these viruses in nasal swabs and tracheal washes was generally weak, indicating that sampling location may affect detection of infection.

We next characterized the virome of bovine pneumonia lungs collected in western Canada using metagenomic sequencing on the Illumina MiSeq, and combined this information with bacterial

culture and targeted real-time PCR results from the same samples to determine the relationships between the microorganisms detected and different types of pneumonia defined by histopathological examination. Detection of *Histophilus somni* and *Pasteurella multocida* was associated with suppurative bronchopneumonia (SBP) and concurrent bronchopneumonia and bronchointerstitial pneumonia (BP&BIP), respectively. None of these viruses, however, was significantly associated with a particular type of pneumonia. Unconventional viruses such as IDV and BRBV were detected, although sparsely, consistent with our previous findings in upper respiratory tract samples.

In the third chapter, we used IDV as a representative BRD-associated virus to examine the feasibility of using metagenomic sequencing for detection of viruses in clinical bovine respiratory samples. We compared results of long-read sequencing on the Oxford Nanopore GridION platform and previously generated Illumina MiSeq data to the results of an IDV-specific qPCR. We concluded that both MiSeq and Nanopore sequencing were capable of detecting IDV in clinical specimens with a range of Cq values.

In the last chapter, we applied Nanopore metagenomic sequencing to characterize the viromes of cattle upon arrival at nine feedlots in Western Canada, and related the findings to health outcomes of these cattle to determine if the composition of the virome of individual animals could be used to predict the likelihood of their development of BRD. No relationship was found between BRD development and the number of viruses detected, the presence of any specific individual virus, or combination of viruses.

In summary, results of these studies demonstrate the diversity of viruses in bovine respiratory tracts, and highlight the need for further research into prevention and control of BRD development in the context of mixed infections. Meanwhile, our results also demonstrate the potential of

metagenomic sequencing on the Illumina MiSeq and Oxford Nanopore platforms for detection of viruses in clinical samples from naturally infected animals with a wide range of viral loads.

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LIST OF ABBREVIATIONS

B. trehalosi	*Bibersteinia trehalosi*
BAAV	Bovine adeno-associated virus
BAdV3	Bovine adenovirus 3
BADV3	bovine adenovirus 3
BALT	bronchus-associated lymphoid tissue
BAV	Bovine astrovirus
BCV	Bovine coronavirus
BDV	Border disease virus
BHV1	Bovine herpesvirus 1
BHV6	Bovine herpesvirus 6
BIP	Bronchointerstitial pneumonia
BKV	Bovine kobuvirus
BLAST	Basic local alignment search tool
BNV	Bovine nidovirus
BoTV	Bovine torovirus
bp	Base pairs
BPIV3	Bovine parainfluenza virus 3
BPV2	Bovine parvovirus 2
BPV3	Bovine parvovirus 3
BPyV	Bovine polyomavirus
BRAV	Bovine rhinitis a virus
BRBV	Bovine rhinitis b virus
BRD	Bovine respiratory disease
BRSV	Bovine respiratory syncytial virus
BVDV	Bovine viral diarrhea virus
BWA	Burrows-Wheeler Aligner

CD	Calf diarrhea
CI	Confidence interval
cp	Cytopathic
CSFV	Classical swine fever virus
ELISA	Enzyme-linked immunosorbent assays
EM	Electron microscopy
EVE	Enterovirus E
FAT	Fluorescent antibody test
FBP	Fibrinous bronchopneumonia
GB	Gigabyte
H. somni	*Histophilus somni*
HA	Hemagglutinin
HN	Hemagglutinin-neuraminidase
HPIV	Human parainfluenza virus
HRSV	Human respiratory syncytial virus
HTS	High throughput sequencing
IAV	Influenza A virus
IBR	Infectious bovine rhinotracheitis
IBV	Influenza B virus
ICIB	Intracytoplasmic inclusion bodies
ICV	Influenza C virus
ICV	Influenza C virus
IDV	Influenza D virus
IHC	Immunohistochemistry
INIB	Intranuclear inclusion bodies
IP	Interstitial pneumonia
ISH	In-situ hybridization
M. bovis	*Mycoplasma bovis*

M. haemolytica	*Mannheimia haemolytica*
MALDI-TOF	Matrix-Assisted Laser Desorption and Ionization Time-of-Flight
MD	Mucosal disease
MDBK	Madin-Darby bovine kidney
MLV	Modified live vaccine
mNGS	Metagenomic next-generation sequencing
MVSK	QIAamp MinElute Virus Spin Kit
NA	Neuraminidase
NCBI	National Center for Biotechnology Information
ncp	Noncytopathic
NGS	Next-generation sequencing
OLC	Overlap layout consensus
P. multocida	*Pasteurella multocida*
PCR	Polymerase chain reaction
PI	Persistently infected
qRT-PCR	Quantitative real-time – polymerase chain reaction
RI	Respiratory infection
RMK	RNeasy Mini Kit
RPA	Recombinase polymerase amplification
RPMK	RNeasy Plus Micro Kit
SBP	Suppurative bronchopneumonia
SGS	Second-generation sequencing
SOAP2	Short oligonucleotide alignment program 2
Spp.	Species
SPSS	Statistical Package for the Social Sciences
ssCDV	Single stranded cDNA virus
TGS	Third-generation sequencing
UBPV6	Ungulate bocaparvovirus 6

UTPV1	Ungulate tetraparvovirus 1
VRMK	QIAamp Viral RNA Mini Kit
WD	Winter dysentery

CHAPTER 1. Literature review

1.1. Introduction of bovine respiratory disease (BRD) and its economic impact

In the 1960s, the Golden Age of Cattle Feeding appeared on the High Plains in the United States due to favorable combined conditions of cattle, corn, transportation, irrigation and friendly weather (Smith et al., 2020). Later on, new technology and universities with skilled veterinarians and nutritionists geared up the cattle industry with additional cattle management programs (Smith et al., 2020). Despite the favorable combinations of environment, knowledge and commitment, BRD is still a plague to the cattle industry. BRD is mainly a disease of the lower respiratory tract of cattle, typically multifactorial in origin and always manifested as respiratory signs clinically with characteristic pneumonia (Hilton, 2014).

BRD has been the most common and significant cause of enormous economic losses for the cattle industry worldwide including United States (Loneragan et al., 2001), Canada (Church and Radostits, 1981), Switzerland (Mehinagic et al., 2019), Australia (Hay et al., 2014), and Europe (Nicholas and Ayling, 2003) due to high morbidity, mortality, reduced growth performance and carcass quality, and massive metaphylactic and therapeutic usage of antimicrobial drugs (Fulton, 2009; Hilton, 2014; Portis et al., 2012; Smith et al., 2020). BRD accounts for 44% - 67% mortality and 70% - 80% morbidity in the United States (Jensen et al., 1976a; Loneragan et al., 2001; Edwards, 2010), 54% - 66% mortality in feedlots in Ontario (Gagea et al., 2006; Martin et al., 1981; Martin et al., 1982) and 10% - 61% mortality in western Canadian feedlots (Church and Radostits, 1981; Ribble et al., 1995; Gagea et al., 2006). In the United States, the economic loss due to BRD is estimated to be over one billion dollars annually (Griffin, 1997b; Mitra et al., 2016) and in Canada, over 80% of the vaccines licensed for cattle are applied for prevention and control of BRD (Bowland and Shewen, 2000; Zhang et al., 2019; Edwards, 2010). In Australia, 40 million

dollars are spent on BRD annually (Cusack et al., 2020). In the United Kingdom, based on 1995 figures, approximately 1.9 million cattle were affected annually by BRD with estimated costs of £54 million and additionally, up to 0.16 million calves have died annually due to pneumonia and related disease with an estimated cost of £99 million to the cattle industry (Nicholas and Ayling, 2003; Gourlay et al., 1989b; Reeve-Johnson, 1999). Furthermore, it is estimated that approximately 16 million cattle with total costs of 576 million euros are impacted annually across Europe (Nicholas and Ayling, 2003; Reeve-Johnson, 1999). A study (Vogel et al., 2015) of over 73 million beef cattle showed that mortality due to respiratory disease averaged 0.091% monthly from 2005 to 2007, 0.097% from 2008 to 2010, and 0.127% from 2011 to 2013, which represented a 35% monthly increase over the last eight years period (Smith et al., 2020).

Over the last 50 years, morbidity and mortality rates caused by BRD have increased in spite of massive amounts of antimicrobials, vaccines and anti-inflammatory agents used (Hilton, 2014; Smith et al., 2020). The answer to why there has been increasing morbidity and mortality of feedlot cattle is as complicated as the etiology of BRD (Hilton, 2014). Hilton et al., listed a few likely possibilities: more and more calves commingled from various sources; younger calves with immature immunity being transported to the feedlot; more unweaned and non-vaccinated calves being auctioned; viral exposure being increased due to calves crowding in pens, and poor husbandry and care of animals (Hilton, 2014).

1.2. Pathogenesis and etiology of BRD

Despite extensive evidence of the involvement of various infectious agents in BRD, experimental challenges with bacteria or viruses alone generally do not reproduce the common respiratory clinical manifestations (Taylor et al., 2010). Additionally, bacterial pathogens associated with BRD including *Mannheimia haemolytica* (*M. haemolytica)*, *Histophilus somni* (*H. somni)*,

Pasteurella multocida (*P. multocida), Bibersteinia trehalosi* (*B. trehalosi)* and *Mycoplasma bovis* (*M. bovis)* (Griffin et al., 2010) can readily be isolated or detected in the nasopharyngeal tracts of both healthy and sick cattle (McMullen et al., 2019). It is now widely accepted that predisposing factors including commingling, long-distance shipping, dehydration, sudden and extreme weather change, dehorning and castration after arrival increase BRD risks (Taylor et al., 2010). In addition, viral infections can disrupt the integrity of epithelial barriers and damage the mucocilliary apparatus leading to increased viscosity in the trachea, failure to clear bacteria in the upper respiratory tract, and deposition of bacteria in alveoli (Ellis, 2009). Viral pathogens most often implicated in BRD are bovine herpesvirus 1 (BHV1), bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus 3 (BPIV3) and bovine coronavirus (BCV), and more recently there is increasing interest in influenza D virus (IDV) (Ellis, 2009; Murray et al., 2016; Zhang et al., 2019; Ng et al., 2015; Mitra et al., 2016; Fulton, 2020). The following section introduces the viral and bacterial pathogens implicated in BRD and describes evidence of their contribution to disease pathogenesis.

1.3. Pathogens associated with BRD

1.3.1 Bovine respiratory syncytial virus (BRSV)

BRSV together with human respiratory syncytial virus (HRSV) belongs to the genus *Pneumovirinae* in the family *Paramyxoviridae* and is a negative-sense, non-segmented, single-stranded, enveloped RNA virus with characteristic lability and poor growth *in vitro* (Sarmiento-Silva et al., 2012). Cattle are universally recognized as the principal reservoir of BRSV, although sheep are also reported to be infected (Sacco et al., 2014; Klem et al., 2014; Sarmiento-Silva et al., 2012). Glycoprotein (G protein) and fusion protein (F protein) of BRSV can induce neutralizing antibodies and their sequences can be used to classify BRSV strains (Brodersen, 2010).

Phylogenetic analysis based on the highly variable G protein showed that many BRD associated BRSV isolates belong to subgroup VII, which indicated that current BRSV vaccines based on strains from subgroup II and III are highly unlikely to provide protection (Valarcher et al., 2000; Bertolotti et al., 2018). BRSV is able to replicate in the presence of detectable antibodies and reinfection occurs in cattle as does HRSV reinfection in humans (Beem, 1967; Valarcher et al., 2000), and so BRSV vaccines may prevent disease, but not the circulation of the virus (Valarcher et al., 2000). Mild or often no disease in cattle infected with tissue culture passaged BRSV has been consistently documented; in contrast, successful reproduction of clinical respiratory disease and typical pulmonary lesions were reported using calf lung passaged BRSV, suggesting attenuation with *in vitro* passage (Ellis, 2017; Ellis et al., 2005). Data from challenge models provides estimates of the disease duration and shedding period in natural infections or outbreaks (Grissett et al., 2015). BRSV shedding begins 1-5 days (median 3 days) post challenge with resolution at 7-14 days (median 9 days), and presentation of clinical signs at 1-6 days (median 3 days) post challenge with resolution at 7-17 days (median 12 days) (Grissett et al., 2015). Seroconversion of BRSV infected animals occurs anywhere between 5 and 21 days (median 9 days) post challenge (Grissett et al., 2015).

1.3.2 Bovine parainfluenza virus 3 (BPIV3)

BPIV3 together with human parainfluenza virus (HPIV) and Sendai virus belongs to the genus *Respirovirus* in the family *Paramyxoviridae* and is a negative-sense, non-segmented, single-stranded, enveloped RNA virus with three genotypes, A (BPIV3a), B (BPIV3b) and C (BPIV3c) (Maidana et al., 2012; Albayrak et al., 2019; Ellis, 2010). The BPIV3c genotype has been previously reported in China, South Korea, Japan and recently Argentina (Albayrak et al., 2019; Maidana et al., 2012). BPIV3a genotype has been isolated in US, Egypt, China and Japan, but the

BPIV3b genotype has been detected only in Australia and Argentina (Albayrak et al., 2019; Maidana et al., 2012). Intriguingly, one of the features of paramyxoviruses is cross-species transmission, which makes it impossible to determine virus strain without sequencing the isolates (Ellis, 2010). BPIV3 exhibits agglutination due to the hemagglutinin-neuraminidase (HN) envelope protein, which also facilitates attachment and entry into host cells (Ellis, 2010). A variety of cell types including tracheal, bronchial, bronchiolar cells and type I and II pneumocytes have sialic acid receptors for BPIV3, enabling ubiquitous infection of the respiratory tract (Ellis, 2010). Successful reproduction of pneumonia and clinical respiratory signs has been documented using an intranasal inoculation route (Bryson et al., 1979). Primary inoculation with BPIV3 followed by *M. haemolytica* induced fibrinous pneumonia, but inoculation with either BPIV3 or *M. haemolytica* alone did not (Jericho, 1979; Smith et al., 2020). In experimental infections, BPIV3 shedding occurs 1-2 days post challenge with resolution at 10-13 days (median 10 days), and presentation of clinical signs starts 2-3 days post-challenge with resolution on day 14 based on only one study without further available data from other studies about the resolution of clinical signs (Grissett et al., 2015). Characterization of the host response to BPIV3 provides a better understanding of this virus when it is associated with BRD, which leads to a better challenge model design and improved estimations of the timing of appearance of clinical signs (Ellis, 2010; Grissett et al., 2015).

1.3.3 Bovine coronavirus (BCV)

Bovine coronavirus (BCV) together with canine respiratory coronavirus and human HCoV-OC43 and HKU1 belongs to genus *Betacoronavirus* in the family *Coronaviridae* of order *Nidovirales* (Saif, 2010). BCV has a worldwide distribution and is a positive-sense, single-stranded, enveloped RNA virus with a 32 kb genome (Decaro and Lorusso, 2020). BCV can cause calf diarrhea (CD),

winter dysentery (WD) in adults, and respiratory infections (RI) in cattle of various ages (Saif, 2010; Fulton et al., 2013). No specific genetic or antigenic markers of BCV have yet been associated with these different clinical presentations (Saif, 2010). Identification of BCV-like human enteric coronavirus (HCoV-4408/USA/1994) in a child with acute diarrhea raised public health concerns over possible transmission between ruminants and human (Suzuki et al., 2020; Zhang et al., 1994). Intranasal, intratracheal or combination routes of infection with BCV strains isolated from diarrhea or respiratory disease cases have been attempted to experimentally reproduce disease but have resulted in diarrhea only (Ellis, 2019). Despite the lack of clinical respiratory signs in experimentally infected animals, the features of interstitial pneumonia were reported histologically (Ellis, 2019; Ridpath et al., 2020b).

Evidence of the association of BCV with BRD is provided by both experimental infection studies and clinical investigations. Initial infection with BCV followed by dual infection with BCV, *M. haemolytica* and *P. multocida* resulted in clinical respiratory signs and lung lesions characteristic of BRD (Storz et al., 2000a). BCV antigen has been detected in respiratory epithelial cells of animals with inflammation in bronchi, bronchioles and alveoli in concert with *Pasteurella* spp. infection (Storz et al., 2000b). PCR detection of BCV has also been positively correlated with BRSV and BHV1 in post-mortem lung samples (Fulton et al., 2009). Recently, sequential challenge with BVDV and BCV using field strains resulted in gross lung lesions in all dual-infection groups with the most prominent lesions in animals challenged with BVDV followed by BCV after 6 days (Ridpath et al., 2020b). In this study, BCV challenge alone resulted in interstitial pneumonia with fibrin strands, increased pulmonary alveolar macrophages and hypertrophy of type II pneumocytes histologically without clinical respiratory signs and gross lung lesions (Ridpath et al., 2020b).

1.3.4 Bovine viral diarrhea virus (BVDV)

Bovine viral diarrhea virus (BVDV) together with border disease virus (BDV) and classical swine fever virus (CSFV) belongs to the genus *Pestivirus* in the family *Flaviviridae* (Brodersen, 2014). BVDV is a positive-sense, single-stranded, enveloped RNA virus with two genotypes, BVDV1 and BVDV2, further classified as noncytopathic (ncp) or cytopathic (cp) based on cell culture morphology (Brodersen, 2014; Pecora et al., 2019). Among subtypes, BVDV1b is the most predominant and its natural host is cattle (Fulton, 2020). Fetus infection with an ncp strain followed by postnatal infection with a cp strain leads to persistently infected (PI) animals with development of alimentary disease known as mucosal disease (MD) (Brodersen, 2014). Acutely transient postnatal infection may mimic the lesions of MD, making differentiation of PI animals and acutely infected animals extremely difficult (Brodersen, 2014). BVDV infection may predispose animals to secondary infections with other pathogens including BPIV3, BHV1, BRSV and *M. haemolytica,* enhancing the severity of respiratory disease (Fulton et al., 2000; Potgieter, 1997). In addition, experimental dual infections with BVDV and BCV have successfully reproduced clinical respiratory manifestations and gross lung lesions, which suggests BVDV has a synergistic effect and might act as a potentiator for other pathogens (Ridpath et al., 2020a). The mechanism of immunosuppression by BVDV is related to extensive lymphocyte apoptosis, particularly affecting $CD4^+$ T cells (Chase, 2013; Peterhans et al., 2003). In experimental infections, BVDV shedding occurs 1-5 days (median 2 days) post challenge with resolution at 11-12 days (median 12 days), and presentation of clinical signs starts at 1-6 days (median 2 days) post challenge with resolution at 6-23 days (median 15 days) (Grissett et al., 2015). Seroconversion occurs 15-19 days (median 17 days) post challenge (Grissett et al., 2015).

1.3.5 Bovine herpesvirus 1 (BHV1)

BHV1 also known as infectious bovine rhinotracheitis virus (IBRV) belongs to subfamily *alphaherpesvirinae* in the family *Herpesviridae* (Jones and Chowdhury, 2010). The first isolation of BHV1 in 1958 and first report of respiratory IBR in 1961 was documented in Canada (Yates, 1982). BHV1 can be classified into three subtypes: subtype 1, 2a and 2b, based on genetic and antigenic characteristics (Metzler et al., 1985). Subtype 1 strains are distributed in North America, South America and Europe, subtype 2a in Brazil and subtype 2b is prevalent in Australia and Europe (van Oirschot, 1995). BHV1 can cause respiratory disorders, abortion and immunosuppression, the latter of which is a crucial step in development of BRD and occurs through induction of apoptosis of $CD4^+$ T cells (Jones and Chowdhury, 2010). BHV1 has the ability to establish latency in trigeminal neurons and pharyngeal tonsils with periodic reactivation resulting in virus transmission following virus shedding (Jones and Chowdhury, 2010). Challenge of calves with aerosolized BHV1 followed by *M. haemolytica* successfully caused fibrinous pneumonia, which was similar to cases of shipping fever reported in the field (Jericho et al., 1976; Jericho and Langford, 1978). Not only synergistic with *M. haemolytica*, BHV1 was also able to facilitate *P. multocida* and *H. somni* infections to cause pneumonia (Jones and Chowdhury, 2007; Yates, 1982). BHV1 modified live vaccines are available, but unfortunately these strains can establish latency and can be subsequently reactivated, leading to outbreaks of BRD in beef cattle and cow-calf operations (Jones and Chowdhury, 2010). In experimental infections, BHV1 shedding occurs 1-3 days (median 2 days) post challenge with resolution at 7-17 days (median 14 days), and presentation of clinical signs starts 2-5 days (median 2 days) post challenge with resolution at 10-15 days (median 14 days) (Grissett et al., 2015). Seroconversion can occur anywhere from 7 to 28 days (median 17.5 days) post challenge (Grissett et al., 2015).

1.3.6 Influenza D virus (IDV)

Influenza D virus (IDV) together with influenza A, B and C virus (IAV, IBV and ICV) belongs to the family *Orthomyxoviridae* and is a negative-sense, single-stranded, segmented RNA virus, with each segment encoding a single transcript (Hause et al., 2014). There is less than 50% amino acid identity between IDV and its closest human pathogen relative (ICV), and no cross-reactivity between IDV and human ICV antisera (Collin et al., 2015; Hause et al., 2014; Hause et al., 2013). IDV has been identified in many countries worldwide and cattle are considered to be the primary natural reservoir of IDV in spite of its original identification being in pigs (Collin et al., 2015; Su et al., 2017). Both IDV and ICV genomes consist of seven genome segments in contrast to the eight genome segments of IAV and IBV. In IDV and ICV, one hemagglutinin-esterase fusion (HEF) gene encodes a protein that combines the function of hemagglutinin (HA) and neuraminidase (NA) proteins of IAV and IBV (Hause et al., 2014). There is a conserved non-coding region at both ends of each segment of IDV with the 3' terminus (3'-C/UCGUAUUCGUC-5') being 11 nucleotides and the 5' terminus (5'-AGCAGUAGCAAG-3') being 12 nucleotides (Hause et al., 2014; Su et al., 2017). A human seroprevalence study showed that 97% of people working with cattle were seropositive for IDV, raising public concerns about zoonosis of IDV (White et al., 2016). Results of a few studies using metagenomic next-generation sequencing (mNGS) have indicated a statistically significant association of IDV with BRD (Mitra et al., 2016; Ng et al., 2015; Zhang et al., 2019). To address whether IDV could cause BRD, Ferguson et al., challenged three dairy calves intranasally with IDV (Ferguson et al., 2016). Minimal respiratory signs were observed in inoculated animals, with neutrophil infiltration in tracheal mucosa and submucosa observed histologically, and IDV antigen detected with immunohistochemistry in tracheal epithelial cells (Ferguson et al., 2016). Subsequently, an inactivated IDV vaccine was

shown to provide partial protection from homologous challenge and to cause significant reduction of virus shedding in nasal and tracheal samples (Hause et al., 2017).

1.3.7 Bacterial pathogens

In the progression of BRD development, primary viral infection is often followed by secondary bacterial infection, which complicates the diagnosis especially at the time of necropsy of animals with severe or chronic disease (Confer, 2009; Fulton, 2009; Griffin et al., 2010). Secondary bacterial infections generally dominate the pathological presentation in these animals, which presents a significant challenge to identifying the underlying causes of the development of BRD (Mehinagic et al., 2019; Zhang et al., 2020b; Booker et al., 2008).

Histophilus somni (*H. somni*) is a Gram-negative bacterium with up to 40% prevalence in post-mortem pneumonic lungs and 42% prevalence in nasal tracts of beef cattle before export (Murray et al., 2017; Murray et al., 2016). *H. somni* is a part of the normal microbiota of the genital and nasal mucosa and can persist in the bovine lung for long periods without any associated clinical signs (Gogolewski et al., 1989; Murray et al., 2016; Gershwin et al., 2005). Synergism between *H. somni* and BRSV in the development of BRD has been demonstrated in inoculation studies, and clinical signs, gross and histological lesions are significantly more severe in cattle challenged with *H. somni* six days after BRSV challenge than in animals challenged with either pathogen alone (Gershwin et al., 2005). In naturally infected animals, isolation of *H. somni* from postmortem lung samples is positively correlated with isolation of *Mycoplasma* spp. (Fulton et al., 2009). A western Canadian study of associations between histopathological findings and microorganisms found that 80% (12/15) of animals positive for *H. somni* were also positive for *M. bovis* (Booker et al., 2008). The role of *H. somni* in the pathogenesis of BRD is thought to involve the activation of platelets

with subsequent promotion of thrombi formation when the bacteria adhere to endothelial cells and induce apoptosis of these cells via lipooligosaccharide (Murray et al., 2016).

Pasteurella multocida (*P. multocida*) is a Gram-negative bacterium with a broad host range that includes dogs, cats, cattle, fowl, pigs and humans (Miyoshi et al., 2012; Peng et al., 2018). In cattle, the prevalence of *P. multocida* detected by bacterial culture varies from 42% in diseased calves to 26% in tracheobronchial samples from healthy calves (Autio et al., 2007b). In nasal swabs of healthy cattle, 17% prevalence was reported using PCR; however, the prevalence in pneumonic lungs was approximately 38% in contrast to 14% prevalence of normal lungs when using both bacterial culture and PCR method (Hotchkiss et al., 2010; Murray et al., 2017). In postmortem lung samples from BRD affected cattle, the isolation of *P. multocida* was positively correlated with isolation of *H. somni* and *Mycoplasma* spp. (Fulton et al., 2009). Applied bacterial culture and quantitative real-time PCR (qRT-PCR) on 637 healthy calves selected throughout Scotland, *P. multocida* was found to be significantly associated with BPIV3 and *Mycoplasma*-like organisms (Hotchkiss et al., 2010; Murray et al., 2017). In a study including 84 calves with BRD in Finland, significant association was found between isolation of *P. multocida* and elevated levels of acute phase proteins including fibrinogen, haptoglobin, serum amyloid-A, lipopolysaccharide binding protein and α-1 acid glycoprotein, consistent with a pathologic role for *P. multocida* in BRD (Nikunen et al., 2007).

The bacterial pathogen perhaps most strongly associated with BRD is *M. haemolytica*, a Gram-negative bacterium that exists as a commensal component of the nasal and nasopharyngeal mucosa of cattle (Rice et al., 2007). Reported prevalence of *M. haemolytica* in postmortem bovine lung samples range from 25% to 47%, and it is frequently isolated from pneumonic lung samples in the absence of other pathogens (Zhang et al., 2020b; Fulton et al., 2009). In a study of associations

between microorganisms and pathologic processes in pneumonic lungs of western Canadian cattle, co-infection of *M. haemolytica* and BVDV occurred in 96% (25/26) of cases based on an IHC test (Booker et al., 2008). *M. haemolytica* inoculation following inoculation with BHV1 has been reported to induce classical BRD signs and macroscopic lesions including purulent rhinitis, necrotizing tracheitis and lobar pneumonia (Burciaga-Robles et al., 2010; Jericho and Langford, 1978). When steers were exposed to BVDV PI animals for 72 hours followed by *M. haemolytica* challenge, animals had increased rectal temperature within 24 hours, increased whole blood cell count within 36 hours and an elevated total neutrophils and lymphocytes count within 72 hours post *M. haemolytica* challenge (Burciaga-Robles et al., 2010). The authors concluded that the association of BVDV and *M. haemolytica* in inducing BRD was successfully established (Burciaga-Robles et al., 2010). Unlike other bacterial pathogens associated with BRD, challenge with *M. haemolytica* on its own has been reported to cause respiratory disease, but only in immunocompromised calves (Bassel et al., 2019). In this study, clean-catch colostrum-deprived calves were challenged with 1×10^{10} colony forming units of *M. haemolytica* by aerosol, and went on to develop bronchopneumonia characterized by oat cells and coagulative necrosis (Bassel et al., 2019). Leukotoxin is considered to be the main virulence factor of *M. haemolytica.* This member of the RTX family of Gram-negative exotoxins induces pneumonia through destruction of leukocytes, which impairs bacterial clearance (Rice et al., 2007).

Mycoplasma bovis (*M. bovis*) is a bacterium without a cell wall that occurs worldwide in cattle causing pneumonia, mastitis, arthritis, abortion and keratojunctivitis (Caswell and Archambault, 2007; Nicholas and Ayling, 2003). Cattle are a natural reservoir and can shed mycoplasma for months and even years with or without clinical signs (Gourlay et al., 1989a; Caswell and Archambault, 2007). It is extremely difficult to eradicate *M. bovis* once it is introduced into a herd

by clinically healthy but shedding cattle (Gourlay et al., 1989a). The prevalence of *M. bovis* can be up to 23% in pneumonic lungs, and it can be detected in 30% of cattle herds (Zhang et al., 2020b; Nicholas and Ayling, 2003); however, necropsy studies showed that *M. bovis* can be identified in 46% of lungs with normal gross and histological features (Gagea et al., 2006). Coinfection of *M. bovis* with *P. multocida* and *H. somni* was detected in up to one third of pneumonic lungs in some studies (Nicholas and Ayling, 2003; Zhang et al., 2020b), leading to the conclusion that mycoplasma infection may be primary with other bacteria complicating the pneumonic process (Nicholas and Ayling, 2003). Although few calves experimentally challenged with *M. bovis* develop caseonecrotic pneumonia, the infiltration of alveoli and bronchioles by neutrophils clearly demonstrates that *M. bovis* can induce bronchopneumonia without progress to caseous lesions (Nicholas and Ayling, 2003). Mixed infections of *M. bovis* with *P. multocida*, BRSV, BPIV3, BVDV and BHV1 have also been frequently reported (Stipkovits et al., 2000; Zhang et al., 2020b; Oliveira et al., 2019).

1.4. Current approaches for diagnosis of BRD

In veterinary medicine, steps of infectious disease investigation include examination of affected animals, gaining a history and understanding of the epidemiology, and more importantly, evaluation of affected organs by histopathology and identification of the pathogenic agents and contributing factors (Caswell et al., 2012). Challenges facing owners, veterinarians and diagnosticians include identifying the causes of BRD in calves that have recovered after treatment, and how to interpret the complex relationships between various infectious agents and BRD (Fulton and Confer, 2012). Given its complex etiology and presentation, BRD diagnosis requires comprehensive and robust diagnostic tools (Caswell et al., 2012). Correspondingly, there are a

range of ante- and post-mortem diagnostic approaches used, which are described in the following sections.

1.4.1 Diagnosis based on clinical presentation

Clinical diagnosis of BRD is hindered by poor diagnostic criteria that are not predictive of pneumonia; however, it is extremely important to diagnose as early as possible to prevent economic loss (McGuirk, 2008). Therefore, a few clinical scoring systems have been developed to help owners, their workers and veterinarians (Buczinski et al., 2018; McGuirk, 2008; Love et al., 2014). Viral and bacterial pathogens generally cause similar clinical respiratory signs including any of the following: fever, anorexia, dry muzzle, depression, tachypnea, dyspnea, nasal discharge, coughing and death (Caswell JL, 2016b). In one scoring system, rectal temperature, nasal discharge, ocular discharge, cough and ear position are each scored from zero to four, representing normal, slightly abnormal, abnormal and severely abnormal, respectively (McGuirk, 2008). This system was assessed for detection of BRD with 62.4% sensitivity and 74.1% specificity (McGuirk, 2008; Buczinski et al., 2015). Another easy-to-implement scoring system including six clinical signs (coughing, nasal discharge, ocular discharge, ear drop or head tilt, fever $\geq$ 39.2°C, and abnormal respiration), dichotomizes animals into normal and abnormal categories (Love et al., 2014). The accuracy of this scoring system was assessed with 95.4% and 88.6% of cases being correctly classified into BRD and healthy categories, respectively (Love et al., 2014).

1.4.2 Diagnosis based on postmortem examination

1.4.2.1 Diagnosis of BRSV and BPIV3 infections

Gross lesions of BRSV and BPIV3 infections are similar, characterized by cranioventral atelectasis and consolidation with deep-red discoloration and rubbery structure (Ellis, 2010; Caswell JL,

2016b); however, variations in the gross lesions with red discoloration and generalized rubbery texture between cranial and caudal lung lobes occur commonly (Caswell JL, 2016b). The feature of interlobular emphysema with formation of bullae may differentiate BRSV from BPIV3 (Ellis, 2010). Histologically, necrotizing bronchitis and bronchiolitis, formation of epithelial syncytial cells and eosinophilic intracytoplasmic inclusion bodies are characteristic features of both BRSV and BPIV3 infections (Ellis, 2010; Caswell JL, 2016b). Histological lesions vary from attenuated bronchiolar epithelium, necrotic or sloughed bronchiolar epithelial cells, infrequent hyaline membrane and inflammatory cells including macrophages, neutrophils and lymphocytes in bronchiolar or alveolar lumens of acute infections to thickened inter-alveolar septa with type II pneumocytes and bronchiolar epithelial hyperplasia, and bronchiolitis obliterans in subacute to chronic infections (Caswell JL, 2016b).

1.4.2.2 Diagnosis of BHV1 infection (IBR)

Gross lesions of IBR are characterized by petechiae, erosions, ulcerations with fibrinonecrotic or suppurative exudate strictly localized to nasal mucosa, larynx and trachea (Caswell JL, 2016b). Systemic gross lesions of BHV1 infection include multiple, well-demarcated, pinpoint foci of necrosis in oral cavity, esophagus, rumen, liver, kidney and spleen (Caswell JL, 2016b). Histologically, cytoplasmic vacuolation, pyknotic and karyolytic nuclei and eosinophilic intranuclear inclusion bodies (INIB) in the nasal mucosa, tracheal glands and bronchi are often seen; in contrast, INIB are commonly seen at the periphery of the necrotic foci accompanied by neutrophils infiltration in systemic BHV1 infection (Caswell JL, 2016b).

1.4.2.3 Diagnosis of BCV infection

Gross lesions of BCV infection in experimentally infected calves are normally minimal with histologically mild interstitial pneumonia characterized by small amounts of fibrin in alveoli, type

II pneumocytes hyperplasia and increased macrophages in alveoli and alveolar septa (Ridpath et al., 2020b). BCV can cause enteric infections with development of villus atrophy, villus fusion, mononuclear infiltration of villi and crypt necrosis (Caswell JL, 2016b).

1.4.2.4 Diagnosis of BVDV infection

Gross lesions of BVDV as a primary pneumonic pathogen are minimal with histological aggregates of monocytes around bronchioles and blood vessels or suppurative exudate in bronchiolar lumens (Caswell JL, 2016b). BVDV and BCV dual infection can result in lung lesions of multiple, pale, firm foci up to 1 cm in diameter throughout the lung lobes, consistent with interstitial pneumonia histologically (Ridpath et al., 2020b). Gross lesions of BVDV infection in mucosal disease and acute postnatal infection consist of erosions or ulcerations throughout the gastrointestinal tract with lymphoid depletion, myocardial necrosis, abortion and cerebellar hypoplasia (Brodersen, 2014).

1.4.2.5 Diagnosis of bacterial infection

Gross lesions of acute infection with *M. haemolytica* or *H. somni* consist of "cranioventral lobar or lobular fibrinous bronchopneumonia, foci of coagulative necrosis, and variable fibrinous pleuritis in contrast to lack of focal coagulative necrosis and abundant fibrinous exudate by *P. multocida* infection" (Caswell JL, 2016b). Bronchopneumonia is characterized by sharp demarcation from the rest of the lung, cranioventral consolidation, and dark red discoloration of the lesions (Caswell JL, 2016b). "Sequestra, bronchiectasis, abscess, and fibrous pleural adhesions" (Caswell JL, 2016b) are common sequelae following acute infections. Histologically, infiltration of neutrophils and macrophages admixed with fibrin, proteinaceous fluid, erythrocytes, necrotic cellular and nuclear debris and bacteria aggregates in alveoli and bronchioles are commonly observed (Caswell JL, 2016b). Leukocyte necrosis and oat cells with appearance of a

streaming chromatin of leukocytes are also characteristic features in *M. haemolytica* and somewhat less *H. somni* infection (Caswell JL, 2016b). In contrast, *P. multocida* infection tends to have minimal or no fibrin exudate in alveoli and interlobular septa with viable neutrophils (Caswell JL, 2016b).

Gross lesions of *M. bovis* are characterized by gray-white, raised, sharply demarcated, friable, multifocal to coalescing foci of caseous necrosis in lung parenchyma, although sometimes it is difficult to differentiate suppurative bronchopneumonia induced by *M. bovis* from other bacteria based on cranioventral consolidation and reddening grossly (Caswell JL, 2016b). Histologically, eosinophilic coagulum with retained cellular outlines and surrounded by degenerative neutrophils and macrophages and outermost fibrous layer is characteristic (Caswell JL, 2016b).

1.4.3 Diagnosis based on laboratory tests

Virus isolation of BRSV and BPIV3 using Madin-Darby bovine kidney (MDBK) cells is not recommended currently for routine diagnosis due to the lability of these viruses in storage and transportation, long turn-around time and challenges with timing of sampling (Caswell JL, 2016b; Ellis, 2010; Brodersen, 2010). In most of the cases of calves that have died of BRD, at least one treatment was attempted, and so BRSV or BPIV3 would be gone by the time the animals are sent for necropsy (Brodersen, 2010; Ellis, 2010). For more stable DNA viruses like BHV1, virus isolation is more appropriate as a definitive diagnosis, and provides the advantage of potential isolation of novel strains from nasal, tracheal or lung samples (Caswell JL, 2016b).

Fluorescent antibody test (FAT) on frozen tissues has been commonly used in diagnostic laboratories despite its low sensitivity and specificity and rapid decay of the fluorescence (Brodersen, 2010; Fulton and Confer, 2012). In contrast to FAT, immunohistochemistry (IHC) that is applied on formalin-fixed paraffin-embedded tissues results in permanent staining and

shows the association between lesions and viral antigens. These advantages have made IHC a common tool in veterinary diagnostic laboratories (Fulton and Confer, 2012). A limitation of IHC is the lack of specific monoclonal antibodies, particularly for newly identified viruses (Fulton and Confer, 2012). IHC has been commonly used for rapid diagnosis of BRSV, BPIV3, BVDV, BCV and BHV1 in veterinary diagnostic laboratories (Caswell JL, 2016b). Another alternative is *in-situ* hybridization (ISH), in which direct binding between a nucleic acid probe and complementary sequence of an infectious agent within tissues is detected (Fulton and Confer, 2012). ISH is not as sensitive as qRT-PCR but potentially more sensitive than FAT and IHC, and it can be applied in cases where antibodies are not available, but the genome sequence of the agent is at least partially known (Fulton and Confer, 2012).

Serology tests such as enzyme-linked immunosorbent assays (ELISA) have been widely used in veterinary diagnostic laboratories to monitor status of infections within cattle herds (Fulton and Confer, 2012). These methods provide information regarding seroconversion suggestive of exposure rather than detection of pathogens or clinical disease (Fulton and Confer, 2012). Seroconversions to BRD associated viruses like BRSV, BPIV3, BVDV, BCV and BHV1 have been reported in cattle without clinical respiratory signs (Grissett et al., 2015; Caswell et al., 2012). Antibody levels against these viral agents were measured to differentiate vaccine-induced cattle from those naturally infected; however, further studies have not supported this approach since antibody levels vary among animals due to differential antibody production and vaccine potency (Fulton and Confer, 2012).

qRT-PCR has become the most common molecular test in veterinary diagnostic laboratories due to fast turnaround time, cost-effectiveness and high sensitivity and specificity (Fulton and Confer, 2012). One of the best examples of application of qRT-PCR is testing BVDV PI animals using ear

tip skin samples, which are simple to collect (Smith et al., 2020). Multiplex qRT-PCR tests for BVDV, BPIV3, BCV, BVDV and BHV1 have been developed to reduce costs (Horwood and Mahony, 2011; Thanthrige-Don et al., 2018). In a recent study, a novel multiplex PCR-electronic microarray assay, which deployed an automated platform with additional flexibility to change the detection probe panel, was developed to detect and differentiate four bacteria (*M. haemolytica*, *H. somni*, *P. multocida*, and *M. bovis*) and five viruses (BRSV, BPIV3, BCV, BVDV and BHV1) simultaneously with high analytical sensitivity and specificity (Thanthrige-Don et al., 2018). Recombinase polymerase amplification (RPA) assays offer a sensitive and accurate alternative diagnostic approach for multiplex detection of bacterial BRD pathogens including *M. haemolytica*, *P. multocida*, *H. somni* and *M. bovis* in deep nasal swabs (Conrad et al., 2020). Positive results from amplification based methods, however, can occur without the presence of infectious viruses (false positives) and point mutations within probe annealing sites may lead to false negatives (Fulton and Confer, 2012). Another drawback of qRT-PCR tests in general is the lack of standard protocols for the interpretation of results (Fulton and Confer, 2012).

Electron microscopy (EM) has one significant advantage for virus detection since it does not require agent-specific reagents for identification of viruses (Goldsmith and Miller, 2009). This is particularly important for new and unknown viruses without available antibodies and known sequences (Goldsmith and Miller, 2009). EM can also be applied to nasal swabs or tissues for identification of viruses (Fulton and Confer, 2012). Limitations also exist including requiring highly skilled professionals to recognize the agents, and inappropriate sample preparation may distort the virus and cellular ultrastructure leading to misidentification (Goldsmith and Miller, 2009).

Laboratory identification of BRD bacterial pathogens is often based on bacterial culture and isolation, which can be applied to various samples including nasopharyngeal, tracheal, lung samples and even frozen tissues (Goldsmith and Miller, 2009; Wolfger et al., 2015). *M. bovis* is an exception since its low biosynthetic capacity makes it fastidious and extremely difficult to isolate in the laboratory (Parker et al., 2018). When combined with other tests such as Matrix-Assisted Laser Desorption and Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry, isolated bacteria are readily identified (Fulton and Confer, 2012; Zhang et al., 2020b). A few considerations must be kept in mind when results are unexpected (Fulton and Confer, 2012). Firstly, BRD pathogens can be commensals, so their detection on its own is not diagnostic of BRD. Secondly, previous antibiotic therapy may lead to false negative isolation results.

1.5 Potential future approaches for diagnosis of BRD

1.5.1 Introduction of high throughput sequencing (HTS)

The concept of "You only find what you are looking for" (Fulton and Confer, 2012) is applicable to current tests used in veterinary diagnostic laboratories. Detection of nucleotide sequences of pathogens is still an important solution to many diagnostic testing problems, but despite various methods available for detection of viruses and bacteria, there is an ongoing demand for development of new technologies that provide fast, inexpensive and accurate sequence information to resolve biological and diagnostic questions of interest (Parker and Chen, 2017; Köser et al., 2012). The Sanger sequencing method, developed by Sanger in 1975, is considered a "low throughput sequencing or first-generation sequencing" technology due to small numbers of DNA molecules processed per unit time (Sanger and Coulson, 1975). The average length sequenced by Sanger technology is 800 bases with potential extension to 1000 bases (Schadt et al., 2010). However, sequencing the first human genome using Sanger technology took approximately three

billion dollars and 10 years due to its low throughput and extremely high cost (Schadt et al., 2010; Venter et al., 2001). To address these issues, new sequencing technologies were developed, referred to as high throughput sequencing (HTS) or next-generation sequencing (NGS) (Buermans and den Dunnen, 2014; Chiu and Miller, 2019). These technologies including second-generation sequencing (SGS) and third-generation sequencing (TGS) and they made it possible to sequence a large number of DNA molecules in parallel (Parker and Chen, 2017; Schadt et al., 2010). Over the past several years, HTS has revolutionized the genomics field (Metzker, 2010; Cheng et al., 2018; Parker and Chen, 2017; Quick et al., 2017; Tyler et al., 2018; Zhu et al., 2020). HTS with its extremely high throughput and low cost per identified base has become a promising technology to detect nucleic acids of infectious agents (Mitra et al., 2016; Ng et al., 2015; Caswell, 2014; Timsit et al., 2016). HTS has many advantages in the identification of non-culturable or difficult-to-culture organisms, in identification of novel pathogens, or variants of known pathogens such as SARS-COV-2 in the current global pandemic (Cheng et al., 2018; Parker and Chen, 2017; Quick et al., 2017; Tyler et al., 2018; Zhu et al., 2020; Quick et al., 2016). Furthermore, HTS can also advance the understanding of infectious diseases through pathogen discovery, detection of drug resistance, pathogen variability and genotyping as well as microbiome analysis (Köser et al., 2012; Kafetzopoulou et al., 2018; Gu et al., 2019; Chiu and Miller, 2019). However, every coin has two sides. A major challenge to application of these HTS approaches is storage of the huge amounts of data generated (Stephens et al., 2015). It is estimated that HTS data will increase approximately five orders of magnitude by 2025, significantly exceeding that associated with the domains of astronomy, YouTube and Twitter (Stephens et al., 2015).

1.5.2 Illumina sequencing as a representative of SGS

Sequencing by synthesis (Bentley et al., 2008), popularized by Illumina (San Diego, CA), involves single-stranded DNA molecules from multiple samples annealed to a flow cell that is precoated with oligonucleotides complementary to the adaptors added to the ends of the DNA fragments (Hodkinson and Grice, 2015; Ravi et al., 2018). Template DNA fragments can be PCR amplicons (e.g. metabarcoding targets such as 16S rRNA or cpn60) or fragmented genomic DNA from specific organisms or metagenomic samples such as tissues or other clinical specimens (Lu et al., 2016). The oligonucleotides on the flow cell anchor the template DNA and act as primers for subsequent amplification (Lu et al., 2016). Amplification occurs on the glass plate by a process known as "bridge amplification", in which individual single-stranded molecules attach at both ends to primers on the glass plate (Hodkinson and Grice, 2015; Ravi et al., 2018). Thousands to millions of clusters of molecules are generated after successive rounds of PCR amplification and are subsequently sequenced (Radford et al., 2012; Schadt et al., 2010). Current reagents can generate paired-end reads that are from 36 bases up to 300 bases in length (Hodkinson and Grice, 2015). The PCR amplification steps involved in amplicon generation or during library preparation of genomic DNA fragments can introduce amplification errors and bias (Schadt et al., 2010). Short DNA sequences can also be challenging for downstream bioinformatics processes and lead to errors in mapping to reference sequences due to ambiguous highly repetitive genomic regions (Buermans and den Dunnen, 2014; Schadt et al., 2010).

1.5.3 Oxford Nanopore sequencing as a representative of TGS

Nanopore sequencing (Oxford Nanopore Technologies) overcomes some of the limitations of the short DNA sequences generated by SGS technologies (Lu et al., 2016). SGS instruments commonly adapt sequencing by synthesis technology that depends on amplification to generate a

large number of clusters of DNA template molecules (Hodkinson and Grice, 2015; Ravi et al., 2018). In contrast, TGS platforms directly process single-stranded DNA molecules without requirement of amplification and synthesis (Lu et al., 2016). One of the devices supporting Nanopore sequencing is the MinION, a portable single-stranded molecule genome sequencing instrument no larger than a typical smartphone (Loman et al., 2015; Lu et al., 2016). The data are collected from the MinION device by a laptop through a Universal Serial Bus (USB) from which the instrument also draws power (Lu et al., 2016). Passage of a single-stranded DNA molecule through a protein pore in the flow cell results in frequent electrical current changes, which are detectable, and dependent on the nucleotide sequence (Quick et al., 2016; Lu et al., 2016). Each MinION flow cell is equipped with 512 channels and each channel is connected to four wells, which are tested at the beginning of a sequencing run using "Mux" procedure (Lu et al., 2016). Ranking the activity of each of the four wells is conducted with the most active well providing data within the first 24 hours and the data being collected equally from the rest of the wells within the next 24 hours (default 48 hour run time) (Lu et al., 2016). The GridION instrument has the capacity to run five flow cells individually or concurrently, providing more flexibility and higher throughput to Nanopore sequencing (https://nanoporetech.com/products/gridion).

A series of steps is carried out to prepare purified nucleic acids for sequencing ("library preparation"). Briefly, after end-repair and dA-tailing, adapters are ligated, followed by bead-based purification (Tyler et al., 2018). Two adaptors: the leader adapter also known as Y adapter (it has a Y structure appearance) and hairpin adapter referred to as HP adapter, are attached to each end of the dsDNA, respectively (Lu et al., 2016). When the motor protein unzips the dsDNA, sequencing starts at the 5' end of Y adapter, followed by the template strand, HP adapter and the complementary strand with speed controlled by the motor protein (Lu et al., 2016). When sufficient

data is accumulated, basecalling can be performed using MinKNOW integrated Guppy (https://nanoporetech.com/).

As this instrument directly sequences native, individual DNA molecules, it is able to sequence extremely long DNA molecules; however, MinION has a much higher error rate (up to 15%) than high throughput sequencing that sequences clonal copies of DNA molecules (Churko et al., 2013; Quick et al., 2016).

1.5.4 Challenges of HTS

1.5.4.1 Host depletion and viral enrichment

The major challenge of detecting viruses in clinical samples (especially tissues) using HTS is that the majority of genetic material in the sample is host derived and viral genetic materials are just like "a needle in a haystack" (Zhang et al., 2020c; Conceição-Neto et al., 2015). The lack of universally conserved regions in viral genomes hinders the application of universal primers like those that can be used to enrich bacterial sequences (Conceição-Neto et al., 2015). Increasing proportional abundance of viral nucleic acids in a sample correspondingly increases the chances of them being sequenced and thus the probability of being detected when the resulting sequence data is aligned to a reference database (Hall et al., 2014; Zhang et al., 2020c). The most commonly used methods for viral enrichment from clinical samples include centrifugation, filtration, or nuclease treatment, and the combination of these three methods significantly increases the proportion of viral sequences (Hall et al., 2014). The centrifugation and/or filtration steps remove host cells and microorganisms such as bacteria and fungi and nuclease (DNase or RNase) treatment digests genetic materials not protected by a viral envelope or capsid (Hall et al., 2014).

Different viral nucleic acid extraction kits have been evaluated for detection of various viruses in clinical samples using HTS (Zhang et al., 2018a). In this study, viral RNA extraction kits including QIAamp Viral RNA Mini Kit (VRMK), QIAamp MinElute Virus Spin Kit (MVSK), RNeasy Mini Kit (RMK), and RNeasy Plus Micro Kit (RPMK) were all suitable for extraction of nucleic acids from DNA viruses for HTS. The different viral extraction kits had varying extraction efficiencies (Zhang et al., 2018a). RPMK was found to be the most suitable kit for RNA virus extraction and MVSK yielded the highest concentration of adenovirus and human parainfluenza virus (HPIV3) nucleic acids (Zhang et al., 2018a).

1.5.4.2 Bioinformatics pipelines

Particular biases and sequencing errors are inherent to any sequencing technology and are unavoidable (Hodkinson and Grice, 2015; Senol Cali et al., 2019). However, data errors can be minimized or removed using bioinformatic approaches to achieve the final goal.

1.5.4.2.1 Basecalling

Basecalling is the process of translating the raw current signal from Nanopore sequencing into four bases A, T, C and G to generate DNA sequences, which is the most important step of the pipeline for the subsequent analysis (Senol Cali et al., 2019; Zeng et al., 2020; Rang et al., 2018). Various basecalling tools have been developed and currently Guppy integrated with or without MinKNOW is most commonly used (Rang et al., 2018). Guppy basecaller, however, is trained on sequence data from *E. coli*, *Saccharomyces cerevisiae* and *Homo sapiens* without inclusion of any viruses, which could lead to errors when it is applied to data from organisms outside the training set (Rang et al., 2018; Zeng et al., 2020). It has been reported that higher basecalling accuracies were obtained when novel *E. coli* sequence data was interpreted using an *E. coli* training dataset compared to a human training dataset (Stoiber and Brown, 2017). High error rates in

homopolymers are common due to disruption of the relationship between homopolymer length and detection time and the Scrappie basecaller may address these homopolymer errors in the future (Senol Cali et al., 2019; Stoiber and Brown, 2017).

1.5.4.2.2 Genome assembly

Genome assembly for short reads generated by MiSeq generally uses a de Bruijn graph approach that splits the reads into short overlapping k-mers and then connections between all k-mers are joined (Compeau et al., 2011). In contrast, genome assembly for long reads generated by Nanopore sequencing commonly deploys overlap layout consensus (OLC) approach due to the high error rates and long reads spanning repetitive regions of the genome (Li et al., 2012). Genome assembly is divided into *de novo* assembly and reference-based assembly, the former of which constructs the genome without needing a reference genome (Rausch et al., 2009). Trinity (Haas et al., 2013), Canu (Koren et al., 2017), Miniasm (Li, 2016), etc. are widely applied for *de novo* assembly with Trinity for short reads and Canu and Miniasm for long reads. Canu performs an initial error-correction step followed by overlap identification, which improves the accuracy of the reads but with a huge computational cost (Koren et al., 2017). Miniasm constructs the genome from uncorrected overlap reads with relatively lower accuracy than Canu (Koren et al., 2017; Li, 2016).

1.5.4.2.3 Taxonomic classification

To taxonomically classify metagenomic data, which contains millions of sequences from viruses, bacteria, archaea and various hosts in a comprehensive manner, all the data must be compared against reference databases containing sequences from thousands to millions of organisms (Corvelo et al., 2018; Kim et al., 2016; Kumar et al., 2018; Langmead and Salzberg, 2012; Li, 2018; Wood et al., 2019; Wood and Salzberg, 2014). This operation is hugely computer-intensive, because querying performance is largely dependent on the size of database (Kim et al., 2016) and

the challenge in using a large database is the large number of unique k-mers that must be indexed (Wood et al., 2019). This scenario necessitates a demand for classifying these sequences quickly and accurately, however, most of the current metagenomic classification programs suffer from slow classification speed, a large index size or computer memory requirements (Corvelo et al., 2018; Kim et al., 2016; Langmead and Salzberg, 2012; Li, 2018; Wood et al., 2019; Wood and Salzberg, 2014).

Kraken's k-mer-based approach provides a fast taxonomic classification of metagenomic data, but its large computer memory requirements may be a limitation for application (Wood et al., 2019). Fortunately, Kraken 2 has been developed and significantly reduces the memory usage as well as maintaining high accuracy and speed; furthermore, Kraken 2 also introduces a translated search mode, which shows similar accuracy compared to Kaiju (a protein classifier) (Wood et al., 2019). Centrifuge's efficient indexing scheme makes it possible to index the NCBI nucleotide database, which contains nearly 40 million nonredundant sequences (~ 110 billon bp) collected from viruses, archaea, bacteria and eukaryotes into an index size of 69 GB (Kim et al., 2016). In contrast, if Kraken requires 100 GB space for its index of a database, BLAST requires 25 GB for the same database and Centrifuge requires only 4 GB to store and index the same database with Bowtie2 requiring 21 GB (Corvelo et al., 2018; Kim et al., 2016; Langmead and Salzberg, 2012; Wood et al., 2019; Wood and Salzberg, 2014; Altschul et al., 1990). The overall accuracy and speed of Kraken and Centrifuge is similar, but Centrifuge is 10 times faster than Bowtie2 and 3500 times faster than MegaBLAST and BLAST when classifying reads (Corvelo et al., 2018; Kim et al., 2016; Wood et al., 2019).

Bowtie2 permits gapped alignment, and the alignment gaps can result either from sequencing errors or from true insertions and deletions (Langmead and Salzberg, 2012). Bowtie2 is faster than

all BWA programs and shows more correct alignments than Burrows-Wheeler Aligner (BWA) or short oligonucleotide alignment program 2 (SOAP2) (Langmead and Salzberg, 2012). In general, BWA, Bowtie2 or SOAP2 are designed for short reads alignment (Langmead and Salzberg, 2012). Minimap2 is specifically designed for the long, noisy reads that are generated by Oxford Nanopore technologies; however, it also works with short reads of >100 bp and assembled contigs (Li, 2018). For aligning short genomic reads, Minimap2 is faster than Bowtie2 and BWA-MEM and more accurate than Bowtie2 as well (Li, 2018). For long reads, Minimap2 is faster at higher accuracy than mainstream long-read mappers like minialign and GraphMap (Li, 2018). In addition, Minimap2 has a lower memory usage than GraphMap (Li, 2018).

1.5.5 Current application of HTS to BRD research

Only two studies prior to our research have investigated nasopharyngeal viromes associated with BRD using Illumina HiSeq and MiSeq, respectively (Mitra et al., 2016; Ng et al., 2015). Results of these studies indicated some unconventional viruses such as bovine adenovirus 3 (BADV3), bovine rhinitis A virus (BRAV) and IDV were significantly associated with BRD (Mitra et al., 2016; Ng et al., 2015). Bacterial microbiome studies on BRD have been extensively explored with most emphasis on SGS as well (Amat et al., 2019; Guo et al., 2020; Holman et al., 2015; Holman et al., 2017; Timsit et al., 2016; Timsit et al., 2018). At the time of writing, no TGS has been applied to investigate the virome of BRD to the best of our knowledge, and reports of the relative performance of HTS and conventional diagnostic methods such as qRT-PCR for viruses are scarce (Kafetzopoulou et al., 2018).

1.6 Prevention and control of BRD

Both inactivated and modified live vaccines are available for BRSV, BPIV3, BHV1 and BVDV (Fulton, 2020; Bowland and Shewen, 2000). The components of common vaccines used in the

cattle industry are often combined and contain BRSV, BPIV3, BVDV1 and 2, IBR and *M. haemolytica* toxoid (Zhang et al., 2019; Bowland and Shewen, 2000). The efficacy of vaccines is highly variable leading to mixed success in vaccination strategies to reduce or prevent BRD. A recent meta-analysis study (Theurer et al., 2015) concluded that there were no significant differences in morbidity and mortality between control and vaccinated groups when evaluating a modified live BRSV vaccine in experimentally challenged animals. Therefore, research and further development regarding BRSV vaccines must continue (Ellis, 2017). Evaluation of BPIV3 vaccine efficacy from various laboratory studies is challenging and inconsistent (Ellis, 2010). One of the challenges is the frequent failure to reproduce the respiratory disease in inoculation studies (Ellis, 2010). It has been proposed that the best approach to controlling BPIV3 is priming calves using intranasal vaccination followed by a parental vaccine to boost (Ellis, 2010). BVDV vaccines incorporated both type 1 and type 2 once the type 2 BVDV was recognized, which greatly expanded the spectrum of immune responses and increased the likelihood of protection against BVDV infection (Smith et al., 2020; Chase, 2013; Peterhans et al., 2003). In addition to vaccination, removal of PI animals identified with improved diagnostic tests has been found to significantly enhance BVDV control (Brodersen, 2014). Modified live or inactivated vaccines for BHV1 greatly decreased abortion risk as shown in a meta-analysis study (Newcomer et al., 2017). Although there is currently no licensed BCV vaccine for BRD control and prevention, a modified live BCV vaccine was reported to significantly reduce BRD treatment and cross-protection was induced against respiratory BCV regardless of strains from different clinical origin (Saif, 2010; Plummer et al., 2004).

Various types of *M. haemolytica* vaccines including bacterin, toxoid, and inactivated or live vaccines have been developed over the last decades (Klima et al., 2014; Waldner et al., 2019). A

meta-analysis indicated that a significant difference in morbidity but not mortality was found between vaccinated and control groups of feedlot cattle (Larson and Step, 2012). The same study showed that currently available vaccines for *H. somni* did not affect BRD development, which is consistent with previous findings of mixed effects on BRD morbidity and no effect on mortality for feedlot cattle (Larson and Step, 2012; Smith et al., 2020). Despite the observation that quadrivalent killed vaccines containing BRSV, BPIV3, *M. bovis* and *M. dispar* showed significant protection against BRD, no licensed *M. bovis* vaccines are available currently (Dudek and Bednarek, 2018; Howard et al., 1987). In a recent study using a saponin-inactivated *M. bovis* vaccine, respiratory signs, lung lesions and spread to internal organs were significantly reduced compared to the unvaccinated group, indicating protection against a virulent *M. bovis* strain (Nicholas et al., 2002).

In addition to vaccines, antibiotics such as tulathromycin, florfenicol, oxytetracyclines, tilmicosin, enrofloxacin and tylosin are used globally to reduce and alleviate secondary bacterial infections; however, emergence of resistant bacterial strains has frequently been reported, raising concerns about contribution of livestock production practices to antimicrobial resistance (Nicholas and Ayling, 2003; Holman et al., 2019). Efforts have also been put into the host, management and environmental factors that contribute to the BRD occurrence (Fulton, 2009; Taylor et al., 2010). Preconditioning programs including castration, dehorning and vaccination prior to weaning, purchasing calves from farm sources, and weaning at the original farms have dramatically reduced BRD morbidity and mortality (Hilton, 2014).

OBJECTIVES

Objective 1: Establish a metagenomic sequencing method to detect multiple viruses simultaneously using the MiSeq platform, and apply the method to determine the prevalence and association of viruses in nasal and tracheal samples from cattle with or without BRD.

Objective 2: Determine the relationship of viruses and BRD associated bacteria to histopathology patterns in bovine pneumonia lung samples.

Objective 3: Establish a method to apply Nanopore sequencing for viral detection and compare performance of Nanopore, MiSeq and real-time qPCR for detection of IDV in BRD samples.

Objective 4: Determine if the composition of the deep nasal virome at the time of arrival at the feedlot predicts future BRD development using Nanopore sequencing.

CHAPTER 2: Respiratory viruses identified in western Canadian beef cattle by metagenomic sequencing and their association with bovine respiratory disease

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Citation

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Chapter transition

High throughput sequencing (HTS) has been widely applied to identify viruses in both human and veterinary medicine. However, the application of HTS to identify viruses associated with BRD is rare. There were only two previous studies investigating the association of viromes with BRD. Neither of these studies, however, included samples from the lower respiratory tract to determine whether the viruses identified in the nasal tract are representative of the entire respiratory tract virome. The objective of our study was to use metagenomic sequencing to characterize and compare the upper and lower respiratory tract viromes of Canadian feedlot cattle, with or without BRD.

2.1 Abstract

Bovine respiratory disease (BRD) causes considerable economic losses in North America. The pathogenesis involves interactions between bacteria, viruses, environment and management factors. Primary viral infection can increase the risk of secondary fatal bacterial infection. The objective of this study was to use metagenomic sequencing to characterize the respiratory viromes of paired nasal swabs and tracheal washes from western Canadian feedlot cattle, with or without BRD. A total of 116 cattle (116 nasal swabs and 116 tracheal washes) were analyzed. The presence of influenza D virus (IDV), bovine rhinitis A virus (BRAV), bovine rhinitis B virus (BRBV), bovine coronavirus (BCV) and bovine respiratory syncytial virus (BRSV) was associated with BRD. Agreement between identification of viruses in nasal swabs and tracheal washes was generally weak, indicating that sampling location may affect detection of infection. This study reported several viruses for the first time in Canada and provides a basis for further studies investigating candidate viruses important to the prevention of BRD.

Keywords: bovine respiratory disease (BRD), metagenomic sequencing, virome, influenza D virus, bovine rhinitis virus, bovine coronavirus, bovine respiratory syncytial virus

2.2 Introduction

Bovine respiratory disease (BRD) is one of the most costly and commonly diagnosed diseases in the beef industry. The disease results in economic losses from morbidity, mortality, cost of therapy, and reduced performance (Griffin, 1997a; Fulton, 2009). Approximately 75% of the morbidity and 50% of the mortality in feedlots in United States is caused by BRD (Edwards, 2010). In Canada, over 80% of the vaccines licensed for cattle are applied for control and prevention of BRD (Edwards, 2010; Bowland and Shewen, 2000). Bovine respiratory disease is considered multifactorial, involving complex interactions between the animal, the pathogens and the

environment, which poses significant challenges to its prevention and control (Murray et al., 2016). It is generally proposed that management practices such as shipping may compromise the immune system, and predispose the animals to viral and bacterial infections (Mosier, 2014). Viral infection can interfere with the immune system and damage the mucocilliary escalator mechanism and lung parenchyma, which in turn facilitates translocation of bacteria and establishment of infection in the lower respiratory tract (Taylor et al., 2010).

To date, the following bacteria and viruses are considered as major BRD pathogens: *Histophilus somni, Pasteurella multocida, Mannheimia haemolytica*, bovine herpesvirus 1 (BHV-1), bovine viral diarrhea virus (BVDV), bovine parainfluenza 3 virus (PI3V), and bovine respiratory syncytial virus (BRSV) (Fulton, 2009). Although vaccines for these pathogens are commercially available, mass medication with antimicrobial on arrival at the feedlot (also known as metaphylaxis in North America) is still needed for effective prevention, which raises major public health concerns regarding antimicrobial usage and resistance (Hilton, 2014; Timsit et al., 2017; Bowland and Shewen, 2000; Ellis, 2009). Furthermore, despite aggressive use of antibiotics and vaccines, BRD morbidity and mortality rates among feedlot cattle have remained steady (Hilton, 2014).

High throughput sequencing (HTS) has recently been applied to identify viruses in specimens from humans and animals (Parker and Chen, 2017; Shan et al., 2011b). A metagenomic study in BRD cases in dairy calves in the USA demonstrated the association of this disease with the presence of bovine adenovirus 3 (BAdV3) and influenza D virus (IDV) in nasopharyngeal and pharyngeal swabs (Ng et al., 2015). Results of another US study suggested IDV as a potential etiologic agent for BRD after metagenomic sequencing of nasal swabs from Mexican and American steers (Mitra et al., 2016). Neither of the above studies, however, included samples from the lower respiratory

tract to determine whether the viruses identified in the nasal tract are representative of the entire respiratory tract virome.

The objective of this study was to use metagenomic sequencing to characterize and compare the upper and lower respiratory tract viromes of Canadian feedlot cattle, with or without BRD.

2.3 Materials and Methods

Sample collection

The study design and sample collection were described previously (Timsit et al., 2018). Cattle with BRD (n = 58) and control cattle (n = 58) were enrolled in this study. These cattle were from four different feedlots in Southern Alberta, Canada. Samples were collected from November 2015 to January 2016. On arrival, all cattle were vaccinated with modified live vaccines against IBR, BVDV types I and II, BPIV3 and BRSV (Pyramid FP 5 + Presponse SQ, Boehringer Ingelheim, Burlington, ON, Canada). The vaccination was repeated 30 days later. Experienced pen-checkers and veterinarians observed cattle daily for signs of respiratory disease and collected the samples after the animals arrived at the feedlots. Cattle with at least one BRD sign (depression, nasal and ocular discharge, cough or dyspnea), a rectal temperature ≥ 40 °C, abnormal lung sounds, a serum haptoglobin concentration ≥ 0.25 g/L and no prior treatment against BRD or other diseases were enrolled as cases. Cattle without any of the above-mentioned signs were defined as control. Deep nasal swab (DNS) and trans-tracheal aspirates (TTA) were collected from these animals (Timsit et al., 2013; Timsit et al., 2018). Control steers were removed from the study if they became sick within 30 days of enrollment.

This study was conducted in strict accordance with the recommendations of the Canadian Council of Animal Care (Olfert et al., 1993). The research protocol was reviewed and approved by the University of Calgary Veterinary Sciences Animal Care Committee (AC15-0109).

Sample preparation

Swabs and tracheal washes were centrifuged at 13,000 × g for 5 min. A subsample of supernatant (160 µl) from each sample and negative control (molecular biology grade water) was incubated with 20 µl of TURBO DNase buffer and 24 units of DNase (Life Technologies, USA) and 20 units of RNase ONE Ribonuclease (Promega) at 37 °C for 90 min to remove host nucleic acids. Viral nucleic acids were then extracted using a viral nucleic acid purification kit (QIAamp MinElute virus spin kit, Qiagen, CA, USA) according to the manufacturer's instructions, and eluted with 30 µl nuclease-free water. Reverse transcription was performed with primer FR26RV-N (5´-GCC GGA GCT CTG CAG ATA TCN NNN NN-3´) (Allander et al., 2005) using a Superscript III First-Strand synthesis kit (Life Technologies). Following termination of the reaction and digestion with RNase H, complementary strand synthesis was carried out using Sequenase DNA polymerase (Affymetrix, Ohio, USA). The resulting double-stranded cDNA and DNA were selected and purified using NucleoMag NGS beads (Macherey-Nagel Inc., Germany) with a volume ratio of 1:1, to remove all fragments less than 200 bases. Purified DNA was subsequently amplified using primer FR20RV (5´-GCC GGA GCT CTG CAG ATA TC-3´) (Allander et al., 2005). The randomly amplified DNA was subjected to NucleoMag NGS clean-up and size selection (Macherey-Nagel Inc., Germany). Quantification was performed using a Qubit 2.0 fluorometer (Invitrogen, Waldbronn, Germany) with the Qubit dsDNA BR assay kit (Invitrogen, Waldbronn, Germany) before proceeding to library preparation.

Library preparation and sequencing

DNA (1 ng) from each individual sample used as input for library preparation using the Nextera XT library preparation kit (Illumina Inc., San Diego, CA, USA*)* according to the manufacturer's protocol. The fragmented DNA was amplified via a limited-cycle PCR program to add index primers at both ends. NucleoMag NGS beads were used to purify and size-select the library DNA. Undiluted library (1 µl) from each sample was analyzed using an Agilent Technology 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to confirm the fragment size distribution of the library. Library normalization was performed according to the bead based normalization method to ensure equal library representation in the pooled samples. The pooled library (24 µl) was mixed with 576 µl hybridization buffer and heated for 2 min at 96 °C. After the incubation, the library was transferred immediately to an ice bath for 5 min and then loaded into the MiSeq reagent cartridge and sequenced using a Miseq V2 500 cycle kit (Illumina Inc., San Diego, CA, USA).

Bioinformatic analysis

Demultiplexed raw data was trimmed for quality with Trimmomatic-0.32 (Bolger et al., 2014) using the following parameters: minimum length of 50 and Phred score of 20. Quality trimmed reads were mapped on to the *Bos taurus* reference genome (PRJNA33843, PRJNA32899) using Bowtie2 (Langmead and Salzberg, 2012) and unmapped reads were identified using samtools (Li et al., 2009). Unmapped reads were extracted from the original fastq files using cdbyank. *De novo* assembly of unmapped reads was performed for each sample using Trinity (Grabherr et al., 2011) with default parameters. Assembled contigs were aligned to the virus Reference Sequence (RefSeq) database (Brister et al., 2015) using BLASTn. As an initial screen of contigs for virus-like sequences, contigs yielding alignments of at least 100 base pairs in length with the expectation

(e) values < 10^{-3} were analyzed further. Contig sequences from each sample that passed this initial screen were examined manually by BLASTx comparison to the Genbank non-redundant protein sequence database to confirm the nucleotide sequence-based identification and remove any contigs with spurious matches such as vector sequences. The total number of reads of each virus in each sample library corresponding to the assembled contigs was determined by Bowtie2 mapping of reads from each sample on to the assembled contig sequences. All original data files were submitted to Sequence Read Archive (SRA) database of the National Center for Biotechnology Information (NCBI) under the accession number SRP157955.

Statistical analysis

Associations between detection of viruses and BRD status were analyzed by logistic regression using generalized linear mixed models (GLMM) in IBM SPSS Statistics (Version 25). Cattle BRD status was set as the dependent variable, and presence of different viruses and combination of different viruses were independent variables in various models. Home pens within feedlot were regarded as random effects. Individual cattle were defined as the experimental unit.

Agreement between nasal swabs and tracheal washes was determined by Cohen's Kappa statistic (Cohen, 1960). The strength of agreement for Kappa coefficient was interpreted as follows: values ≤ 0 = no agreement, 0.01 - 0.20 = slight, 0.21 - 0.40 = fair, 0.41 - 0.60 = moderate, 0.61 - 0.80 = substantial and 0.81 - 1 = almost perfect agreement (Landis and Koch, 1977).

Statistical significance was defined as $P < 0.05$, and statistical trend as $P < 0.1$.

2.4 Results

Identification of viruses

A total of 82.7 million reads were generated. After removing low quality reads and subtracting host derived reads, 33.6 million reads remained, including 9.6 million from nasal swabs and 24.0 million from tracheal washes. A total of 1.8 million high-quality viral reads were obtained, accounting for 2.19% of the total reads generated. A total of 21 viruses were identified from nasal swab and tracheal wash samples (Table 2.1 and Supplementary Table 2.4S). The largest contig assembled for each virus varied from 351 to 7,513 bases, which mapped to different regions of the viral genomes (Table 2.1). When all assembled contigs were considered, the genome coverage of each individual virus varied from as low as 2% (BPIV3) to virtually complete coverage (>99%, IDV). The number of reads mapping to each virus was correspondingly variable, ranging from minimally 11 to maximally 1,061,037 reads. No viruses were identified in negative controls. No statistical analyses were performed on the viruses identified in less than three cattle.

Statistical analyses

When different viruses alone were analyzed as independent variables, influenza D virus (IDV), bovine rhinitis B virus (BRBV), bovine respiratory syncytial virus (BRSV) and bovine coronavirus (BCV) showed significant association with BRD (Table 2.2). There was a statistical trend between the presence of BRAV and BRD (Table 2.2). Furthermore, the presence of at least one of the following viruses - IDV, BRAV, BRBV, BRSV and BCV – was used as a dichotomous variable for analysis and showed significant association with BRD (Table 2.3). Ungulate tetraparvovirus (UTPV1) and ungulate bocaparvovirus 6 (UBPV6) were the two most prevalent viruses identified

in this study, but they were not positively associated with BRD. Other viruses identified in this study also lack positive associations with BRD (Table 2.2).

The agreements of the identification of viruses between nasal swabs and tracheal washes were generally slight to moderate. IDV, BRBV and BRAV were mainly identified in nasal swabs. In contrast, the majority of BRSV was identified in tracheal washes, while BCV was identified in both nasal and tracheal regions (Figure 2.1 and Supplementary Table 2.4S). BRBV and BRSV were present in all four feedlots and each feedlot had at least two of the five viruses described above that were associated with BRD (Figure. 2.2).

Table 2. 1. Viruses identified by metagenomics.

Virus	Family	Genome Size(bp)	Largest contig size (bp) from any individual sample	Largest contig % AA identity (protein)	Total number of reads from all samples[§]
IDV	*Orthomyxoviridae*	12,546	1,587	99 (PB2)	17,297
ICV	*Orthomyxoviridae*	12,555	1,010	100 (PB1)	307
BRBV	*Picornaviridae*	7,556	2,431	99 (polyprotein)	38,648
BRAV	*Picornaviridae*	7,245	1,296	100 (polyprotein)	1,022
EVE	*Picornaviridae*	7,414	3,186	98 (polyprotein)	20,124
BRSV	*Paramyxoviridae*	15,140	1,169	98 (RdRp)	121,005
BPIV3	*Paramyxoviridae*	15,537	279	99 (M)	49
BCV	*Coronaviridae*	31,032	7,513	99 (ORF1ab)	197,921
BNV	*Coronaviridae*	20,261	4,782	99 (PP1a/b)	86,392
BPV2	*Parvoviridae*	5,610	1,149	93 (nonstructural protein)	1,427
BAAV	*Parvoviridae*	4,693	1,096	99 (Cap)	1,002
UTPV1	*Parvoviridae*	5,108	4,375	98 (NS1, VP1 and VP2)	1,061,037
UBPV6	*Parvoviridae*	5,224	4,518	99 (nonstructural protein)	263,902
BVDV1	*Flaviviridae*	12,258	602	97 (NS5b)	12
HCV	*Flaviviridae*	8,850	528	97 (core protein)	11
BAdV3	*Adenoviridae*	34,446	366	99 (284R)	14
BAV	*Astroviridae*	6,233	1,220	98 (NSP1ab)	884
ssCDV	*Genomoviridae*	2,300	676	91 (Rep)	158
WUPyV	*Polyomaviridae*	5,229	731	77 (large T antigen)	337
PBCV	*Phycodnaviridae*	331,00	351	96 (CVM1)	288
HPV	*Papillomaviridae*	7,966	763	100 (major capsid protein)	174

Note: IDV – influenza D virus, ICV – influenza C virus, BRBV – bovine rhinitis B virus, BRAV – bovine rhinitis A virus, EVE – enterovirus E, BRSV – bovine respiratory syncytial virus, BPIV3 – bovine parainfluenza virus 3, BCV – bovine coronavirus, BNV – bovine nidovirus, BPV2 – bovine parvovirus 2, BAAV – bovine adeno-associated virus, UTPV1 – ungulate tetraparvovirus 1, UBPV6 – ungulate bocaparvovirus 6, BVDV1 – bovine viral diarrhea virus 1, HCV – bovine hepacivirus, BAdV3 – bovine adenovirus 3, BAV – bovine astrovirus, ssCDV – single stranded cDNA virus, WUPyV – WU polyomavirus, PBCV - paramecium bursaria chlorella virus, HPV –

human papillomavirus type 40, bp – base pairs, AA – amino acids. § Out of 1.8 million virus sequence reads from all samples.

Table 2. 2. Prevalence of different viruses and their association with BRD. ("*" represents the statistical significance and "ǂ" represents the statistical trend)

Virus	No. of positive cattle/total BRD or control cattle (% positive)		Odds ratio	95% CI for odds ratio	*P* value
	BRD	Control			
IDV	13 (22)	3 (5)	6.145	1.435 - 26.310	0.015*
BRBV	16 (28)	6 (10)	3.836	1.245 - 11.821	0.020*
BRSV	10 (17)	1 (2)	13.422	1.454 - 123.885	0.022*
BCV	11 (19)	2 (3)	7.392	1.354 - 40.346	0.021*
BRAV	7 (12)	2 (3)	5.659	0.982 - 32.602	0.052ǂ
BPV2	7 (12)	3 (5)	3.289	0.682 - 15.865	0.137
BNV	4 (7)	23 (40)	0.078	0.021 - 0.288	0.000
ICV	0 (0)	6 (10)	---	---	0.967
BAV	5 (9)	1 (2)	4.485	0.459 - 43.798	0.195
UTPV1	26 (45)	16 (28)	1.878	0.812 - 4.348	0.140
UBPV6	8 (14)	20 (34)	0.296	0.108 - 0.814	0.019
WUPyV	3 (5)	6 (10)	0.421	0.081 - 2.185	0.300
EVE	6 (10)	2 (3)	4.258	0.704 - 25.740	0.113
BAdV3	1 (2)	2 (3)	0.339	0.025 - 4.619	0.414

Note: IDV – influenza D virus, BRBV – bovine rhinitis B virus, BRSV – bovine respiratory syncytial virus, BCV – bovine coronavirus, BRAV – bovine rhinitis A virus, BPV2 – bovine parvovirus 2, BNV – bovine nidovirus, ICV – influenza C virus, BAV – bovine astrovirus, UTPV1 – ungulate tetraparvovirus 1, UBPV6 – ungulate bocaparvovirus 6, WUPyV – WU polyomavirus, EVE – enterovirus E, BAdV3 – bovine adenovirus 3.

Table 2. 3. Association between presence of at least one of the following five viruses and BRD. ("*****" represents the statistical significance)

Virus	No. of cattle positive for at least one of the five viruses		Odds ratio	95% CI for odds ratio	*P*
	BRD	Control			
IDV BRBV BRSV BCV BRAV	38	13	7.988	3.077-20.737	0.0001*

Note: IDV – influenza D virus, BRBV – bovine rhinitis B virus, BRSV – bovine respiratory syncytial virus, BCV – bovine coronavirus, BRAV – bovine rhinitis A virus

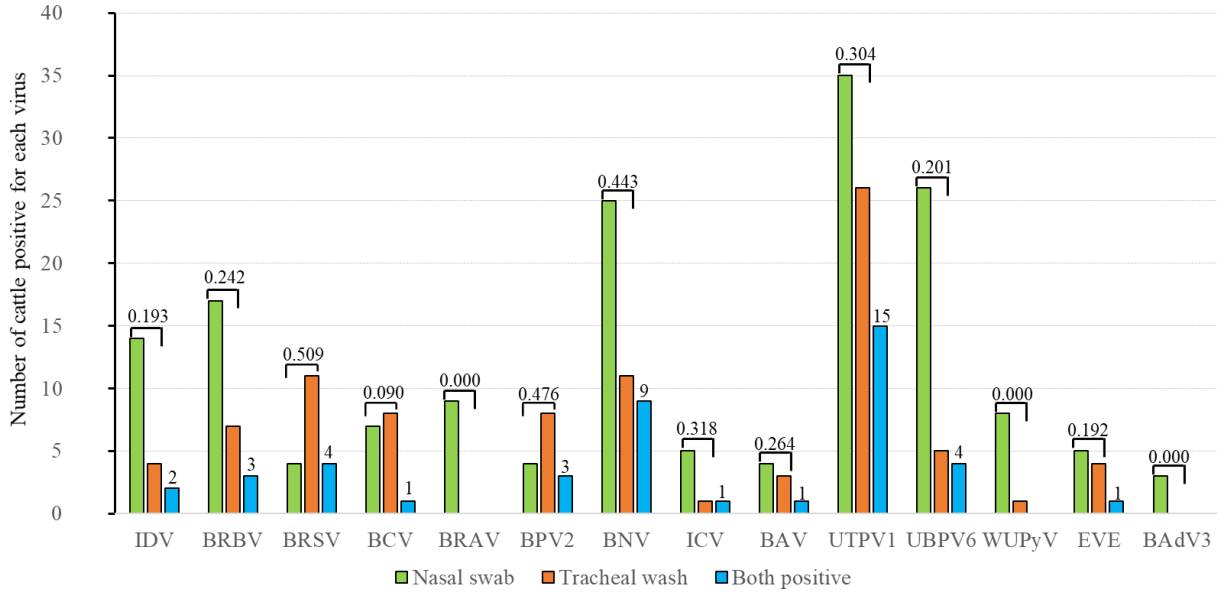

Figure 2. 1. The number of cattle positive for viruses in nasal (nasal swabs = 116) and tracheal (tracheal washes = 116) tracts with *Kappa* coefficient. The number of cattle positive for both regions are labelled at the top.

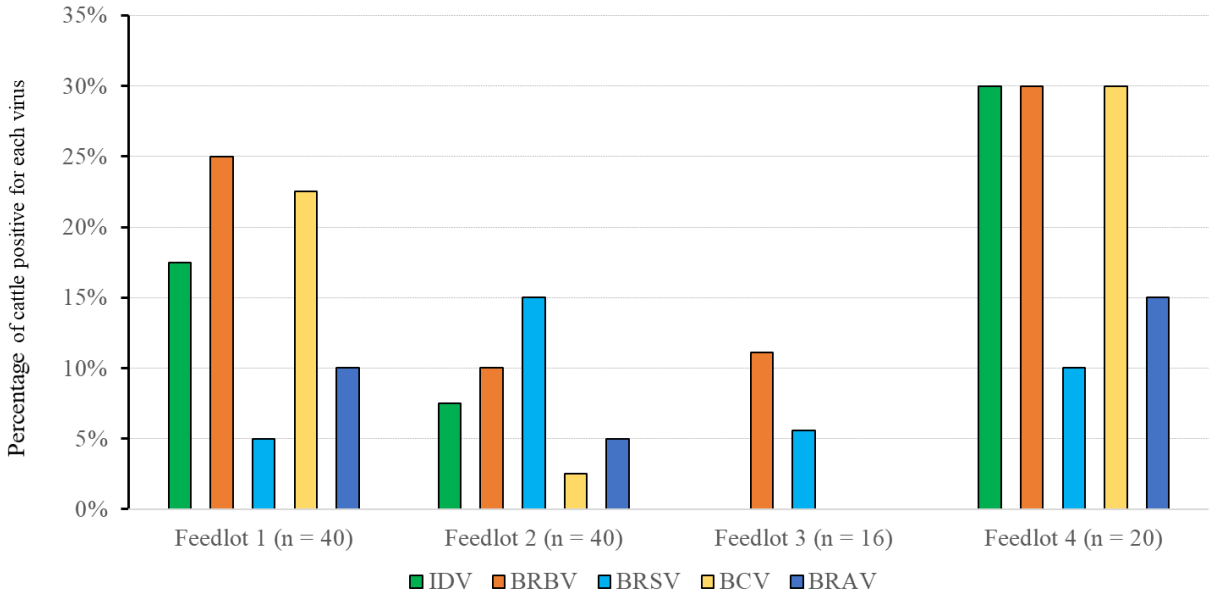

Figure 2. 2. Percentages of cattle positive for IDV, BRBV, BRSV, BCV and BRAV in samples per feedlot.

2.5 Discussion

In this study, some viruses that are not included in the current BRD vaccines were identified, furthermore, these viruses were significantly associated with the BRD.

All the cattle in this experiment were vaccinated for bovine herpesvirus 1 (BHV1), bovine viral diarrhea virus 1 and 2 (BVDV1 and 2), BRSV and bovine parainfluenza virus 3 (BPIV3). BHV1 was not identified in any of the samples, while BVDV1 and BPIV3 were only identified in one and two samples, respectively. This may indicate that the efficacies of vaccines for BVDV, BHV1 and BPIV3 are generally satisfactory, or the level of exposure to these viruses in this population was low. In contrast, BRSV was detected in 17 % of BRD cases and 2 % of control cattle, with a significant group difference in the single variable analysis. The detected BRSV may be vaccine strains, but the data generated in this study was not sufficient to differentiate vaccine BRSV strains from field strains. On the other hand, regardless of the strains identified in this study, its association with BRD should not be simply overlooked.

There is increasing evidence that BCV is associated with BRD (Lathrop et al., 2000; Storz et al., 2000a) and results of the current study provided support for this notion. BCV was significantly associated with BRD in this study in the single variable analysis (Table 2.2). The role of BCV in the pathogenesis of BRD is not well characterized. In an inoculation study, lung lesions were mild after BCV inoculation. However, degenerative changes were noted in the bronchi (Storz et al., 2000a). Accordingly, it is possible that BCV affects the mucocilliary clearance function of the upper respiratory tract and facilitate secondary bacterial infection (Storz et al., 2000a).

To our knowledge, this is the first report of identification of IDV in western Canada. IDV was initially identified in swine in the United States, and then was found to be prevalent in cattle of the United States, Luxembourg, Ireland, France, Japan and China (Flynn et al., 2018; Mekata et al., 2018; Snoeck et al., 2018; Su et al., 2017). IDV may be an emerging pathogen in Canadian cattle; or, on the other hand, the lack of Canadian reports before may be due to the unavailability of a diagnostic assay. IDV was also recently identified in sheep and goats (Quast et al., 2015). Although there is evidence that IDV can infect humans, whether it can cause disease is unclear at this point and the risk of zoonosis is considered to be low (Su et al., 2017). Preliminary evidence showed that IDV could be potentially associated with BRD (Mitra et al., 2016; Ng et al., 2015), and our study provided additional evidence for the role of IDV in BRD as its detection was significantly associated with BRD in single variable analysis. In a previous study, IDV was transmitted efficiently through direct contact, causing mild respiratory signs and the virus can be detected in the lung of affected animals by PCR (Ferguson et al., 2016). However, in that same study, the lack of pulmonary lesions and negative immunohistochemical staining suggested that IDV might mainly act in the upper respiratory tract (Ferguson et al. 2016). An inactivated IDV vaccine was developed recently, providing partial protection in cattle from mild respiratory disease, which

further supports an etiological role for IDV in BRD (Hause et al., 2017). The samples of our study were paired nasal swabs and tracheal washes and the majority of positive IDV samples were nasal swabs, which again suggested IDV might mainly cause upper respiratory tract infection.

BRAV and BRBV belong to genus *Aphthovirus*, family *Picornaviridae* (Hollister et al., 2008). BRAV is composed of two serotypes, BRAV1 and BRAV2, while BRBV consists of one serotype, BRBV1. Both viruses are common in cattle in the United States (Hause et al., 2015). In this study, BRBV was significantly associated with BRD and there was a statistical trend of association between BRAV and BRD (Table 2.2). To the best of our knowledge, these two viruses have not previously been reported in Canada. The current data represent early evidence that these two viruses may play a role in BRD development. Further research is needed to verify the current data and study the mechanism by which BRAV and BRBV may be implicated in BRD development.

Even though Enterovirus E (EVE) was found not to be significantly associated with BRD in our current study, a novel strain of EVE was detected in a recent report from cattle with severe respiratory and enteric disease (Zhu et al., 2014a). However, the pathogenesis of EVE is not well understood at this point.

This was the first report of influenza C virus (ICV) in Canadian cattle. Interestingly, the detections were from cattle without respiratory disease, which was inconsistent with the report from the United States that ICV was detected in cattle with respiratory disease (Zhang et al., 2018b). Further investigation is needed to understand the impact of ICV infection in cattle.

In cattle, six species of parvovirus have been reported: ungulate bocaparvovirus 1 (UBPV1), bovine adeno-associated virus (BAAV), ungulate erythroparvovirus 1 (UEPV1), ungulate tetraparvovirus 1 and 2 (UTPV1 and 2), and ungulate copiparvovirus 1 (UCPV1) (Cotmore et al., 2014). Four of these species (BAAV, UTPV1, UBPV6 and BPV2) were detected in this study.

UTPV1, previously known as bovine hokovirus (Cotmore et al., 2014), was the most prevalent virus in our study, detected in 35.3% of the cattle tested. UBPV6, the second most prevalent virus, previously known as bovine parvovirus 1, was present in 23.5% of the total cattle. BAAV, UTPV1, UBPV6 and BPV2 have not been established as pathogenic agents related with respiratory diseases (Schmidt et al., 2004; Cibulski et al., 2016a).

Although individually, the prevalence of IDV, BRAV, BRBV, BCV and BRSV were not high across all cattle, 44% of the cattle were infected by at least one of these viruses. Presence of these viruses in the respiratory tract was shown to be significantly associated with BRD. This indicates that, not one single virus, but a group of viruses may be important for the development of BRD.

Also worth noting is that the agreements of detection between nasal swabs and tracheal washes were generally low. This may be an indication that virus populations differ in the various locations of the respiratory tract. These findings emphasize the diagnostic challenges of BRD, because the common practice is to test samples from only one location (almost always nasal swabs), which compromises the ability to obtain accurate diagnoses. On the other hand, it is not practical to collect tracheal washes for diagnostic purpose due to the laborious procedures. Therefore, caution should be taken when interpreting negative diagnostic results based on only one location of the respiratory tract.

Overall, our work did demonstrate that the upper and lower respiratory tract viromes of cattle with or without BRD are diverse and variable, and that samples from the upper respiratory tract may not be representative of the lower respiratory tract. Several viruses that are not currently targeted in diagnostic investigations of BRD, namely IDV, BRAV and BRBV, may play important roles in this clinical syndrome. Determination of their roles in BRD pathogenesis will require further

studies, including inoculation experiments. Results of these studies could lead to improved diagnostic strategies and identification of targets for vaccine development to reduce BRD.

Acknowledgement

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Conflict of interest

The authors declare no competing interest. This study was funded by Agriculture Development Fund (ADF), Alberta Livestock and Meat Agency Ltd., and Genome Alberta. M.Z. is supported by a China Scholarship Council (CSC).

Table 2.4S. Supplementary table (.xls). Number of viruses identified in nasal (BRD, n = 58; Control, n = 58) and tracheal (BRD, n = 58; Control, n = 58) samples.

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CHAPTER 3. The pulmonary virome, bacteriological and histopathological findings in bovine respiratory disease from western Canada

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Citation

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Author contributions: Conceptualization: MZ, JEH and YH; methodology: MZ, MG and DLG; formal analysis: MZ and JEH; data curation: MZ and JEH; writing—original draft preparation: MZ; writing—review and editing: JEH, CF, MG, DLG and YH; supervision: YH and JEH; project administration: YH; funding acquisition: YH.

Chapter transition

In my first study I applied metagenomic sequencing to identify unconventional viruses associated with BRD and several unconventional viruses in beef cattle, such as influenza D virus (IDV), bovine rhinitis virus A and B (BRAV and BRBV) were identified. This study and those conducted by other research groups, however, focused on the virome of *ante mortem* samples from the upper respiratory tract (nasal cavity and trachea). To the best of our knowledge, the virome of lungs from cattle with BRD has not been characterized, although lung is the primary location where significant BRD lesions are usually found, and a common diagnostic specimen. The objective of this study was to characterize the virome of bovine pneumonia lungs collected in western Canada using metagenomic sequencing, and to combine this information with bacterial culture and targeted real-time PCR results from the same samples to determine the relationships between the microorganisms detected and different types of pneumonia defined by histopathological examination.

3.1 Abstract

The etiology and pathogenesis of bovine respiratory disease (BRD) is complex and involves the interplay of infectious agents, management and environmental factors. Previous studies of BRD focused on *ante mortem* samples from the upper respiratory tract and identified several unconventional viruses. The lung, however, is the primary location where significant BRD lesions are usually found, and is a common *post mortem* diagnostic specimen. In this study, results of high throughput virome sequencing, bacterial culture, targeted real-time PCR and histological examination of 130 bovine pneumonic lungs from western Canadian cattle were combined to explore associations of microorganisms with different types of pneumonia. Fibrinous bronchopneumonia (FBP) was the predominant type of pneumonia (46.2%, 60/130), and was associated with the detection of *Mannheimia haemolytica*. Detection of *Histophilus somni* and *Pasteurella multocida* were associated with suppurative bronchopneumonia (SBP) and concurrent bronchopneumonia and bronchointerstitial pneumonia (BP&BIP), respectively. Sixteen viruses were identified, of which bovine parvovirus 2 (BPV2) was the most prevalent (11.5%, 15/130) followed by ungulate tetraparvovirus 1 (UTPV1, 8.5%, 11/130) and bovine respiratory syncytial virus (BRSV, 8.5%, 11/130). None of these viruses, however, were significantly associated with a particular type of pneumonia. Unconventional viruses such as influenza D virus (IDV) and bovine rhinitis B virus (BRBV) were detected, although sparsely, consistent with our previous findings in upper respiratory tract samples. Taken together, our results show that while virus detection in *post mortem* lung samples is of relatively little diagnostic value, the strong associations of *H. somni* and *M. haemolytica* with SBP and FBP, respectively, indicate that histopathology can be useful in differentiating bacterial etiologies.

Keywords: Bovine pneumonia, bovine parvovirus 2, influenza D virus, metagenomic sequencing, *Mycoplasma bovis*, pulmonary virome

3.2 Introduction

Bovine respiratory disease (BRD) is one of the most common and costly diseases in both beef and dairy cattle worldwide (Fulton, 2009; Mehinagic et al., 2019). *Mannheimia haemolytica* (*M. haemolytica*), *Pasteurella multocida* (*P. multocida*), *Histophilus somni* (*H. somni*), *Bibersteinia trehalosi* (*B. trehalosi*) and *Mycoplasma bovis* (*M. bovis*) are major bacterial pathogens, and their pathogenicity can be greatly enhanced by viral infections, which can damage the respiratory epithelium (Griffin et al., 2010). Bovine respiratory syncytial virus (BRSV) (Brodersen, 2010), bovine herpesvirus 1 (BHV1) (Jones and Chowdhury, 2010), bovine viral diarrhea virus (BVDV) (Brodersen, 2014), and bovine parainfluenza virus 3 (BPIV3) (Ellis, 2010) are considered to be the most common and pathogenic viral pathogens associated with BRD. Killed and modified live virus vaccines for BRSV, BVDV, BHV1 and BPIV3 are available for BRD prevention in North America (Fulton et al., 2016). Unfortunately, assessment of the effectiveness of bacterial vaccination for BRD has indicated variable to little benefit (Griffin et al., 2010; Larson and Step, 2012), and excessive antimicrobial usage for metaphylaxis raises concerns of increasing antibiotic resistance for both animals and humans (Portis et al., 2012). BRD morbidity and mortality rates among feedlot cattle are not reduced despite viral vaccination and antimicrobial usage (Hilton, 2014).

Metagenomic sequencing has been shown to be a powerful tool for detection of viruses (Buermans and den Dunnen, 2014) and previous studies have applied metagenomic sequencing to identify unconventional viruses associated with BRD (Mitra et al., 2016; Ng et al., 2015; Zhang et al., 2019). As a result of these work, several unconventional viruses associated with BRD in dairy and

beef cattle, such as influenza D virus (IDV), bovine rhinitis virus A and B (BRAV and BRBV) have been identified (Mitra et al., 2016; Ng et al., 2015; Zhang et al., 2019). These studies have focused on the virome of *ante mortem* samples from the upper respiratory tract (nasal cavity and trachea). To the best of our knowledge, the virome of lungs with BRD has not been investigated, although this is the primary location where significant BRD lesions are usually found, and a common diagnostic specimen.

The complex, multifactorial nature of BRD complicates not only the understanding of disease pathogenesis and development of strategies for disease management, but also making an etiological diagnosis in cattle affected by the disease. The objective of this study was to characterize the virome of bovine pneumonia lungs collected in western Canada using metagenomic sequencing, and to combine this information with bacterial culture and targeted real-time PCR results from the same samples to determine the relationships between the microorganisms detected and different types of pneumonia defined by histopathological examination.

3.3 Materials and Methods

Ethical statement

Ethical statement is not applicable in this study because the bovine lung samples were submitted to Prairie Diagnostic Services Inc. (PDS, Saskatoon, Canada) for routine diagnostic purposes.

Sample collection and histology

Cases of pneumonia (n = 130) submitted to PDS from September 2017 to December 2018 were conveniently selected. The proportion of beef cattle was 90.8% (118/130) compared to 9.2% (12/130) dairy cattle. Histology and H&E staining were performed routinely on formalin-fixed

tissues. Fresh lung samples were stored at -20°C for up to 6 weeks according to routine laboratory practice, and then transferred to - 80°C for longer term storage prior to metagenomic sequencing.

Metagenomic sequencing

Lung tissues (about 5 ×5 ×5 mm) were homogenized with silicon beads (0.1 mm diameter, BioSpec Products) in a Tissue Lyser II (30 cycles/sec for 5 min, Qiagen, Netherlands). After centrifugation at 13, 000 × g for 5 min, the supernatant was filtered (Filtropur S 0.2 µm, Sarstedt, Germany). Library preparation for metagenomic sequencing was performed as previously described (Zhang et al., 2019). Briefly, filtered supernatants were incubated with 24 units of DNase (Life Technologies, USA) and 20 units of RNase ONE Ribonuclease (Promega, USA) at 37°C for 90 min. Viral nucleic acids were extracted using the QIAamp MinElute virus spin kit (Qiagen, Netherlands) according to the manufacturer's instructions. Reverse transcription was performed using a Superscript III First-Strand synthesis kit (Life Technologies, USA) with primer FR26RV-N 5'-GCC GGA GCT CTG CAG ATA TCN NNN NN- 3' (Allander et al., 2005). Second strand synthesis was carried out using Sequenase DNA polymerase (Affymetrix, USA). Double stranded DNA was purified using NucleoMag NGS beads (Macherey-Nagel Inc., Germany), and subsequently amplified using primer FR20RV 5'-GCC GGA GCT CTG CAG ATA TC- 3' (Allander et al., 2005). cDNA was quantified using Qubit dsDNA BR assay kit (Invitrogen, Germany) and 1 ng cDNA from each sample was used for library preparation using the Nextera XT library preparation kit (Illumina, CA, USA) according to manufacturer's instructions. After confirmation of the fragment size distribution of the libraries with Agilent Technology 2,100 Bioanalyzer (Agilent Technologies, CA, USA) and normalization of the libraries, the pooled barcoded libraries were sequenced using a MiSeq V2 500 cycle kit (Illumina, CA, USA).

Bioinformatic analysis

Bioinformatic analysis was performed as previously described (Zhang et al., 2019). In brief, quality trimming was performed using Trimmomatic-0.32 (Bolger et al., 2014) with minimum length of 50 and Phred score of 20. Bowtie2 (Langmead and Salzberg, 2012) was used to map the trimmed reads to the host (*Bos taurus*) reference genome (PRJNA33843, PRJNA32899) and SAMtools (Li et al., 2009) was implemented to identify the unmapped reads, which were subsequently extracted from the original fastq files using cdbyank. The extracted unmapped reads were assembled into contigs with default parameters using Trinity (Grabherr et al., 2011). The assembled reads were compared to the virus Reference Sequence (RefSeq) database (Brister et al., 2015) using BLASTn. Virus-like contigs aligning over a minimum length of 100 base pairs with expect value $< 10^{-3}$ to the RefSeq database were further examined manually by BLASTx comparison to the NCBI non-redundant protein database to identify any spurious matches. The total number of reads corresponding to each identified virus was determined from the Bowtie2 SAM results files.

Histological examination

Cases were classified as suppurative bronchopneumonia (SBP), fibrinous bronchopneumonia (FBP), interstitial pneumonia (IP), bronchointerstitial pneumonia (BIP), bronchopneumonia+ bronchointerstitial pneumonia (BP&BIP) and bronchiolitis using features described in Table 1, which were adapted from Caswell (Caswell JL, 2016a).

Routine diagnostic tests for conventional pathogens associated with BRD

Various adjunct tests were performed by PDS according to submitting veterinarians' requests as part of the diagnostic investigation in individual cases.

DNA and RNA were extracted using DNeasy Blood & Tissue Kit (Qiagen) and RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Viral real-time PCR was performed on 34 lung samples targeting common viruses including BHV1, BRSV, BVDV, BPIV3 and BCV following standardized protocols (Wang et al., 2008; Hakhverdyan et al., 2005; Mahlum et al., 2002; Horwood and Mahony, 2011; Decaro et al., 2008). Real-time PCR for *Mycoplasma bovis* was performed on 120 lung samples (Clothier et al., 2010).

To isolate *M. haemolytica*, *H. somni*, *P. multocida*, and *B. trehalosi*, lung samples were cultured on 5% Columbia sheep blood and Chocolate agar plates, incubated at 35 °C with 5% CO_2 for 18 hours. Characteristics of bacterial colonies were observed such as yellow pigment for *H. somni*, β-hemolysis for *M. haemolytica*, and mucoid appearance for *P. multocida*. Matrix Assisted Laser Desorption and Ionization Time of Flight (MALDI-TOF) Mass Spectrometry (MALDI Biotyper 3.1.66) was used for bacterial identification, with scores equal or greater than 2.0 considered positive identification (Karunarathna et al., 2017).

Statistical analysis

Binary logistic regressions using IBM SPSS Statistics (Version 25) were implemented to analyze the association between microorganisms and types of pneumonia. Linear regressions using IBM SPSS Statistics (Version 25) were used to evaluate associations between the presence of the numbers of viruses and the presence of different bacteria. Specificity, sensitivity and agreement of detection of BHV1, BRSV, BVDV, BCV and BPIV3 between metagenomic sequencing and qPCR were determined using 2 × 2 tables. $P < 0.05$ is defined as statistical significance.

Samples were clustered according to prevalence of pathogens using UPGMA (Unweighted Pair Group Method with Arithmetic Mean), and relationships visualized as a heatmap generated using heatmap.plus 1.3 in R (Williams et al., 2019). The distance matrix was created for hierarchical

clustering using hclust function in R. The optimal number of clusters was determined by the elbow method and further confirmed with gap statistics using fviz_nbclust and combining clustering methods using NbClust function in R.

3.4. Results

Cases description and histopathological patterns

130 cases were included in our study. The age of the cattle included ranged from 1 week to 8 years with a median age of 10 months. 26.7% (35/130) of the cases were collected in November, the month with most cases, followed by December with 9.9% (13/130). Most (62.3%, 81/130) of the cases were from Alberta and 31.5% (41/130) from Saskatchewan, with 3.9% (5/130) from Manitoba, and 2.3% (3/130) from British Columbia. Typical examples of the histopathological features corresponding to the classification system described in Table 3.1 are shown in Figure 3.1. FBP (46.2%, 60/130) was the most frequent type of pneumonia, followed by SBP (16.9%, 22/130), BP&BIP (13.8%, 18/130), BIP (11.5%, 15/130), IP (6.2%, 8/130). Cases showing only bronchiolitis (5.4%, 7/130) without pneumonia were least frequently diagnosed.

Table 3. 1. Definition of histopathological criteria for different types of pneumonia.

Types of pneumonia	Features
Suppurative bronchopneumonia (SBP)	The exudate predominantly composed of neutrophils within the lumen of bronchi, bronchioles and alveoli with hyperemia, cellular debris or hyperplasia of BALT or fibrosis
Fibrinous bronchopneumonia (FBP)	The exudate predominantly composed of fibrin within the lumen of bronchi, bronchioles and alveoli with edema of interlobular septa or coagulative necrosis or fibrosis
Interstitial pneumonia (IP)	Thickening of the alveolar septum by edema, congestion or hypercellularity or hyperplasia of type II pneumocytes with hyaline membranes, interstitial fibrosis or BALT hyperplasia
Bronchointerstitial pneumonia (BIP)	The presence of features of both bronchiolar necrosis and IP
Bronchopneumonia + bronchointerstitial pneumonia (BP+BIP)	The presence of features of both BP (SBP or FBP) and BIP
Bronchiolitis	Necrosis of epithelial cells in bronchi or bronchioles with purulent or fibrinous exudates or accumulation of inflammatory cells, epithelial hyperplasia (squamous metaplasia)

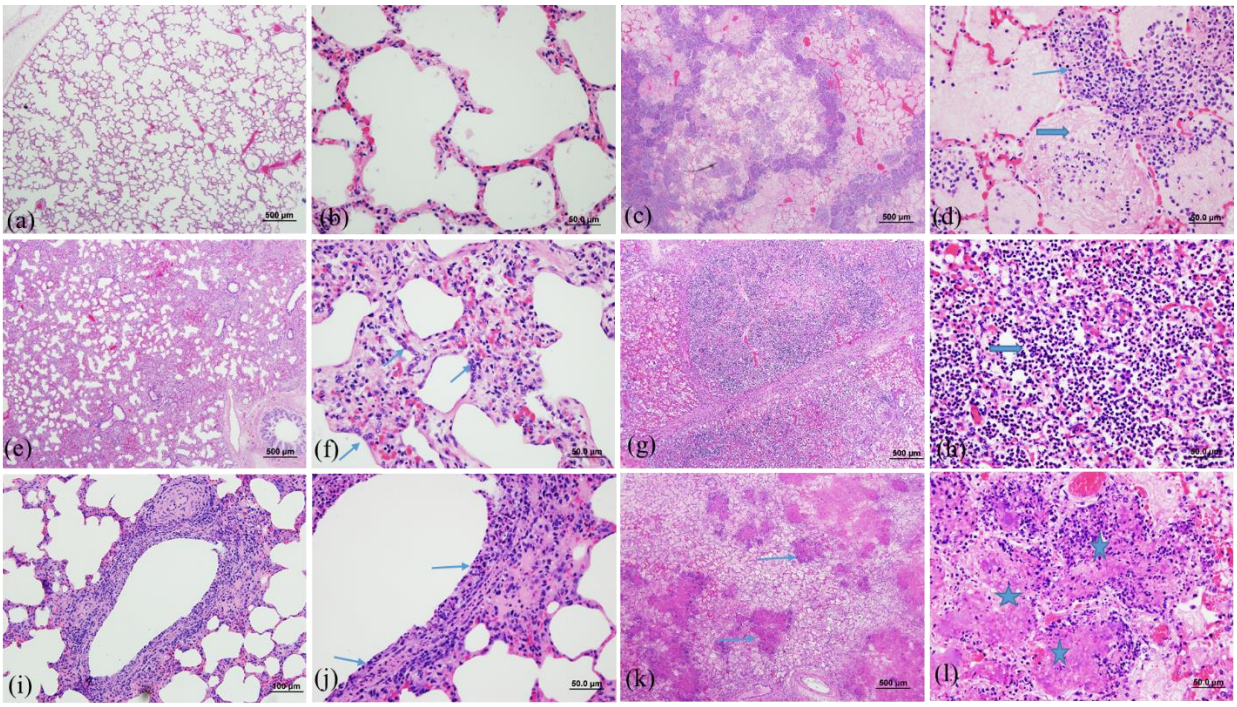


Figure 3. 1. Histopathological features of bovine lungs with different types of pneumonia. (a) Normal lung in low magnification (LM) and (b) in high magnification (HM). (c) FBP with multifocal areas of necrosis, congestion and edema and (d) infiltration of large numbers of oat cells (thin arrow) and fibrin (thick arrow) in alveoli. (e) IP with thickening of alveolar septa and infiltration of inflammatory cells and (f) type II pneumocytes hyperplasia (arrows). (g) and (h) SBP with predominate infiltration by large numbers of neutrophils (thick arrow). (i) and (j) bronchiolitis with necrotic epithelial cells, loss of cilia and inflammatory cells infiltrated mucosa and submucosa. (k) and (l) showed multifocal areas of coagulative necrosis characterized by hypereosinophilic materials and necrotic cellular debris (thin arrows and stars). Hematoxylin and eosin stain. (a), (c), (e), (g), (k): low magnification, 500 um; (i): low magnification, 100 um; (b), (d), (f), (j), (l): high magnification, 50 um.

Metagenomic identification of viruses

A total of 30.9 million sequence reads were generated. After removing low-quality and host-derived reads, 8.9 million reads remained. Viral reads of high quality (0.19 million) accounted for 0.61% of the total reads generated, and 2.13% of the total high quality reads. A total of 16 viruses were identified. The largest viral contigs assembled from individual samples varied from 234 to 6,126 bp (Table 3.2). Similarity of the assembled contigs to reference sequences from the RefSeq virus database based on BLASTx were all above 93.7% amino acid identity, and most (9/16) were >97% identical to their closest match in the database. The numbers of reads aligned to each virus ranged from 2 to 77,240 reads, and there was a corresponding range of genome coverage observed for each virus in each individual sample with the proportion of the genome covered ranging from as low as 0.54% to almost complete coverage (>98%, UTPV1). Only viruses identified in at least two samples were included in statistical analyses.

Table 3. 2. Viruses identified by metagenomics.

Virus	Family	Genome size (bp)	Largest contig size (bp) from any individual sample	Largest contig % AA identity (protein)	Total No. of reads from all samples
FAdV-A	*Adenoviridae*	43,804	235	100 (short fiber)	2
BAV	*Astroviridae*	6,233	6,126	96.23 (nsp1a)	23,350
PeSV	*Betaflexiviridae*	8,041	344	95.61 (replicase)	8
BCV	*Coronaviridae*	308,845	4,952	98.35 (polyprotein)	77,240
BVDV1	*Flaviviridae*	12,258	1,125	94.93 (polyprotein)	1,452
BVDV2	*Flaviviridae*	12,476	1,113	99.5 (polyprotein)	158
IDV	*Orthomyxoviridae*	12,546	251	98.8 (PB2)	2
BRSV	*Paramyxoviridae*	15,140	2,244	100 (nucleocapsid)	13,167
BPIV3	*Paramyxoviridae*	15,537	2,151	95.66 (phosphoprotein)	312
BPV2	*Parvoviridae*	5,610	1,670	94.24 (capsid)	262
BPV3	*Parvoviridae*	5,100	612	99.4 (nonstructural)	20
UTPV1	*Parvoviridae*	5,108	5,010	99.89 (minor capsid)	71,761
AAAV	*Parvoviridae*	4,684	203	96 (rep)	4
EVE	*Picornaviridae*	7,414	251	98.8 (polyprotein)	4
BRBV	*Picornaviridae*	7,556	234	93.65 (polyprotein)	9
BPyV	*Polyomaviridae*	4,697	285	99.6 (agnoprotein)	8

Note: FAdV-A: Fowl aviadenovirus; BAV: Bovine astrovirus; PeSV: Pea streak virus; BCV: Bovine coronavirus; BVDV 1 and 2: Bovine viral diarrhea virus 1 and 2; IDV: Influenza D virus; BRSV: Bovine respiratory syncytial virus; BPIV3: Bovine parainfluenza virus 3; BPV 2 and 3: Bovine parvovirus 2 and 3; UTPV1: Ungulate tetraparvovirus 1; AAAV: Avian adeno-associated virus; EVE: Enterovirus E; BRBV: Bovine rhinitis virus B; BPyV: Bovine polyomavirus; AA: amino acid; bp: base pairs.

For additional confirmation of virome sequencing results, available real-time PCR results for BVDV, BPIV3, BRSV, BHV1 and BCV for 34 samples were compared to metagenomic sequencing results (Supplementary Table 3.3S). In all cases where one or more of these viruses

was detected by metagenomic sequencing, the detection was confirmed by real-time PCR, however in four cases, a virus was detected by real-time PCR that was not detected in the metagenomic data from that sample. When real-time PCR was considered as the gold standard, the specificity of metagenomic sequencing was 100% and sensitivity was 60%. The agreement of identification of viruses between MiSeq and real-time PCR was 91%.

Prevalence of microorganisms and their associations with BRD

The prevalence of viruses was generally low, with at least one virus detected in only 36.9% (48/130) of samples. Overall, bovine parvovirus 2 (BPV2) was the most prevalent virus (11.5%, 15/130), followed by ungulate tetraparvovirus 1 (UTPV1) and BRSV (both 8.5%, 11/130) (Figure 3.2). The conventional BRD viruses BRSV (8.5%, 11/130), BVDV1 and 2 (2.3%, 3/130 and 3.8%, 5/130, respectively), and BPIV3 (2.3%, 3/130) were identified infrequently. Six animal viruses (bovine rhinitis B virus (BRBV), influenza D virus (IDV), fowl aviadenovirus (FAdV-A), avian adeno-associated virus (AAAV), and bovine polyomavirus (BPyV)) were only identified in one sample each (Figure 3.2). The most prevalent virus in each type of pneumonia was BPV2 (6.9%, 9/130) in FBP, bovine astrovirus (BAV) (3.1%, 4/130) in SBP, BRSV (1.5%, 2/130) in IP, UTPV1 (1.5%, 2/130) in BIP, BVDV (1.5%, 2/130) in bronchiolitis, and BRSV and UTPV1 (1.5%, 2/130) in BP&BIP (Figure 3.3a). However, for every type of pneumonia, samples in which no virus was detected was the largest category (Figure 3.3a). Only BPV2 and BRSV were detected in all pneumonia categories with BPV2 detection rates varying from 0.8% to 6.9% and those of BRSV varying from 0.8% to 3.1% (Figure 3.3a).

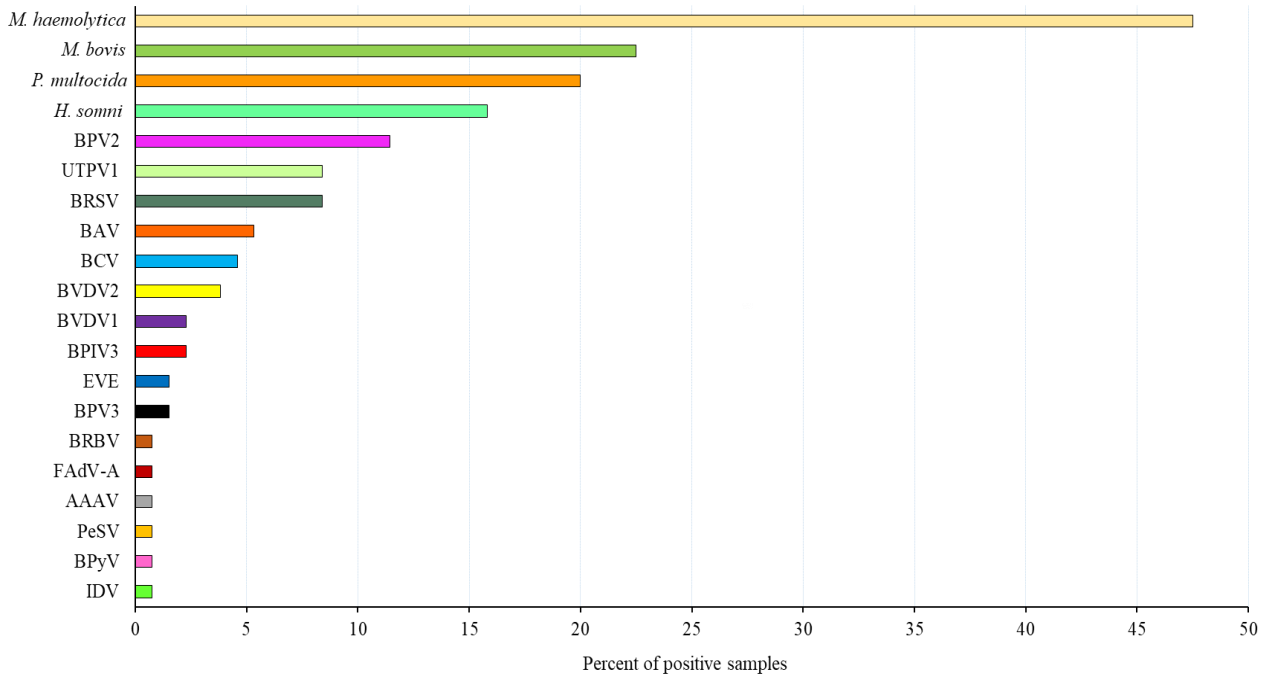
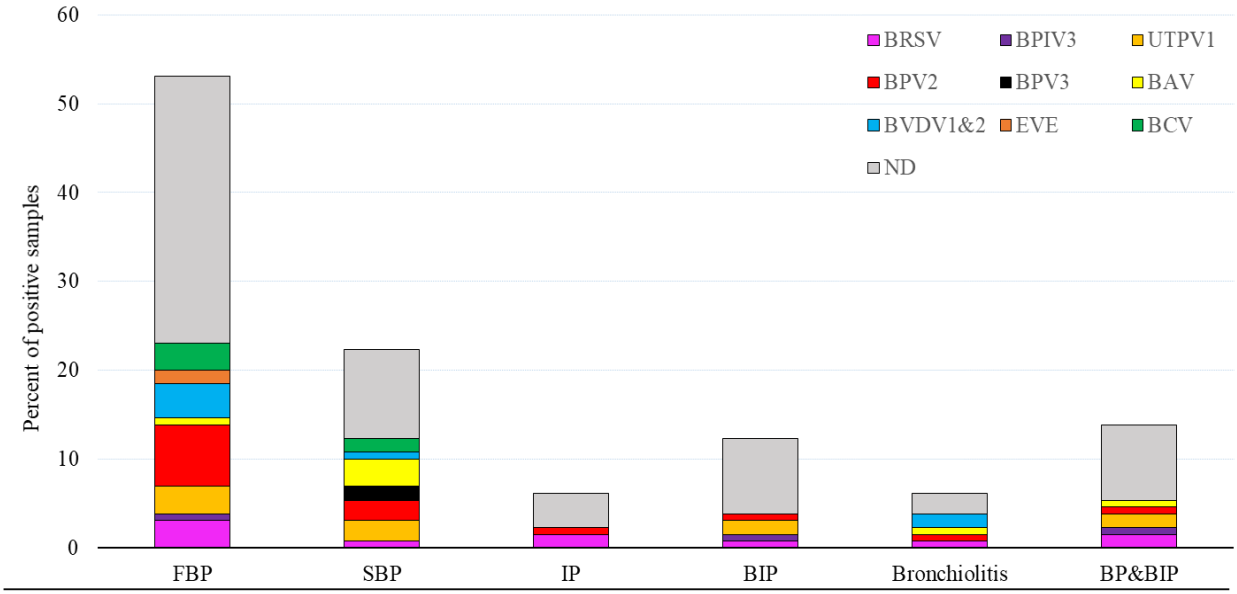
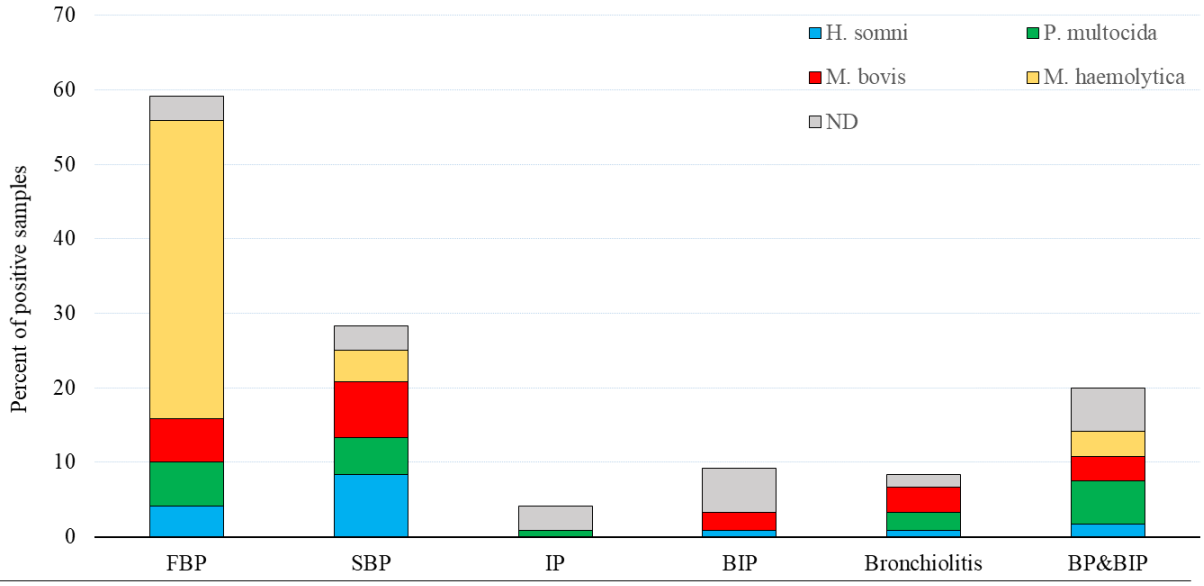


Figure 3. 2. Prevalence of viruses and bacteria in bovine pneumonia lung samples. Viruses were investigated in 130 samples but bacterial results were only available for 120. FAdV-A: Fowl aviadenovirus; BAV: Bovine astrovirus; PeSV: Pea streak virus; BCV: Bovine coronavirus; BVDV 1 and 2: Bovine viral diarrhea virus 1 and 2; IDV: Influenza D virus; BRSV: Bovine respiratory syncytial virus; BPIV3: Bovine parainfluenza virus 3; BPV 2 and 3: Bovine parvovirus 2 and 3; UTPV1: Ungulate tetraparvovirus 1; AAAV: Avian adeno-associated virus; EVE: Enterovirus E; BRBV: Bovine rhinitis virus B; BPyV: Bovine polyomavirus; *M. haemolytica*: *Mannheimia haemolytica*; *M. bovis*: *Mycoplasma bovis*; *P. multocida*: *Pasteurella multocida*; *H. somni*: *Histophilus somni*.



	FBP	SBP	IP	BIP	Bronchiolitis	BP&BIP
BRSV	4 (3.1)	1 (0.8)	2 (1.5)	1 (0.8)	1 (0.8)	2 (1.5)
BPIV3	1 (0.8)	0 (0.0)	0 (0.0)	1 (0.8)	0 (0.0)	1 (0.8)
UTPV1	4 (3.1)	3 (2.3)	0 (0.0)	2 (1.5)	0 (0.0)	2 (1.5)
BPV2	9 (6.9)	3 (2.3)	1 (0.8)	1 (0.8)	1 (0.8)	1 (0.8)
BPV3	0 (0.0)	2 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
BAV	1 (0.8)	4 (3.1)	0 (0.0)	0 (0.0)	1 (0.8)	1 (0.8)
BVDV1&2	5 (3.8)	1 (0.8)	0 (0.0)	0 (0.0)	2 (1.5)	0 (0.0)
EVE	2 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
BCV	4 (3.1)	2 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
ND	39 (30.0)	13 (10.0)	5 (3.8)	11 (8.5)	3 (2.3)	11 (8.5)

Figure 3. 3a. The prevalence of identified viruses in each type of pneumonia (n = 130). The bar charts show the proportion of virus positive and ND samples assigned to each type of pneumonia by color as illustrated in the legend. The lower table shows the number (percent) of virus positive and ND samples out of 130 total samples. BAV: Bovine astrovirus; BCV: Bovine coronavirus; BVDV 1 and 2: Bovine viral diarrhea virus 1 and 2; BRSV: Bovine respiratory syncytial virus; BPIV3: Bovine parainfluenza virus 3; BPV 2 & 3: Bovine parvovirus 2 and 3; UTPV1: Ungulate tetraparvovirus 1; EVE: Enterovirus E; ND: viruses not detected. Only viruses identified in at least two samples are shown.



	FBP	SBP	IP	BIP	Bronchiolitis	BP&BIP
H. somni	5 (4.2)	10 (8.3)	0 (0.0)	1 (0.8)	1 (0.8)	2 (1.7)
P. multocida	7 (5.8)	6 (5.0)	1 (0.8)	0 (0.0)	3 (2.5)	7 (5.8)
M. bovis	7 (5.8)	9 (7.5)	0 (0.0)	3 (2.5)	4 (3.3)	4 (3.3)
M. haemolytica	48 (40.0)	5 (4.2)	0 (0.0)	0 (0.0)	0 (0.0)	4 (3.3)
ND	4 (3.3)	4 (3.3)	4 (3.3)	7 (5.8)	2 (1.7)	7 (5.8)

Figure 3. 3b. The prevalence of bacteria in each type of pneumonia (n = 120). The bar charts show the proportion of bacteria positive and ND samples assigned to each type of pneumonia by color as illustrated in the legend. The lower table shows the number (percent) of bacteria positive and ND samples out of the total cultured. *M. haemolytica: Mannheimia haemolytica; M. bovis: Mycoplasma bovis; P. multocida: Pasteurella multocida; H. somni: Histophilus somni*. ND: bacteria not detected.

Complete bacteriological results including culture and real-time PCR for *M. bovis* were only available for 120/130 lung samples. Among these samples, *M. haemolytica* (47.5%, 57/120) was the most prevalent bacterial species, followed by *M. bovis* (22.5%, 27/120), *P. multocida* (20.0%, 24/120) and *H. somni* (15.8%, 19/120) (Figure 2.2). The most prevalent bacterium in each type of pneumonia was *M. haemolytica* (40.0%, 48/120) in FBP, *H. somni* (8.3%, 10/120) in SBP, *M. bovis* (2.5%, 3/120) in BIP, *M. bovis* (3.3%, 4/120) in bronchiolitis and *P. multocida* (5.8%, 7/120)

in BP&BIP (Figure 3.3b). In contrast to the virus findings, samples in which no bacteria were detected was the smallest category for each type of pneumonia except IP and BIP (Figure 3.3b).

The association between viruses and bacteria and different types of pneumonia was analyzed using binary logistic regression. None of the viruses detected were significantly associated with any type of pulmonary pathology. *M. haemolytica* was positively associated with FBP ($p = 0.001$), and *H. somni* with SBP ($p = 0.003$), and *P. multocida* with BP&BIP ($p = 0.01$) (Supplementary Table 3.4S).

The results from linear regression analysis showed that the number of virus species detected in an individual sample was positively associated with the presence of *M. bovis* ($p = 0.002$) but not the presence of other BRD bacteria (data not shown). Furthermore, the presence of *M. bovis* showed statistically significant association with formation of bronchiolitis obliterans ($p = 0.0001$, data not shown).

In order to explore qualitatively whether any particular combinations of pathogens were associated with pneumonia types, a pathogen "profile" for each sample was created based on positive or negative detection of the 13 viruses or bacteria that were detected in the 120 samples that were screened for both viruses and bacteria. Profiles were clustered using UPGMA and clustering of the distribution patterns was visualized as a heatmap (Figure 3.4). The optimal number of clusters determined based on clustering algorithm was 5. Cluster 1 contained samples representing all pneumonia types in which none of the agents were detected. Cluster 3 (BRSV) and Cluster 5 (mostly mixed profiles including all agents except BRSV) also contained a mixture of samples from all pneumonia types. Cluster 2 consisted of a singleton sample in which only BAV was detected. Consistent with the analysis of associations of individual agents with pneumonia type, most (60/130) FBP profiles were found in Cluster 5, which was characterized by the detection of

M. haemolytica. Interestingly, *M. haemolytica* was frequently observed in the absence of any other agents, unlike *H. somni, P. multocida* and *M. bovis,* which were more frequently detected along with at least one other virus or bacterium (Figure 3.4).

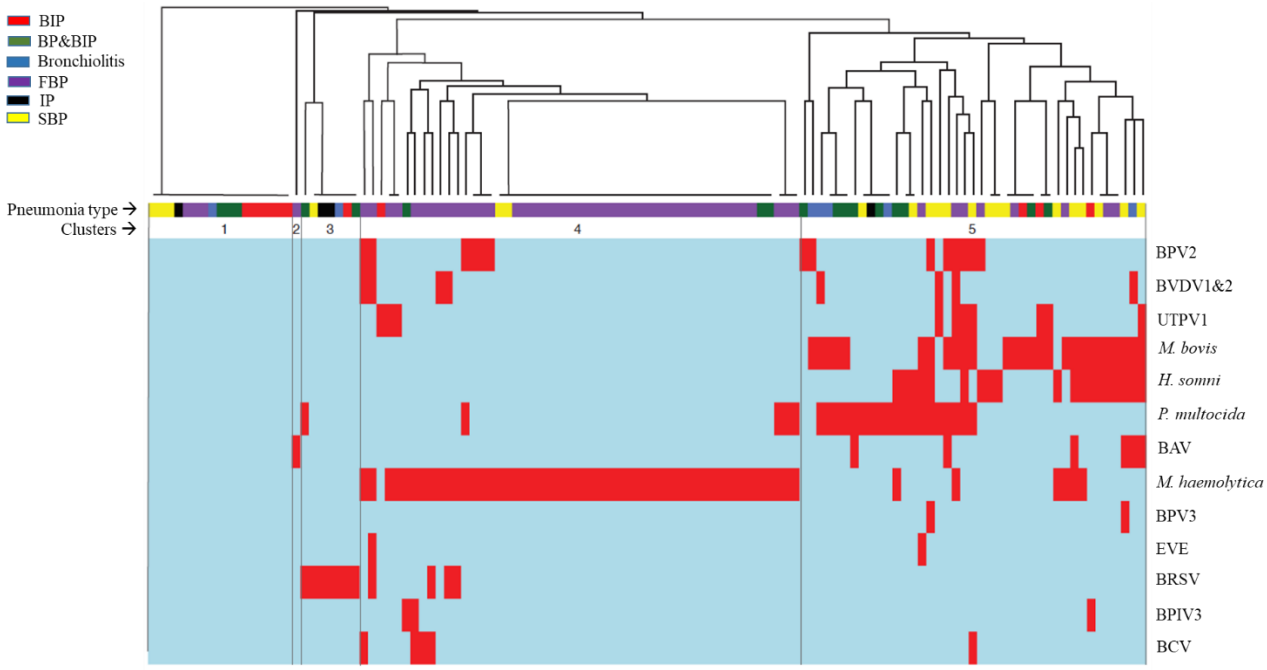


Figure 3. 4. Clustering of 120 samples for which both virome and bacteriology data were available based on microbial profiles. The coloured blocks under the dendrogram indicate pneumonia type according to the legend. The five clustered supported by elbow analysis are indicated. The heatmap below the dendrogram shows pathogen profiles of individual samples including positive (red) or negative (blue) for each microorganism. BAV: Bovine astrovirus; BCV: Bovine coronavirus; BVDV 1 & 2: Bovine viral diarrhea virus 1 and 2; BRSV: Bovine respiratory syncytial virus; BPIV3: Bovine parainfluenza virus 3; BPV 2 & 3: Bovine parvovirus 2 and 3; UTPV1: Ungulate tetraparvovirus 1; EVE: Enterovirus E; *M. haemolytica: Mannheimia haemolytica; M. bovis: Mycoplasma bovis; P. multocida: Pasteurella multocida; H. somni: Histophilus somni*. BIP: bronchointerstitial pneumonia; BP&BIP: bronchopneumonia + bronchointerstitial pneumonia; FBP: fibrinous bronchopneumonia; IP: interstitial pneumonia; SBP: suppurative bronchopneumonia.

3.5. Discussion

Advances in BRD epidemiology, pathogen genomics and proteomics, characterization of the respiratory tract microbiome, and the development of experimental challenge models (Fulton, 2009) have led to an evolution of our understanding of the relationship of pneumonic lesions with infectious pathogens originally reported in the 1970s (Jensen et al., 1976a; Jensen et al., 1976b). Most recently, application of advanced technologies for virus identification has resulted in further insight into the relationships between well-recognized pathogens and BRD (Booker et al., 2008; Mehinagic et al., 2019; Oliveira et al., 2019). Previously, we detected 21 viruses in bovine nasal swabs and tracheal washes from western Canadian beef cattle, among which IDV, BRBV, BRSV and BCV were significantly associated with BRD (Zhang et al., 2019). In dairy calves, metagenomic sequencing showed a significant association between the presence of BRAV, IDV and bovine adenovirus 3 (BAdV3) in nasopharyngeal and pharyngeal samples with BRD (Ng et al., 2015). Furthermore, another study of characterization of virome of BRD in nasal swabs from feedlot cattle suggested IDV as an etiology (Mitra et al., 2016). In order to investigate whether these viruses play direct roles in pulmonary lesions, the metagenomic virome of pneumonic lungs was characterized.

The viruses detected in lung tissue were similar to those previously detected in upper respiratory tract samples; however, the prevalence of viruses in the lungs in this study was lower than that in nasal swabs and tracheal washes. There are three possible explanations for the lower viral prevalence in lungs. Firstly, viruses may have already been cleared by the host by the time the animal died or was euthanized (Fulton et al., 2009). Cattle were usually treated repeatedly before death or the owner decided to euthanize for diagnostic purposes. At that point, viruses, if any, might have already been cleared, leaving only the pulmonary lesions. Secondly, some viruses,

such as IDV (Ferguson et al., 2016) preferentially infect the upper respiratory tract, not the lung. In the current study, IDV was detected in the lung of only 1/130 cattle, compared to 16/116 upper respiratory tract samples in our previous study (Zhang et al., 2019). Similarly, UTPV1 was only detected in 8.5% (11/130) of pneumonic lungs, but was the most prevalent virus in nasal swabs and tracheal washes (36.2%, 42/116) (Zhang et al., 2019). Thirdly, the method used to deplete host DNA may not be as effective in tissues samples compared to nasal swabs and tracheal washes, where there is less host material. In fact, 38.8% of sequence reads from the lung samples were host-derived, compared to only 27.7% of reads from nasal swabs and tracheal washes (Zhang et al., 2019). The relatively larger amounts of contaminating host DNA could contribute to a reduction in the analytical sensitivity for viruses. The accuracy of the virome sequencing results was supported by targeted real-time PCR assays with 91% agreement between the methods.

Apart from the well-documented conventional BRD viruses (BRSV, BVDV, BPIV3, and BHV1), several parvoviruses were identified in lungs including BPV2 (genus *Copiparvovirus*), UTPV1 (genus *Tetraparvovirus*), BPV3 (genus *Erythroparvovirus*) and avian adeno-associated virus (AAAV, genus *Dependoparvovirus*), all of which have been identified and reported in cattle except AAAV (Cibulski et al., 2016b). BPV2 was first reported as a contaminant of commercial bovine sera and BPV3 was frequently detected in common laboratory reagents (Allander et al., 2001). Although various parvoviruses have been detected in cattle, whether these viruses cause disease in cattle remains to be investigated. In our previous study, parvoviruses were not associated with BRD (Zhang et al., 2019).

BAV (5%) was the fourth most prevalent virus in the pneumonia lung virome. Although BAV showed no statistically significant association with any types of pneumonia, recent studies have demonstrated association of BAV with encephalitis in cattle (Selimovic-Hamza et al., 2017) and

it is commonly identified in animal feces (Li et al., 2011; Shan et al., 2011a). Taken together, these findings indicate the potential importance of BAV in other body systems. Because all BAV-positive cases also contained parvoviruses, *M. haemolytica*, *P. multocida*, *H. somni*, or *M. bovis*, it remains possible that BAV synergizes with other viruses and bacteria to aggravate symptoms.

Concerns about IDV have been raised since its discovery and it has now been found worldwide, particularly in cattle (Hause et al., 2014; Su et al., 2017). We first reported the identification of IDV in western Canada and the prevalence was 13.8% in nasal swabs and tracheal washes from cattle with BRD (Zhang et al., 2019). In this study, the single IDV-positive lung had bronchiolitis and was co-infected by BRSV. It has been shown that IDV primarily infects the upper respiratory tract (Ferguson et al., 2016), thus the low prevalence of IDV in this study is not surprising, and does not disprove its potential significance in BRD.

Considering the low rate of virus detection and the lack of association of any of the viruses detected with lung lesion type, *post mortem* virus detection in lung tissue is unlikely to be diagnostically informative and provides low analytical sensitivity relative to *ante mortem* sampling of the upper respiratory tract for virus surveillance.

Bacteria associated with BRD are mostly considered opportunistic pathogens(Caswell JL, 2016a). They are frequently isolated from the nasopharynx of healthy cattle, but can cause disease under some circumstances when host defenses have been compromised by stress or viral infection (Caswell, 2014). Experimental models of bacterial pneumonia have been successfully established by infection with BHV1 or BVDV followed by *M. haemolytica* (Burciaga-Robles et al., 2010; Jericho and Langford, 1978). We observed significant associations of bacteria with particular types of pneumonia. *M. haemolytica* was associated with FBP, consistent with well-established knowledge (Caswell JL, 2016a). We also found that *H. somni* was associated with SBP (but not

FBP), and *P. multocida* was associated with BP&BIP. The strong associations of *H. somni* and *M. haemolytica* with SBP and FBP, respectively, indicates that histopathology can be useful in differentiating different bacterial etiologies. A recent study showed that aerosolized challenge of *M. haemolytica* consistently resulted in development of FBP, which may indicate that *M. haemolytica* can also be a primary pathogen (Bassel et al., 2019) although the study design did not rule out the infection of unconventional viruses such as IDV or BRBV as a contributing factor. Interestingly, in most cases where *M. haemolytica* was detected, no viruses or rare bacteria were detected (Figure 4), which was consistent with results of a previous study (Fulton et al., 2009). The association of *P. multocida* with BP&BIP suggests an opportunistic infection from a primary or concurrent viral infection; because the pathogenesis of BIP is thought to be initiated by epithelial damage by viruses or toxic gases (Caswell JL, 2016b; Autio et al., 2007a), consistent with the idea, in case where *P. multocida* was detected, other viruses were also present (Figure 3.4).

Analysis of the relationship between the number of viruses and infection with different bacteria was conducted to investigate whether infection with multiple viruses predisposes a host to bacterial infection, or vice versa. We found that *M. bovis* infection was associated with the detection of higher numbers of viruses ($p = 0.002$). Further, bronchiolitis obliterans, which is regarded as a chronic lesion, was associated with *M. bovis* infection, consistent with results of a recent study using immunohistochemistry (IHC) to analyze association of histological features with infectious agents in dairy cows (Oliveira et al., 2019). It is possible that chronic *M. bovis* infection can compromise host immunity and lead to infection with multiple viruses, or alternatively, multiple viral infections could predispose cattle to *M. bovis* infection.

Our results demonstrate that there are strong associations between different types of histopathologic lesions and the presence of particular BRD associated bacteria. While the

relatively low prevalence of viruses in pneumonic lungs limits the diagnostic utility of virus detection in this specimen, our results do confirm the effectiveness of the metagenomic sequencing technique itself, and illustrate how its non-targeted nature allows accurate and simultaneous detection of multiple viruses, which presents a distinct advantage over application of multiple targeted assays. Future development of robust methods for simultaneous detection of viruses, bacteria and fungi with metagenomic sequencing will further enable the application of this approach to both pathogenesis research and diagnostic investigations of complex clinical syndromes like BRD.

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Table 3.3S. Supplementary table (.xls). Agreement between results of MiSeq and real-time PCR for BCV, BHV1, BVDV, BPIV3, and BRSV.

Table 3.4S. Supplementary table (.xls). Association of microorganisms with different types of pneumonia. Available at: https://onlinelibrary.wiley.com/doi/full/10.1111/tbed.13419

CHAPTER 4: Assessment of metagenomic sequencing and qPCR for detection of influenza D virus in bovine respiratory tract samples

Citation

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Author contributions: Conceptualization: MZ, JEH and YH; methodology: MZ and JEH; software: MZ and JEH; formal analysis: MZ; data curation: MZ and JEH; sample resources: TWA; writing—original draft preparation: MZ; writing—review and editing: JEH, CF, TWA, DLG and YH; supervision: YH and JEH; project administration: YH; funding acquisition: YH.

Chapter transition

Given the diversity of viruses now known to be associated with BRD and the potential for discovery of novel viruses like IDV, there is increasing interest in application of metagenomic sequencing for diagnostics, since screening for many individual viruses using targeted PCR assays quickly becomes logistically complex, expensive, and time-consuming. The relative performance of metagenomic sequencing compared to PCR in terms of analytical sensitivity, however, has not been widely explored. At the time of writing, there is only one published study focused on the assessment of performance of MiSeq and Nanopore sequencing relative to gold standard qPCR, which included a limited number of samples and a narrow range of viral loads. In this study, IDV was used as a representative BRD-associated virus to examine the feasibility of using metagenomic sequencing for detection of viruses in clinical bovine respiratory samples. We compared results of long-read sequencing on the Oxford Nanopore GridION platform and previously generated Illumina MiSeq data to the results of an IDV-specific qPCR. The objective was to assess IDV detection using all three approaches applied to a set of bovine respiratory tract samples containing a wide range of viral loads.

4.1. Abstract

High throughput sequencing is currently revolutionizing the genomics field and providing new approaches to the detection and characterization of microorganisms. The objective of this study was to assess the detection of influenza D virus (IDV) in bovine respiratory tract samples using two sequencing platforms (MiSeq and Nanopore (GridION)), and species-specific qPCR. An IDV-specific qPCR was performed on 232 samples (116 nasal swabs and 116 tracheal washes) that had been previously subject to virome sequencing using MiSeq. Nanopore sequencing was performed on 19 samples positive for IDV by either MiSeq or qPCR. Nanopore sequence data was analyzed by two bioinformatics methods: What's In My Pot (WIMP, on the EPI2ME platform), and an in-house developed analysis pipeline. The agreement of IDV detection between qPCR and MiSeq was 82.3%, between qPCR and Nanopore was 57.9% (in-house) and 84.2% (WIMP), and between MiSeq and Nanopore was 89.5% (in-house) and 73.7% (WIMP). IDV was detected by MiSeq in 14 of 17 IDV qPCR-positive samples with Cq (cycle quantification) values below 31, despite multiplexing 50 samples for sequencing. When qPCR was regarded as the gold standard, the sensitivity and specificity of MiSeq sequence detection were 28.3% and 98.9%, respectively. We conclude that both MiSeq and Nanopore sequencing are capable of detecting IDV in clinical specimens with a range of Cq values. Sensitivity may be further improved by optimizing sequence data analysis, improving virus enrichment, or reducing the degree of multiplexing.

Keywords: Illumina MiSeq sequencing; influenza D virus; nanopore GridION sequencing; qPCR; diagnostics; bovine respiratory disease

4.2. Introduction

High throughput sequencing is currently revolutionizing the genomics field and providing new approaches to the detection and characterization of viruses. The utilization of metagenomic

sequencing to elucidate genome sequences of viruses, particularly RNA viruses, directly from clinical samples offers several benefits. First, metagenomics enables identification and genomic characterization of unexpected viruses or even novel viruses either as primary pathogens or as co- infectants, without prior knowledge of their clinical significance (Kafetzopoulou et al., 2018; Wamaitha et al., 2018; Parker and Chen, 2017; Tyler et al., 2018). Second, it eliminates the need for ongoing optimization of primers and/or probes for rapidly evolving or highly diverse RNA viruses (Andersen et al., 2015). Third, it facilitates routine surveillance and early detection of outbreaks of novel virus strains that are distinct from currently circulating strains. Finally, the development of portable sequencing devices creates the potential for timely identification of routine cases or outbreaks in the field (Filloux et al., 2018; Quick et al., 2016). Sequencing technology continues to evolve rapidly. With the capability of generating long reads, relatively lower set-up cost and portability, Oxford Nanopore sequencing has attracted increasing attention for its potential advantages in some circumstances over short-read sequencing technologies (Schadt et al., 2010; Greninger et al., 2015).

Metagenomic sequencing has been widely used for non-targeted detection of viruses and has been applied to identify several “new” viruses associated with bovine respiratory disease (BRD) (Buermans and den Dunnen, 2014; Mitra et al., 2016; Ng et al., 2015; Zhang et al., 2019). In dairy cattle, bovine adenovirus 3 (BAdV3), bovine rhinitis A virus (BRAV), and influenza D virus (IDV) showed significant association with BRD (Ng et al., 2015). In beef cattle, bovine rhinitis B virus (BRBV), BRAV, and IDV showed statistical association with BRD (Mitra et al., 2016; Zhang et al., 2019). Among all of the viruses detected by sequencing of the bovine respiratory tract metagenome, influenza D virus (IDV) has been identified as a common virus associated with BRD in both beef and dairy cattle, suggesting the potential contribution of IDV to BRD (Mitra et al.,

2016; Ng et al., 2015; Zhang et al., 2019). Influenza D virus (IDV) belongs to the Orthomyxoviridae, and is a single-stranded, enveloped, segmented and negative-sense RNA virus (Ferguson et al., 2015). Since its discovery in swine in USA in 2011, IDV has been reported all over the world, and cattle are thought to be the natural host reservoir (Dane et al., 2019; Hause et al., 2014; Hause et al., 2013; Su et al., 2017; Zhang et al., 2019). In addition to cattle and swine, IDV has been reported in sheep, goats, laboratory animals (ferrets and guinea pigs), and seropositivity has been detected in humans (White et al., 2016; Zhai et al., 2017; Hause et al., 2013; Sreenivasan et al., 2015). Concerns about interspecies transmission and potential zoonosis have been raised due to the high seroprevalence of IDV antibodies in people exposed to cattle (White et al., 2016).

Given the diversity of viruses now known to be associated with BRD and the potential for discovery of novel viruses like IDV, there is increasing interest in application of metagenomic sequencing for diagnostics, since screening for many individual viruses using targeted PCR assays quickly becomes logistically complex, expensive, and time-consuming. The relative performance of metagenomic sequencing compared to PCR in terms of analytical sensitivity, however, has not been widely explored. At the time of writing, there is only one published study focused on the assessment of performance of MiSeq and Nanopore sequencing relative to gold standard qPCR, which included a limited number of samples and a narrow range of viral loads (Kafetzopoulou et al., 2018).

In this study, IDV was used as a representative BRD-associated virus to examine the feasibility of using metagenomic sequencing for detection of viruses in clinical bovine respiratory samples. We compared results of long-read sequencing on the Oxford Nanopore GridION platform and previously generated Illumina MiSeq data (Zhang et al., 2019) to the results of an IDV-specific

qPCR. The objective was to assess IDV detection using all three approaches applied to a set of bovine respiratory tract samples containing a wide range of viral loads.

4.3. Materials and Methods

Ethics Statement

The samples used in this study were collected as part of a previous study [10]. Collection of the samples was approved by the University of Calgary Veterinary Sciences Animal Care Committee (AC15-0109).

Sample Preparation

The overall sample preparation workflow is shown in Figure 1a. Sample collection and preparation were described previously (Timsit et al., 2018; Zhang et al., 2019). Briefly, paired nasal swabs (n = 116) and tracheal washes (n = 116) were collected from cattle with BRD and healthy controls from four different feedlots in Alberta, Canada between November 2015 and January 2016 (Timsit et al., 2018). The samples were centrifuged and supernatants were treated with DNase (Life Technologies, Carlsbad, CA, USA) and RNase (Promega, Madison, WI, USA), followed by extraction of viral nucleic acids using a commercial kit (QIAamp MinElute virus spin kit, Qiagen, Venlo, Netherlands). A portion (2.5 µL) of extracted total nucleic acids was used directly as a template for IDV qPCR and another portion (7 µL) used to generate cDNA for sequencing. The first strand was reverse transcribed with primer FR26RV-N (5′- GCC GGA GCT CTG CAG ATA TCN NNN NN-3′) using Superscript III enzyme (Life Technologies, Carlsbad, CA, USA), followed by complementary strand synthesis using Sequenase polymerase (Affymetrix, Santa Clara, CA, USA) as per manufacturer's instructions (Allander et al., 2005). Double-stranded DNA was purified using NucleoMag beads (Macherey-Nagel Inc., Bethlehem, PA, USA) and

subsequently subjected to random amplification with primer FR20RV (5′-GCC GGA GCT CTG CAG ATA TC-3′) prior to sequencing library preparation (Allander et al., 2005).

qPCR Confirmation and Quantification

Quantitative real-time PCR for IDV was performed on the extracted total nucleic acids for each sample (total number of samples = 232) using previously described primers and probe specific for IDV (Faccini et al., 2017). The qPCR was carried out using AgPath-ID One-Step RT-PCR reagents (ThermoFisher Scientific, Waltham, USA) in a total volume of 12.5 µL, which included 2.5 µL template, 4 pM forward/reverse primers, 2 pM probe and 0.5 µL AmpliTaq Gold DNA polymerase in a Bio-Rad CFX 96 Real-Time Detection System (Bio-Rad, Hercules, CA). The following cycling conditions were used: reverse transcription phase at 48 °C for 30 min; initial activation phase at 95 °C for 10 min; 40 two-step cycles of denaturation at 95 °C for 15 s; and annealing and extension at 60 °C for 1 min. To obtain a positive control template, a DNA fragment corresponding to a 170 bp portion of the PB1 gene of IDV (accession number: JQ922306) was synthesized and inserted into pUC57-Amp vector (Bio Basic, Markham, ON, Canada). A 10-fold dilution series of the positive control plasmid was used to construct a standard curve to determine the efficiency of the PCR. All samples were tested in duplicate along with the standard curve and no template controls. Samples, for which both of the duplicates gave a sigmoid amplification curve with a Cq (cycle quantification) value, were considered positive.

GridION Library Preparation and Sequencing

Nineteen samples that were IDV positive by either MiSeq virome sequencing or qPCR were selected for Nanopore sequencing. Samples were selected to represent the full range of Cq values observed in the qPCR results. Three batches of six samples were multiplexed and run on individual

flow cells, while one sample (sample 129) was run individually. The DNA used for GridION Nanopore library preparation was from the same randomly amplified DNA that was used for MiSeq sequencing (Figure 1a). Ligation 1D sequencing kit SQK-LSK108 was used for library preparation. End-repair and dA-tailing were performed on randomly amplified DNA for each sample using NEBNext FFPE DNA repair mix and Ultra II End-prep enzyme mix (New England Biolabs, Ipswich, MA, USA). After purification with AMPure XP beads (Beckman Coulter, Brea, CA, USA), native barcode ligation using the EXP-NBD103 barcode kit (Oxford Nanopore Technologies, Oxford, UK) and Blunt/TA Ligase master mix (New England Biolabs, Ipswich, MA, USA) was performed as per manufacturer's instructions. The concentration of barcoded libraries was determined using a Qubit fluorometer (ThermoFisher Scientific, Waltham, MA, USA) and subsequently equimolar amounts of each barcoded library (total amount = 1 µg) were pooled, and adaptors were added using Quick T4 DNA Ligase (New England Biolabs, Ipswich, MA, USA). Each pooled library (14.5 µL) was mixed with 35 µL Priming Buffer and 25.5 µL Loading Beads and loaded dropwise through the sample port into the flow cell (FLO-MIN106) as per manufacturer's instructions. The MinKNOW platform QC check confirmed at least 800 available pores, and the High Accuracy Basecalling (HAC) Flip-flop model was applied.

Bioinformatic Analysis

The workflow of bioinformatic analysis is illustrated in Figure 1b. Once Nanopore raw data were demultiplexed and trimmed using Porechop (https://github.com/rrwick/Porechop) and passed the quality score (Qscore) 7, high quality reads were aligned to the bovine genome (BioProject Accessions PRJNA33843, PRJNA32899) using Minimap2 (Li, 2018), and unmapped reads (i.e., non-host derived reads) from each sample were *de novo* assembled using Trinity (Langmead and Salzberg, 2012; Grabherr et al., 2011). Assembled contigs were mapped to the virus Reference

Sequence (RefSeq) database using BLASTn and virus-like contigs with a minimum alignment length of 100 bp and an expectation (e) value < 10^{-3} were further examined by BLASTx alignment to the GenBank non-redundant protein sequence database to confirm the nucleotide sequence-based identification and to remove any spurious matches (Brister et al., 2015). The total number of viral reads was determined as previously described (Zhang et al., 2019).

Quality filtered reads from the Nanopore sequencing were also uploaded to the EPI2ME (https://epi2me.nanoporetech.com/) platform for analysis with the WIMP (What's in My Pot, version 2.3.7) application for taxonomic classification of reads.

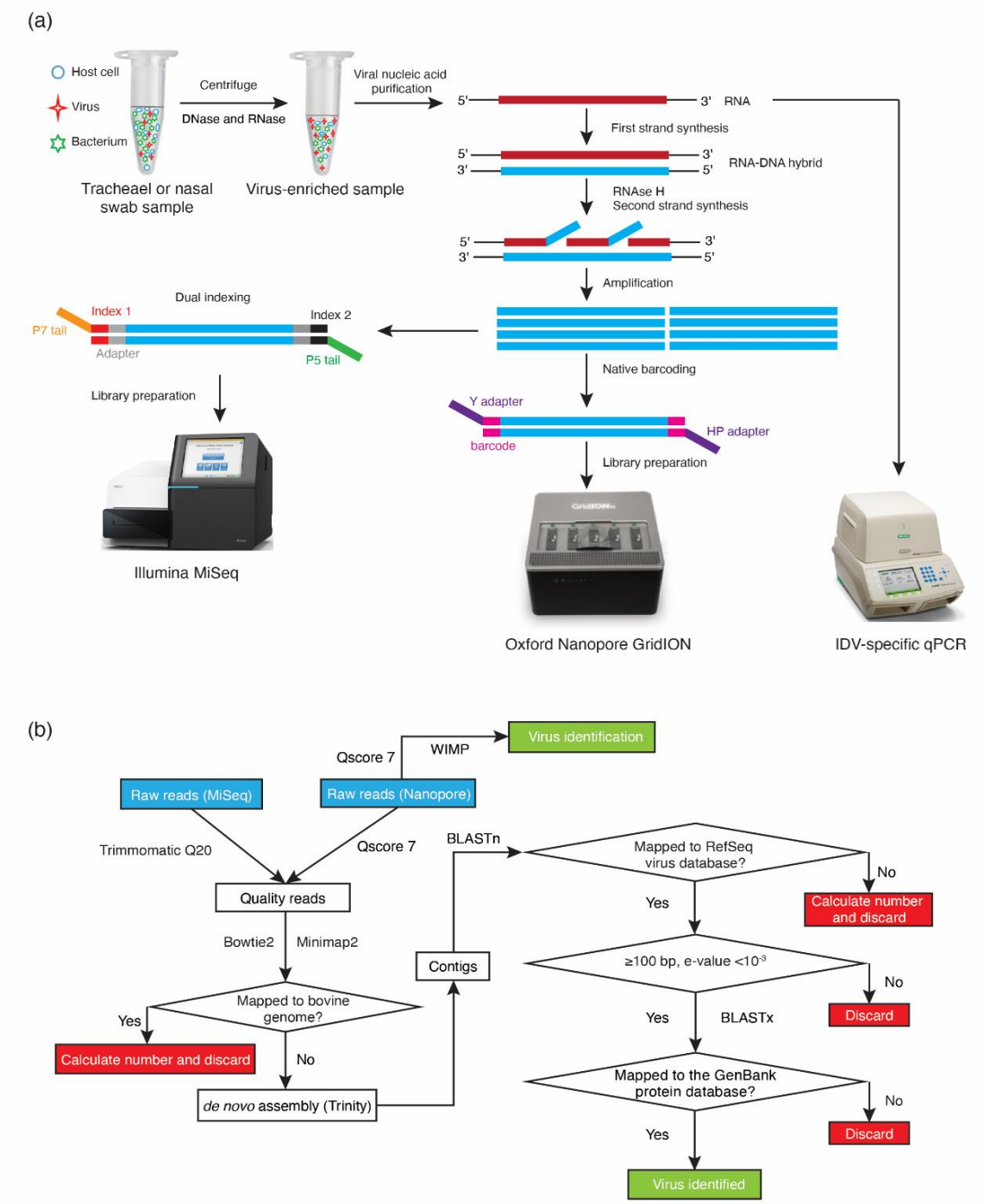

Figure 4. 1. Workflow for GridION Nanopore sequencing, MiSeq sequencing, and qPCR of bovine respiratory tract samples (BRD). (**a**) The workflow illustrates both the sample and library preparation. Extracted RNA was used directly for qPCR, while DNA randomly amplified from the same extracts was used for MiSeq sequencing and GridION Nanopore sequencing; (**b**) Bioinformatic workflow to identify viruses in BRD samples. WIMP (What's In My Pot) analysis

was used only for Nanopore data. The remaining analysis was the same for data from both MiSeq and GridION Nanopore sequencing except Minimap2 was used instead of Bowtie2 for host sequence subtraction.

4.4. Results

Comparison of IDV Detection by MiSeq and qPCR

Samples totaling 232 (116 nasal swabs and 116 tracheal washes) that had been sequenced using MiSeq previously (500 cycle V2 chemistry, libraries of 50 multiplexed samples) were tested by an IDV-specific qPCR (Figure 4.1a) [10]. The detection limit of the PCR was demonstrated to be 62.5 copies per reaction (data not shown). There were 53 IDV positive samples based on the qPCR and the range of Cq values was from 16.99 (6.25×10^7 copies per reaction) to 39.46 (6.88 copies per reaction) with median Cq value being 34.07 (2.91×10^2 copies per reaction). The agreement of IDV detection between qPCR and MiSeq was 82.8%. When qPCR was regarded as the gold standard, the sensitivity and specificity of MiSeq detection were 28.3% and 98.9%, respectively. IDV was detected by MiSeq in 14 of 17 IDV qPCR-positive samples with Cq values below 31 (Figure 4.2), when multiplexing 50 samples in the MiSeq flow cell. Only 1 of 36 IDV qPCR-positive samples with a Cq value above 31 was detected by MiSeq (Figure 4.2). Nineteen samples that were positive for IDV by qPCR or MiSeq, and that represented the full range of qPCR Cq values, were selected for further analysis by Nanopore sequencing.

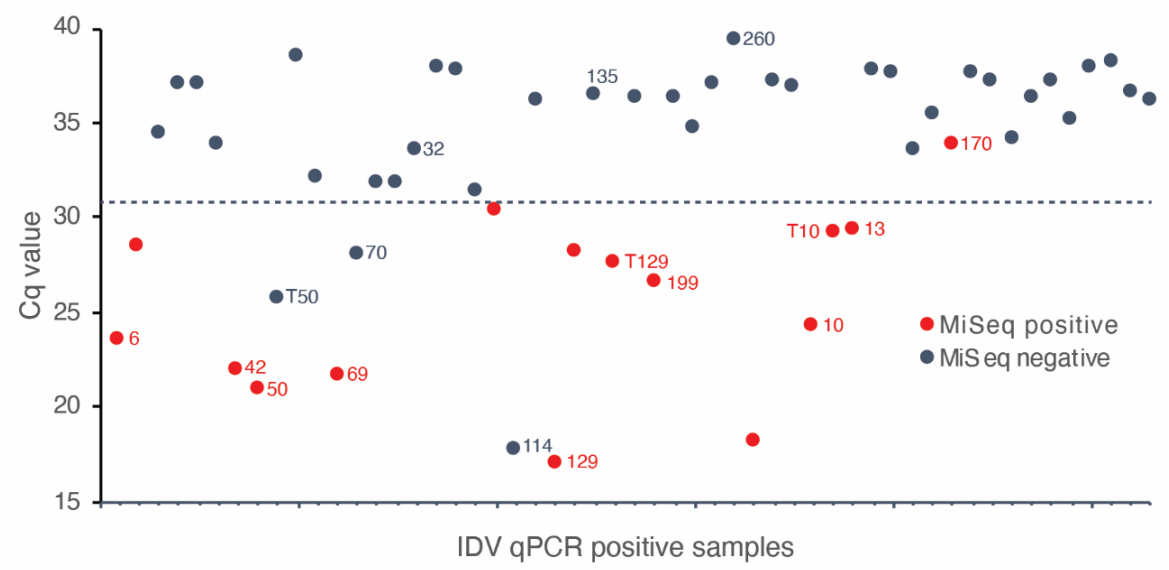

Figure 4. 2. Cq values and MiSeq detection results for 53 samples positive by IDV qPCR. Samples positive for both qPCR and MiSeq are indicated by red dots. Most concurrent detection by MiSeq and qPCR occurred in samples with Cq values <31 (dotted line). Samples selected for Nanopore sequencing are indicated with their respective sample identification number.

Comparison of Nanopore Sequencing Results with Previously Determined MiSeq Data

A total of 82.7 million reads were obtained from MiSeq. After removing low-quality reads and host-derived reads, 33.6 million reads remained. A total of 1.8 million high-quality viral reads was generated, accounting for 2.19% of the total reads obtained from MiSeq (Zhang et al., 2019). A total of 5.9 million raw Nanopore reads was generated, 69.5% of which passed the quality filter (Qscore 7) and were classified taxonomically when the data was uploaded to the EPI2ME platform. These classified reads included a total of 0.41 million viral reads, accounting for 6.9% of filter passed, classified reads (Figure 4.3). The proportion of viral reads per sample was 0.1% to 18.4% (Nanopore, WIMP analysis) compared to 0.03% to 3.1% for the previously generated MiSeq data; however, with both sequencing approaches, the majority of reads obtained were identified as host-derived or other (bacteria, fungi, unclassified) (Figure 4.3).

In addition to WIMP classification of quality-filtered Nanopore reads, we also performed a de novo assembly of the Nanopore reads. The largest IDV contigs assembled for each sample from Nanopore data (using the in-house bioinformatics workflow, Figure 4.1b) were generally longer than those from MiSeq data and ranged from 626 to 2308 bp (Nanopore), and 249 to 1584 bp (MiSeq) (Table 4.1). The genome segment coverage of each largest contig from each sample was from 10.5% to 95.3%. The proportion of Nanopore reads mapped to IDV for each sample by in-house analysis was higher than that from MiSeq except for sample T10 and T30. The proportion of IDV reads identified in the WIMP analysis of the Nanopore data, however, was generally comparable to that from the Nanopore (in-house) workflow (Table 4.1). The proportion of reads identified as IDV in Nanopore (WIMP), Nanopore (in-house) and MiSeq sequencing was generally extremely low (average 2.51%, 17.03%, and 0.46%, respectively). As expected, approximately six times more reads were obtained for the individually sequenced sample 129 than for those from the multiplexed samples (Table 4.1). Sample 129 also had the lowest Cq value in the IDV qPCR (16.99, corresponding to 6.25×10^7 copies per reaction) and the highest proportion of IDV reads in the metagenomic sequencing results (Nanopore-WIMP 14.69%, Nanopore-in-house 27.72%, MiSeq 1.48%) (Table 4.1).

Table 4. 1. Summary of data from Nanopore, MiSeq and qPCR on detection of influenza D virus (IDV) for each individual sample.

Sample *	Cq Value	Copy Number (Per Reaction)	Number (%) of IDV Reads			Largest IDV Contig (bp)		Total Input Reads	
			Nanopore (WIMP)	Nanopore (In-House)	MiSeq	Nanopore (In-House)	MiSeq	Nanopore	MiSeq
129	16.99	6.25×10^7	321,638 (14.69)	606,932 (27.72)	2182 (1.48)	2030	951	2,188,805	147,341
114	17.53	1.31×10^7	8 (<0.01)	ND	ND	N/A	N/A	335,559	136,961
50	20.71	1.25×10^6	1088 (5.74)	1625 (8.57)	162 (1.1)	1161	498	18,966	14,719
69	21.69	4.48×10^5	944 (7.83)	2287 (18.97)	1812 (0.69)	980	842	12,053	263,262
42	22.04	2.86×10^5	608 (0.34)	584 (0.33)	26 (0.07)	986	499	179,559	37,560
6	23.33	2.16×10^5	281 (1.44)	350 (1.8)	183 (0.08)	1034	522	19,512	228,875
10	24.09	1.31×10^5	1656 (1.17)	1650 (1.11)	87 (0.2)	1359	485	148,381	44,433
199	26.01	4.64×10^4	4211 (10.99)	10861 (28.34)	3250 (1.49)	2308	552	38,329	218,800
T50	26.76	2.93×10^4	3 (<0.01)	ND	ND	N/A	N/A	43,193	1,256,918
T129	28.20	8.00×10^3	403 (0.23)	502 (0.28)	34 (<0.01)	843	327	177,706	444,979
70	28.71	5.70×10^3	2 (0.02)	ND	ND	N/A	N/A	12,211	160,704
T10	29.15	5.50×10^3	29 (0.54)	ND	455 (0.03)	N/A	913	5,413	1,415,256
13	29.22	4.15×10^3	116 (0.10)	152 (0.13)	48 (0.04)	626	470	119,073	132,847
32	33.60	2.18×10^2	ND	ND	ND	N/A	N/A	275,387	32,312
170	35.62	8.25×10^1	1080 (2.03)	1991 (3.75)	8167 (0.74)	903	1,584	53,131	1,100,167
135	36.51	4.88×10^1	10 (<0.01)	ND	ND	N/A	N/A	142,737	154,220
260	39.46	6.88	9 (<0.01)	ND	ND	N/A	N/A	164,607	959,935
T30	ND	-	3 (<0.01)	ND	2 (<0.01)	N/A	249	139,037	1,156,213
T52	ND	-	51 (0.1)	52 (0.1)	497 (0.05)	1616	1,341	51,180	930,628

Cq = quantification cycle; ND = not detected; WIMP = What's In My Pot; bp = basepair; * Samples beginning with T are tracheal, all others are nasal swabs.

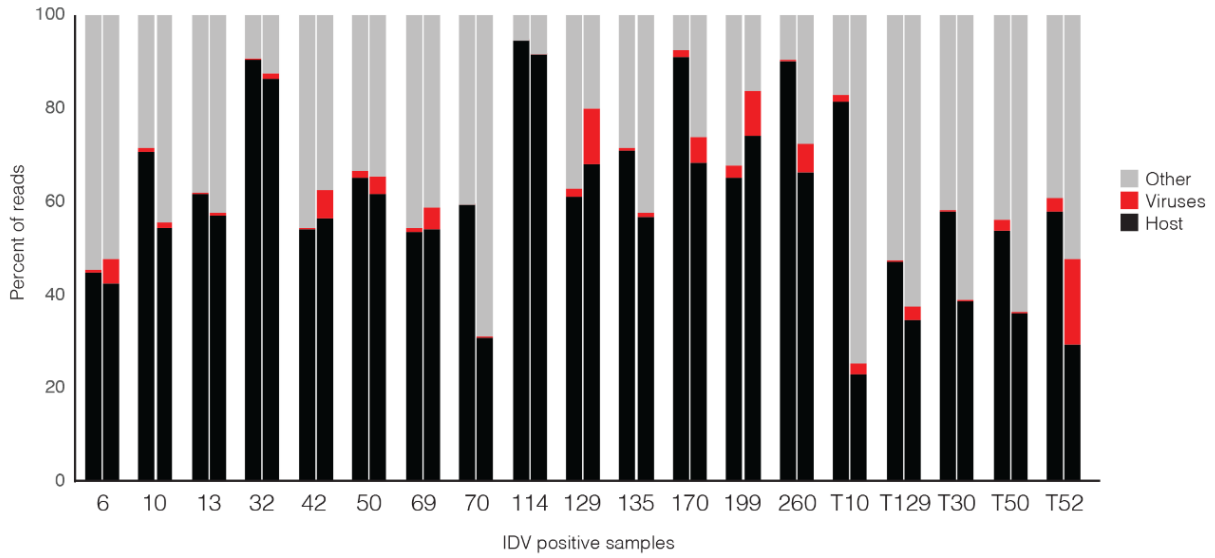

Figure 4. 3. Proportions of reads corresponding to host, viruses or other taxa (bacteria, fungi, unclassified) from 19 IDV-positive samples sequenced using Nanopore sequencing (WIMP) (left bar in each pair) and MiSeq sequencing (right bar). Labels on the *x*-axis indicate individual specimens; tracheal samples are denoted by "T" before animal number.

Comparison of IDV Detection by qPCR, MiSeq and Nanopore Sequencing

The 19 samples selected for sequencing on the Nanopore GridION platform represented a range of IDV concentrations based on qPCR of 6.88 to 6.25 × 10^7 genome copies per reaction, corresponding to Cq values ranging from 39.46 to as low as 16.99 (Table 4.1). The agreement of IDV detection between qPCR and Nanopore was 57.9% (in-house) and 84.2% (WIMP), and that between MiSeq and Nanopore was 89.5% (in-house) and 73.7% (WIMP). IDV was detected in the Nanopore data from all but one (18/19) of the IDV-positive samples when reads were classified using the WIMP application, but this proportion dropped to 11/19 when the in-house read assembly workflow was used. For most (7/8) of the samples with disparate results, 10 or fewer IDV reads were identified in the WIMP analysis. The exception was sample T10 with 29 IDV reads.

In order to explore qualitatively whether detection of other viruses in addition to IDV was comparable between the two metagenomic sequencing platforms, we compared the complete lists of viruses detected by MiSeq or Nanopore in the 19 IDV-positive samples. The number of different viruses detected in each sample varied from none to a maximum of four. The proportion of samples with perfect agreement between MiSeq and Nanopore (in-house) was 52.6%, by MiSeq and Nanopore (WIMP) it was 47.4%; and by Nanopore (in-house) and Nanopore (WIMP) it was 36.8% (Supplementary Table 4.1S).

4.5. Discussion

Metagenomic sequencing is transforming routine detection of viruses from traditional cell culture, antibody–antigen techniques and qPCR to detection of viruses in a target-independent manner. Sequencing approaches have now been widely applied for detection of known and novel agents in various types of clinical specimens in both human and veterinary medicine (Parker and Chen, 2017; Li et al., 2011; Shan et al., 2011a). The potential usefulness of viral metagenomics for virus surveillance and diagnostics is still in debate due to its performance relative to the gold-standard method of real-time qPCR routinely employed in diagnostic laboratories (Filloux et al., 2018). A recent assessment of the performance of Nanopore, MiSeq and qPCR for detection of chikungunya and dengue viruses in serum or plasma samples with relatively high viral loads (Cq values from 14 to 32) demonstrated 100% agreement between these methods (Kafetzopoulou et al., 2018). In this investigation, however, a maximum of 16 samples was multiplexed and sequenced using MiSeq, and each sample was sequenced individually on Nanopore (Kafetzopoulou et al., 2018). This low degree of multiplexing translates to high analytical sensitivity, but correspondingly makes these technologies relatively more expensive per sample and decreases the potential application for routine diagnostics. In our current study, we performed further exploration to assess

the performance of metagenomic sequencing approaches with a higher degree of multiplexing of clinical samples in both MiSeq and Nanopore sequencing. IDV presented an excellent target for this comparison given its association with BRD in beef and dairy cattle, and the availability of specimens with a wider range of viral loads than has been included in previous investigations [19,20,31].

Different viral extraction kits have been demonstrated to have variable extraction efficiencies for different viruses in respiratory clinical samples (Zhang et al., 2018a). The QIAamp MinElute Virus Spin Kit (MVSK) has been found to be generally applicable for isolating nucleic acid for qPCR or metagenomic virus identification of adenovirus, influenza virus A, human parainfluenza virus 3, human coronavirus OC43, and human metapneumovirus in respiratory clinical samples (Zhang et al., 2018a). In our current study, the original nucleic acids extracted with the MVSK were used for both qPCR and metagenomic sequencing, eliminating the influence of different extraction methods and kits on our results (Figure 4.1a).

The IDV-specific qPCR assay detected its target in 22.8% (53/232) of specimens. While the majority of IDV positive samples with Cq value below 31 were detected by MiSeq, only 1/36 samples with a Cq above this threshold were positive by sequencing (Figure 4.2). These results demonstrate that for samples where the viral load exceeds 6.25×10^2 per reaction even a relatively modest MiSeq sequencing effort (50 samples multiplexed in a single flow cell) is sufficient to detect the virus. The agreement between qPCR and Nanopore of 57.9% (in-house) and 84.2% (WIMP) demonstrated that relatively modest Nanopore sequencing effort (six samples multiplexed) is also sufficient to detect the virus. The results from Nanopore sequencing (in-house), however, showed no consistent relationship between viral load and detection by sequencing; furthermore, no consistent relationship between viral load and proportion of viral

reads was observed in either MiSeq or Nanopore sequencing (Table 4.1). For example, the two IDV positive samples 199 and 10 had Cq values of 26.01 and 24.09, respectively; however, sample 199 had a higher proportion of IDV sequence reads in Nanopore and MiSeq than sample 10 (Table 4.1).

There are several possible explanations for differences in both the proportion of IDV reads and total viral reads detected in each sample by MiSeq and Nanopore. First, variation in the amounts of DNA used for sequencing library preparation for the two sequencing platforms may play an important role. Second, the abundance of virus relative to host or bacterial genetic material is a critical determinant of the detection threshold of metagenomic sequencing. A greater proportional abundance of a virus increases the chance that it will be detected by sequencing and improves the genome coverage obtained. Therefore, virus enrichment is commonly applied to clinical samples and enrichment methods such as those used in this study (a combination of centrifugation and nuclease- treatment) should lead to removal of bacteria and host cells, thus improving virus detection (Hall et al., 2014). Virus propagation in cell culture is a less appealing method for virus enrichment since it is time- consuming, requires specific expertise and creates the potential for introduction of mutations (Quick et al., 2017). Reduction of the degree of multiplexing of samples is an alternative way to improve virus detection, but there is a corresponding increase in cost per sample and a corresponding reduction in throughput that are undesirable in research or clinical diagnostic settings. Reduction of the degree of multiplexing of samples also reduces the chances of cross-barcode contamination because barcode reagents are susceptible to cross-contamination (Esling et al., 2015).

Bioinformatic analysis in metagenomic sequencing remains challenging but is crucial for accurate identification of diagnostic targets. We used the comparable pipeline to analyze both data from

MiSeq and Nanopore sequencing (in-house), which showed the feasibility of metagenomic viral whole-genome-sequencing using both Nanopore and MiSeq technology with the assembled contigs covering from 10 to 95% of each IDV genome segment. Although *de novo* assembly was performed on Nanopore sequencing data, for the majority of the samples, the length of the largest contig was that of one single read. Skipping the assembly step in bioinformatic analysis of Nanopore data could provide an advantage for timely identification of potential pathogens. The long reads of Nanopore sequencing are thought to provide good confidence for species level identification, but the low coverage combined with the error rates of this platform preclude its use for strain-level resolution (Tyler et al., 2018).

The detection rates of IDV, the number or proportion of IDV reads (Table 4.1), and other viruses detected (Supplementary Table 4.1S) from Nanopore (WIMP) and Nanopore (in-house) were different, which demonstrates that bioinformatic analysis affects the results of virus detection. Taxonomic classification in WIMP is based on Centrifuge (Kim et al., 2016), which compares query sequences to a (undescribed) reference database with a high speed and space-optimized k-mer-based algorithm. For Nanopore (in-house) and MiSeq analysis, BLAST (Altschul et al., 1990) was used to compare assembled contig sequences to the NCBI RefSeq virus database, which is a more computationally intense process that produces more detailed results. The identification of a match using Centrifuge is based on probabilities of particular k-mer combinations occurring in the query and reference and not a consideration of the entire query sequence, thus increasing the possibility of false positives (Kim et al., 2016). In contrast, Trinity assembly and then BLAST search against a reference database could lead to false negatives if the particular target sequence is very rare (Bolger et al., 2014). If there are very few reads derived from some component of the metagenome, these reads may not be included in the assembly since there is insufficient "evidence"

to support building contigs from them (Altschul et al., 1990; Bolger et al., 2014). The current lack of definition of the reference database or the ability to use custom databases with WIMP make this approach inappropriate for clinical diagnostic applications due to the difficulty of validating such approaches. Our results provide an illustration of the profound effects that post-sequencing analysis can have on results, and the trade-offs associated with each choice. Selection of the most appropriate analysis pipeline must consider the sequencing platform, as well as tolerance for false negatives and false positives, logistical considerations, and the required taxonomic resolution. (*Note added in proof:* A comparison of sequence read mapping methods was conducted following publication of this chapter. Results are described in Appendix 1).

Analytical sensitivity is currently one of the main limitations of metagenomics. In this study, IDV was detected by MiSeq sequencing in specimens with qPCR Cq value as high as 35.62 when 50 samples were multiplexed in comparison to a maximum Cq value of 39.46 using Nanopore with multiplexing of six samples. For the IDV positive samples with low virus loads (e.g., sample 32), targeted qPCR may be preferable given its higher analytical sensitivity. Interestingly, we observed two samples that were IDV positive by both Nanopore (WIMP) and MiSeq but negative by qPCR (Samples T30 and T52, Table 4.1). These cases illustrate a potential advantage of metagenomic sequencing compared to qPCR since a likely explanation for this observation is that these specimens contained strain variants of IDV that were not detected by the qPCR assay. We were unable to determine if this was the case since the IDV sequence reads did not cover the region of the genome targeted by the species-specific qPCR assay. Targeted PCR assays for rapidly evolving RNA viruses require ongoing performance monitoring, and optimization of primers and probes (Andersen et al., 2015). No single method is suitable for application for all pathogens or specimen types, and each one has advantages in different circumstances.

4.6. Conclusion

Taken together our results demonstrate the potential of metagenomic sequencing on the Illumina MiSeq and Oxford Nanopore platforms for detection of viruses, including IDV, in clinical samples from naturally infected animals with a wide range of viral loads. While application of these approaches to screening animal populations or infectious disease research is feasible, their deployment for routine virology diagnostics in clinical settings will require additional research, laboratory and bioinformatic method development, and performance evaluation. Our exploration of two sequencing platforms, different degrees of multiplexing, including samples containing a wide range of virus loads, and comparing to a current diagnostic gold standard is an important step toward achieving these goals. Selection of appropriate methods will continue to require careful consideration of the numerous trade-offs that confront practitioners at each step of the investigation.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4915/12/8/814/s1, Table 4.1S: Summary of viruses detected by Nanopore and MiSeq sequencing.

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CHAPTER 5: The nasal viromes of cattle on arrival at western Canadian feedlots and their relationship to development of bovine respiratory disease

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Citation

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Chapter transition

Metagenomic sequencing from our and other laboratories has been applied to the identification of viruses associated with BRD, and these previous studies were focused on samples collected at the time when cattle showed clinical signs. No studies have been done on the viromes of cattle upon arrival at feedlots. Given the importance of viral infection in the pathogenesis of BRD, the composition of an animal's virome upon arrival at the feedlot might be an important factor in determining health outcomes during the feeding period. In this study, we applied Nanopore metagenomic sequencing to characterize the viromes of cattle upon arrival at nine different feedlots in Western Canada and related the findings to health outcomes of these cattle to determine if the composition of the virome of individual animals could be used to predict the likelihood of their development of BRD.

5.1. Abstract

Bovine respiratory disease (BRD) has a complex pathogenesis and etiology, being the costliest disease affecting the cattle industry in North America. In this study, we applied Nanopore-based viral metagenomic sequencing to explore the nasal virome of cattle upon arrival at feedlot and related the findings to the development of BRD. Deep nasal swabs (DNS) from 310 cattle for which BRD outcomes were known (155 cattle developed BRD within 40 days, and 155 remained healthy) were included. The most prevalent virus in on-arrival samples was bovine coronavirus (BCV) (45.2%, 140/310), followed by bovine rhinitis virus B (BRBV) (21.9%, 68/310), enterovirus E (EVE) (19.6%, 60/310), bovine parainfluenza virus 3 (BPIV3) (10.3%, 32/310), ungulate tetraparvovirus 1 (UTPV1) (9.7%, 30/310) and influenza D virus (7.1%, 22/310). No relationship was found between BRD development and the number of viruses detected, the presence of any specific individual virus, or combination of viruses. Bovine kobuvirus (BKV) was detected in 2.6% of animals (8/310), being the first report of this virus in Canada. Results of this study demonstrate the diversity of viruses in bovine DNS collected upon arrival at feedlot, and highlights the need for further research into prediction of BRD development in the context of mixed infections.

Keywords: Bovine respiratory disease (BRD), bovine coronavirus, influenza D virus (IDV), bovine kobuvirus (BKV), Nanopore sequencing, nasal virome

5.2. Introduction

Bovine respiratory disease (BRD) is the most common and economically important disease of beef cattle. Its high morbidity and mortality necessitate the use of therapeutic and metaphylactic antibiotics, reducing performance in the cattle industry all over the world (Fulton, 2009; Griffin, 1997b; Hilton, 2014). Concerns are increasingly being raised about the variable efficacy of

metaphylaxis in reducing BRD morbidity and mortality, and its relationship to antimicrobial resistance in bovine and human pathogens (Portis et al., 2012). The etiology of BRD is multifactorial and bacterial infection is thought to be secondary, facilitated by viral damage to the mucocilliary respiratory epithelium and lung parenchyma (Griffin et al., 2010; Mosier, 2014). Bovine herpesvirus 1 (BHV1), bovine viral diarrhea virus (BVDV), bovine parainfluenza virus 3 (BPIV3), and bovine respiratory syncytial virus (BRDV) are considered to be primary BRD viruses, and vaccines for these viruses are commercially available (Fulton, 2009; Bowland and Shewen, 2000). Surprisingly, no reduction of prevalence of BRD due to immunization and antimicrobial usage has been observed, which has led to more recent studies using viral metagenomics to identify other viruses as potential primary infections (McVey, 2009). These studies have identified significant associations of influenza D virus (IDV), bovine coronavirus (BCV), bovine rhinitis A and B virus (BRAV and BRBV), and bovine adenovirus 3 (BAdV3) with BRD (Mitra et al., 2016; Ng et al., 2015; Zhang et al., 2019).

Detection of pathogens using culture requires experienced personnel, has a turnaround time of several days to weeks and offers limited sensitivity, while molecular tests only target specific pathogens and have limited ability to differentiate strains or genotypes (Graf et al., 2016). In contrast, metagenomic sequencing presents the advantages of simultaneous detection of virtually any microorganism and enabling genotyping, particularly for detection of respiratory pathogens (Graf et al., 2016). Compared to second-generation sequencing technologies, third-generation sequencing technology, particularly Nanopore sequencing, offers the potential for real-time detection in-field and it generates longer sequences, which enables rapid turnaround for pathogen identification, genotyping and molecular epidemiologic studies (Loose et al., 2016; Buermans and den Dunnen, 2014).

Metagenomic sequencing has been applied to the identification of viruses associated with BRD, these previous studies were focused on samples collected at the time when cattle showed BRD symptoms (Mitra et al., 2016; Ng et al., 2015; Zhang et al., 2019). No studies have been done on the viromes of cattle upon arrival at feedlots. Knowledge of the viral infection status of cattle on arrival would contribute to pathogen surveillance, epidemiological studies, and evaluation of efficacy of vaccination strategies. Given the importance of viral infection in the pathogenesis of BRD, the composition of an animal's virome upon arrival at the feedlot might be an important factor in determining health outcomes during the feeding period. In this study, we applied Nanopore metagenomic sequencing to characterize the viromes of cattle upon arrival at nine different feedlots in Western Canada and related the findings to health outcomes of these cattle to determine if the composition of the virome of individual animals could be used to predict the likelihood of their development of BRD.

5.3. Materials and Methods

Ethics statement

This study was conducted in strict accordance with the recommendations of the Canadian Council of Animal Care (CCAC) (Olfert et al., 1993). The research protocol was reviewed and approved by the University of Calgary Veterinary Sciences Animal Care Committee (AC14-0192).

Study animals

Crossbred beef-bred heifers (n = 116), steers (n = 176) and bull (n = 18) calves (arrival body weight ± SD = 260 ± 87 kg) were enrolled in this study at nine feedlots located in southern Alberta between August 2017 and April 2018. The sources of enrolled cattle were Canada (Alberta, Saskatchewan, British Columbia, and unknown) (72.9%, 226/310), United States (Washington, Idaho, Colorado,

and unknown) (11.6%, 36/310). The origins of 15.5% (48/310) were not reported. On arrival at the feedlots, calves were processed according to standard feedlot protocols and received topical avermectin (Bimectin™, Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada) and a clostridial vaccine (Ultrabac® 7/Somubac®, Zoetis Canada Inc., Kirkland, QC, Canada) (McMullen et al., 2019). Standard feedlot protocols were performed for subsequent animal husbandry as previously described (McMullen et al., 2019). After arrival processing, study calves were commingled with other calves and housed in large, outdoor dirt-floor pens with capacities between ~ 250 and 300 cattle/pen. Calves were fed twice daily a barley-based diet formulated to ensure sufficient nutrient requirements. The diet did not contain any in-feed antimicrobials. Feed bunks were visually inspected and evaluated every day before feeding. Feed deliveries were adjusted accordingly to allow for *ad libitum* consumption.

Study design and case definition

Calves were visually inspected and evaluated daily by experienced feedlot personnel for clinical presentation. Any calves that presented signs associated with BRD such as depression, difficulty breathing, cough, nasal discharge and ocular charge were examined further in the feedlot hospital facility and one apparently healthy pen-mate was selected from the pen and walked to the same hospital facility for further evaluation as previously described (McMullen et al., 2019). Calves that exhibited two or more of the above specific respiratory signs, abnormal lung sounds, a serum haptoglobin concentration $\geqslant$ 0.25 g/L and no prior treatment against BRD or other diseases as well as rectal temperature $\geq$ 40.0 °C were enrolled in the BRD group. Calves that had a rectal temperature < 40.0 °C and did not present any BRD signs were enrolled in the control group. Control calves were removed from the study if they became sick within 40 days of enrollment.

Sampling procedures

Deep nasal swab (DNS) samples were collected on arrival at the feedlots as previously described (McMullen et al., 2019). Briefly, long, guarded swabs (27 cm) with a rayon bud (MW 124, Medical Wire & Equipment, Corsham, United Kingdom) were inserted into nostrils cleaned with paper towel and down into the nasopharynx. The nasopharynx samples were collected by extending the swab beyond the guard and vigorously moving back and forth against the mucosal surface. The entire DNS was removed from the nasal passageway after retracting the swab behind the guard. The tip of the swab was inserted into a transport tube containing liquid Amies transport media, where it was cut from the rest of the swab using scissors. Samples were immediately stored in a polystyrene cooler containing ice and then transported to the Agriculture and Agri-food Canada (AAFC) Lethbridge Research and Development Center in Lethbridge, Alberta within 48 h. Swabs were removed from the transport media and placed into microcentrifuge tubes individually with 1 ml of 20% glycerol/80% brain heart infusion (BHI) broth. The supernatants after centrifugation were transported to the University of Saskatchewan in a polystyrene cooler on dry ice and frozen at -80 °C until use.

Sample preparation

Sample preparation was described previously (Zhang et al., 2019). Briefly, DNS were clarified by centrifugation at 13,000 × g for 5 min and 160 µl supernatant from each sample and negative controls (molecular biology grade water) was incubated with 24 units of DNase and 20 µl of TURBO DNase buffer (Life Technologies, Carlsbad, CA) for 90 min at 37 °C. Viral nucleic acids from each sample and negative control (molecular biology grade water) were extracted using QIAamp MinElute virus spin kit (Qiagen, Venlo, Netherlands). The first strand was reverse-transcribed with Superscript III enzyme (Life Technologies, Carlsbad, CA) using FR26RV-N

primer (Allander et al., 2005), followed by second strand synthesis with Sequenase polymerase (Affymetrix, Santa Clara, CA). After purification of double-stranded DNA with AMPure XP beads (Beckman Coulter, Brea, CA), random amplification using FR20RV primer was conducted before proceeding to Nanopore library preparation (Allander et al., 2005).

Nanopore library preparation and sequencing

Library preparation was performed using Ligation Sequencing 1D (SQK-LSK109). Purified amplified cDNA was subject to end-repair and dA-tailing for each sample using NEBNext FFPE DNA repair mix and Ultra II End repair/dA-tailing Module (New England Biolabs, Ipswich, MA) as per the manufacturer's instructions. After purification with AMPure XP beads, each sample was uniquely barcoded with Native Barcoding Expansion 1-12 (EXP-NBD104, Oxford Nanopore Technologies, Oxford, UK) using Blunt/TA Ligase master mix (New England Biolabs, Ipswich, MA). The concentration of barcoded libraries was measured with a Qubit fluorometer (ThermoFisher Scientific, Waltham, MA) and subsequent pool of equimolar amounts of each barcoded sample (total amount = 1µg) with added Adaptor Mix II was ligated using Quick T4 DNA ligase (New England Biolabs, Ipswich, MA). Each pooled library (12 µl) was mixed with 37.5 µl sequencing buffer and 25.5 µl loading beads and added the library into the flow cell via the SpotON sample port in a dropwise manner. A total of 31 batches of ten samples were multiplexed. Libraries were run on an Oxford Nanopore GridION. The MinKNOW platform QC check confirmed more than 800 available pores for each flow cell.

Bioinformatic analysis

Nanopore raw data were basecalled using MinKNOW software that contains integrated Guppy (https://nanoporetech.com/). Basecalled data were demultiplexed and trimmed using Porechop

(https://github.com/rrwick/Porechop) and quality score (Qscore) 7. For an initial assessment of taxonomic composition, the basecalled data was uploaded to the EPI2ME platform for analysis with WIMP (What's in My Pot, version 3.4.0). For the virome specific analysis, high quality reads were mapped on to the National Center for Biotechnology Information (NCBI) RefSeq virus database (downloaded, 2020-05-11) using Minimap2 (Li, 2018). Mapped reads from each sample were *de novo* assembled using Trinity (Haas et al., 2013). Assembled contigs were aligned to the RefSeq virus database using BLASTn. Contigs with a minimum alignment length of 100 bp and an expectation (e) value < 10^{-3} were further examined by BLASTx alignment to the GenBank non-redundant protein sequence database to confirm the nucleotide sequence-based identification and to remove any spurious matches. The total number of sequence reads from each sample corresponding to the assembled contigs was determined by Minimap2 mapping of reads from each sample on to the assembled contig sequences.

Statistical analysis

Generalized linear mixed models (GLMM) were used to analyze the associations between detection of viruses and BRD development in IBM SPSS Statistics (version 26). Models were fitted using binary logistic regression and the outcome was modelled as a dichotomous variable (BRD = 1 vs healthy = 0) with cattle transportation distance, arrival weight and risk assessment (high risk versus low risk) being random factors. Individual cattle was defined as the experiment unit. Values of $P < 0.05$ were defined as statistically significant.

UPGMA (Unweighted Pair Group Method with Arithmetic Mean) was used to cluster all the samples based on the prevalence of viruses and relationships were visualized as a heatmap using heatmap. plus 1.3 in R (Williams et al., 2019). The distance matrix was determined for hierarchical

clustering using hclust function in R. The optimal number of clusters was determined by the elbow method and further confirmed with gap statistics using fviz_nbclust.

Phylogenetic analysis

Representative sequences of bovine kobuvirus (BKV) were selected from GenBank and used for phylogenetic reconstruction using MEGAx (Kumar et al., 2018). Nucleotide sequences were aligned using MUSCLE with default parameters. Phylogenetic trees were constructed using the Maximum-likelihood method with General Time Reversible model. A total of 500 bootstrap replicates with a gamma distribution for rates among sites were conducted.

5.4. Results

Population data

Deep nasal swabs (DNS) were collected from cattle upon arrival at nine feedlots (8 to 54 animals from each feedlot). Weights of individual animals varied from 102 to 510 kg and there was no difference between BRD and control groups (*t*-test, $p = 0.96$). The average transportation distance was 547 km.

Virome of bovine deep nasal swabs upon arrival on feedlot

A total of 121.5 million reads were generated with an average quality score of 10.0 (range 9.7 to 10.5), and an average read length of 588 (range 531 to 630). The initial WIMP analysis resulted in classification of 61.1 million reads, of which 97.3% were identified as host; however, 1.2 million reads were classified as bacterial and 0.48 million reads were classified as viral, accounting for 1.9% and 0.76 % of the classified reads, respectively. The remaining 0.01% were classified as archaeal.

After demultiplexing, trimming and quality filtering with Porechop, a total of 105 million reads were obtained available for analysis. A total of 0.99 million reads were successfully mapped to the virus RefSeq database, accounting for 0.94% of reads. After *de novo* assembly, BLAST alignment of the contigs to the viral RefSeq database followed by filtration and curation of the results as described in the Methods resulted in a final set of 0.4 million contigs corresponding to 22 viruses (Table 5.1). The largest assembled contig for each virus varied from 479 to 19,557 bases, and these aligned to reference viral genomes with nucleotide identities from 80.0% (BRAV) to 98.9% (BCV) (Table 5.1). When all contigs were considered, the genome coverage of each virus ranging from as low as 1.7% (bovine torovirus, BoTV) to complete coverage (>99.9%, IDV, BCV, BPIV3 and BRBV). The number of reads aligning to each virus was correspondingly variable, ranging from 2 (BoTV) to 278,022 reads (BCV). No viruses were identified in the negative controls.

All original Nanopore data files in this study were submitted to the NCBI Sequence Read Archive (SRA) and are associated with BioProject accession PRJNA634678.

Table 5. 1. Viruses identified in 310 bovine nasal swabs.

Virus	Family	Genome size (bp)	Largest contig (bp) from any individual sample	Largest contig % nucleotide identity to reference	No. of reads from all samples[§]
BCV	*Coronaviridae*	31,032	19,557	98.9	278,022
BRBV	*Picornaviridae*	7,556	7,762	92.6	71,955
BPIV3	*Paramyxoviridae*	15,456	8579	98.7	23,198
IDV	*Orthomyxoviridae*	12,546	1,523	98.6	16,992
EVE	*Picornaviridae*	7,414	3,563	96.0	7,927
BRAV	*Picornaviridae*	7,245	1,539	80.0	1,477
UTPV1	*Parvoviridae*	5,108	3,598	98.5	1,371
BVDV2	*Flaviviridae*	8,850	3813	94.7	544
BNV	*Coronaviridae*	20,261	976	95.0	368
BHV6	*Herpesviridae*	144,898	724	86.6	280
UBPV6	*Parvoviridae*	5,224	1,060	87.6	95
BKV	*Picornaviridae*	8,337	615	89.1	76
BRSV	*Paramyxoviridae*	15,140	655	91.9	72
BAAV	*Parvoviridae*	4,693	680	93.3	45
BVDV1	*Flaviviridae*	12,258	1393	95.3	23
BPV2	*Parvoviridae*	5,610	940	84.9	13
ssCDV	*Genomoviridae*	2,300	714	88.7	13
ICV	*Orthomyxoviridae*	12,555	773	92.3	11
BAV	*Astroviridae*	6,233	550	82.5	10
BPyV	*Polyomaviridae*	5,229	574	96.0	7
BAdV3	*Adenoviridae*	34,446	2066	92.5	6
BoTV	*Coronaviridae*	28,475	479	93.6	2

Note: BAdV3 – bovine adenovirus 3, BAAV – bovine adeno-associated virus, BAV – bovine astrovirus, BCV – bovine coronavirus, BHV6 – bovine herpesvirus 6, BKV – bovine kobuvirus, BNV – bovine nidovirus, BoTV – bovine torovirus, BPIV3 – bovine parainfluenza virus 3, BPV2 – bovine parvovirus 2, BPyV – bovine polyomavirus, BRAV – bovine rhinitis A virus, BRBV – bovine rhinitis B virus, BRSV – bovine respiratory syncytial virus, BVDV1 and 2 – bovine viral diarrhea virus 1 and 2, EVE – enterovirus E, ICV – influenza C virus, IDV – influenza D virus,

ssCDV – single stranded cDNA virus, UBPV6 – ungulate bocaparvovirus 6, UTPV1 – ungulate tetraparvovirus 1, bp – base pairs. § Out of 0.4 million virus sequence reads from all samples.

Prevalence of viruses and their association with BRD development

Statistical analysis was performed on the viruses identified in more than three individual cattle (n = 12). BCV was the most prevalent virus (45.2%, 140/310), identified in 22.3% (69/155) and 22.9% (71/155) of cattle who developed BRD within 40 days and those that remained healthy, respectively (Table 5.2). BRBV was the second most prevalent virus (21.9%, 68/310), followed by enterovirus E (EVE) (19.4%, 60/310), BPIV3 (10.3%, 32/310), ungulate tetraparvovirus 1 (9.7%, 30/310) and IDV (7.1%, 22/310). When each individual virus identified was considered as an independent variable, no viruses showed significant association with BRD development (Table 5.2). Bovine herpesvirus 6 (BHV6) (2.9%, 9/310) and BVDV (1.29%, 4/310) were exclusively identified in cattle that developed BRD, but this association was not significant (p = 0.161, 0.237, respectively) (Table 5.2).

No viruses were detected in 25.5% (79/310) of cattle, while 38.1% (118/310) of cattle had a single virus with similar numbers in the BRD and control groups (Figure 5.1). Co-infections were common, with more than one virus detected in 52.9% (164/310) of animals (Figure 5.1). There was no association of the number of viruses detected with BRD development using generalized linear mixed model ($p > 0.05$, data not shown).

Table 5. 2. Prevalence of viruses in on-arrival nasal samples and their association with future development of BRD.

Virus	Number (%) of cattle positive*		Odds ratio	95% CI for odds ratio	*P* value
	BRD	Control			
BCV	69 (22.26)	71 (22.9)	0.938	0.598 - 1.471	0.779
BRBV	33 (10.65)	35 (11.29)	0.927	0.54 - 1.592	0.784
EVE	28 (9.03)	32 (10.32)	0.847	0.481 - 1.494	0.566
BPIV3	15 (4.84)	17 (5.48)	0.87	0.417 - 1.816	0.709
UTPV1	17 (5.48)	13 (4.19)	1.346	0.628 - 2.884	0.444
IDV	11 (3.55)	11 (3.55)	1	0.419 - 2.388	1
BRAV	9 (2.9)	6 (1.94)	1.531	0.529 - 4.428	0.431
BHV6	9 (2.9)	0 (0)	100.4	0.159 - 63534.6	0.161
BNV	4 (1.29)	5 (1.61)	0.795	0.208 - 3.033	0.736
BKV	1 (0.32)	7 (2.26)	0.137	0.017 - 1.139	0.066
BRSV	3 (0.97)	4 (1.29)	0.745	0.163 - 3.406	0.703
BVDV	4 (1.29)	0 (0)	34.673	0.096 - 12551	0.237

*Only viruses detected in at least three animals are included

Note: BCV – bovine coronavirus, BHV6 – bovine herpesvirus 6, BKV – bovine kobuvirus, BNV – bovine nidovirus, BPIV3 – bovine parainfluenza virus 3, BRAV – bovine rhinitis A virus, BRBV – bovine rhinitis B virus, BRSV – bovine respiratory syncytial virus, BVDV – bovine viral diarrhea virus, EVE – enterovirus E, IDV – influenza D virus, UTPV1 – ungulate tetraparvovirus. CI – confidence interval.

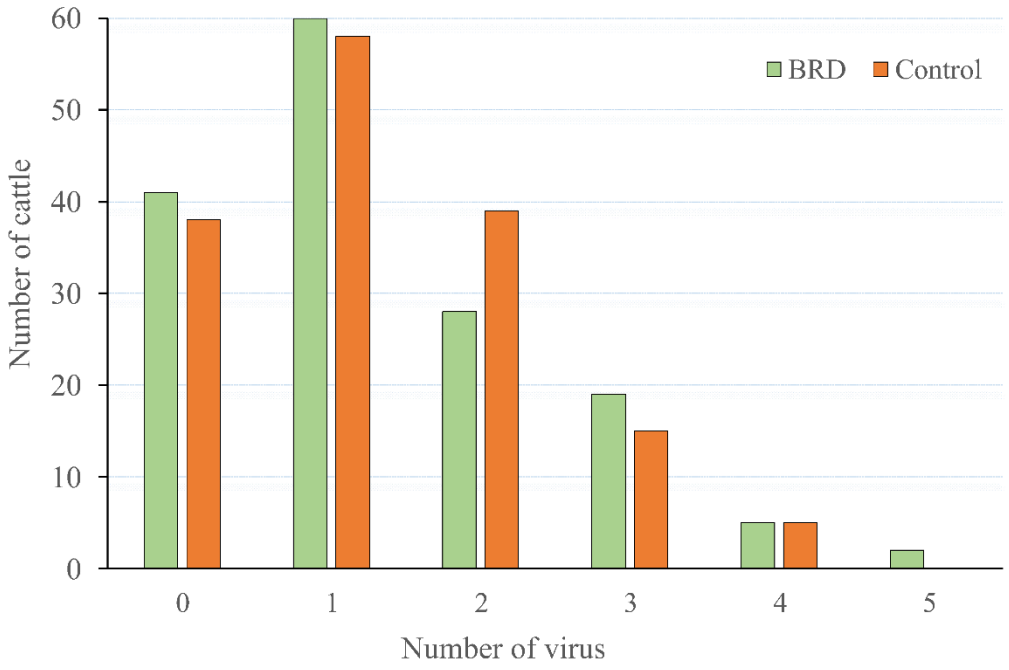

Figure 5. 1. Number of viruses detected in individual cattle in the BRD and control groups.

In order to explore qualitatively whether any particular combinations of viruses were associated with BRD development, a viral profile for each sample was created based on presence or absence of the 12 viruses that were detected in at least three animals. Profiles were clustered using UPGMA, and clustering of the distribution patterns was visualized as a heatmap (Figure 5.2). The optimal number of clusters determined based on clustering algorithm was 10. Cluster 1 contained samples representing BRD and control groups in which no viruses were detected. All clusters with the exception of Cluster 4 (containing only one animal with BVDV from the BRD group), contained a mixture of BRD and control animals.

Surprisingly, bovine kobuvirus (BKV, not previously reported in Canada) was identified in 2.6% (8/310) of animals, most of which were in the healthy control group (2.3%, 7/310).

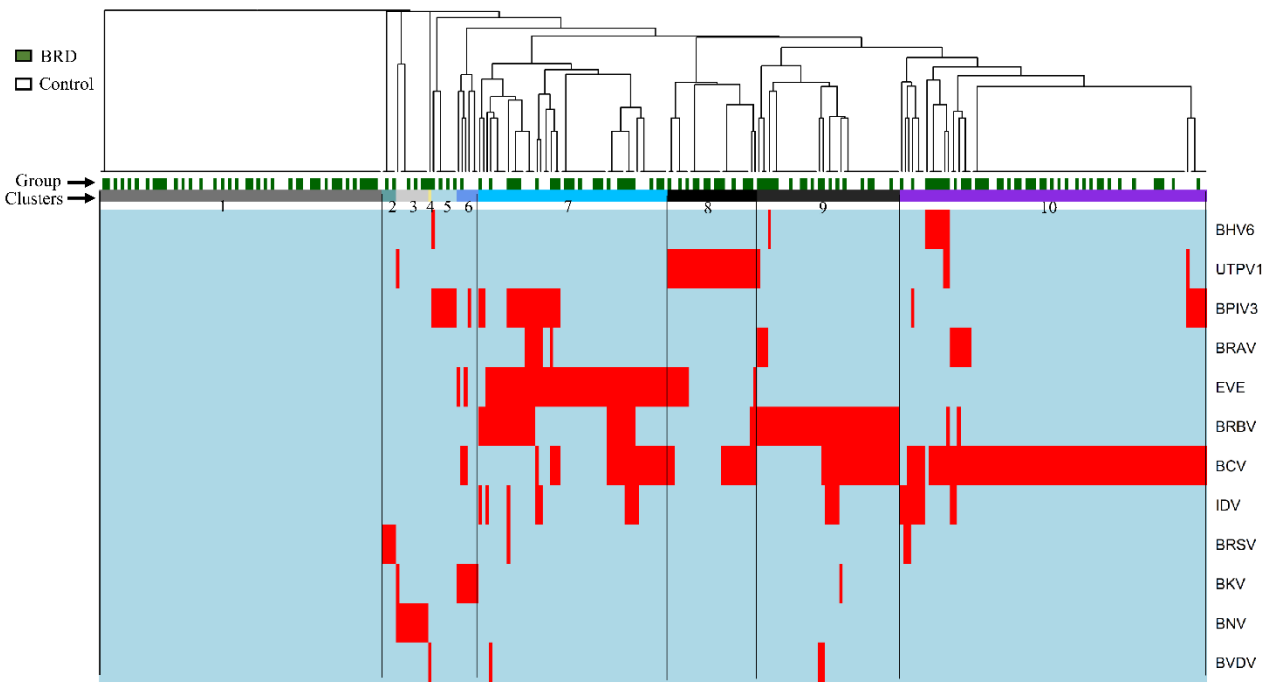

Figure 5. 2. Clustering of 310 samples based on virome profiles. The coloured blocks under the dendrogram indicate BRD and control group according to the legend. The ten clusters supported by elbow analysis are indicated with numbers. The heatmap below the dendrogram shows viral profiles of individual samples based on presence (red) or absense (blue) of each virus. Only viruses detected in at least three animals were included. BCV – bovine coronavirus, BHV6 – bovine herpesvirus 6, BKV – bovine kobuvirus, BNV – bovine nidovirus, BPIV3 – bovine parainfluenza virus 3, BRAV – bovine rhinitis A virus, BRBV – bovine rhinitis B virus, BRSV – bovine respiratory syncytial virus, BVDV – bovine viral diarrhea virus, EVE – enterovirus E, IDV – influenza D virus, UTPV1 – ungulate tetraparvovirus.

Phylogenetic analysis

Since there were no previous reports of BKV in Canada, and in order to explore the relationship of the BKV identified to previously reported sequences from other countries, we performed a phylogenetic analysis based on 261 bp of the viral protein 1 (VP1) gene. Sequence coverage depth of this region of the genome ranged from 20 to 40 in two animals and so sequences from these animals were used in the analysis (GenBank Accessions MT642607, MT642608). The study

sequences grouped with Aichivirus B with good bootstrap support and were at least 92% identical to other bovine Aichivirus B isolates from USA and Japan (Figure 5.3.).

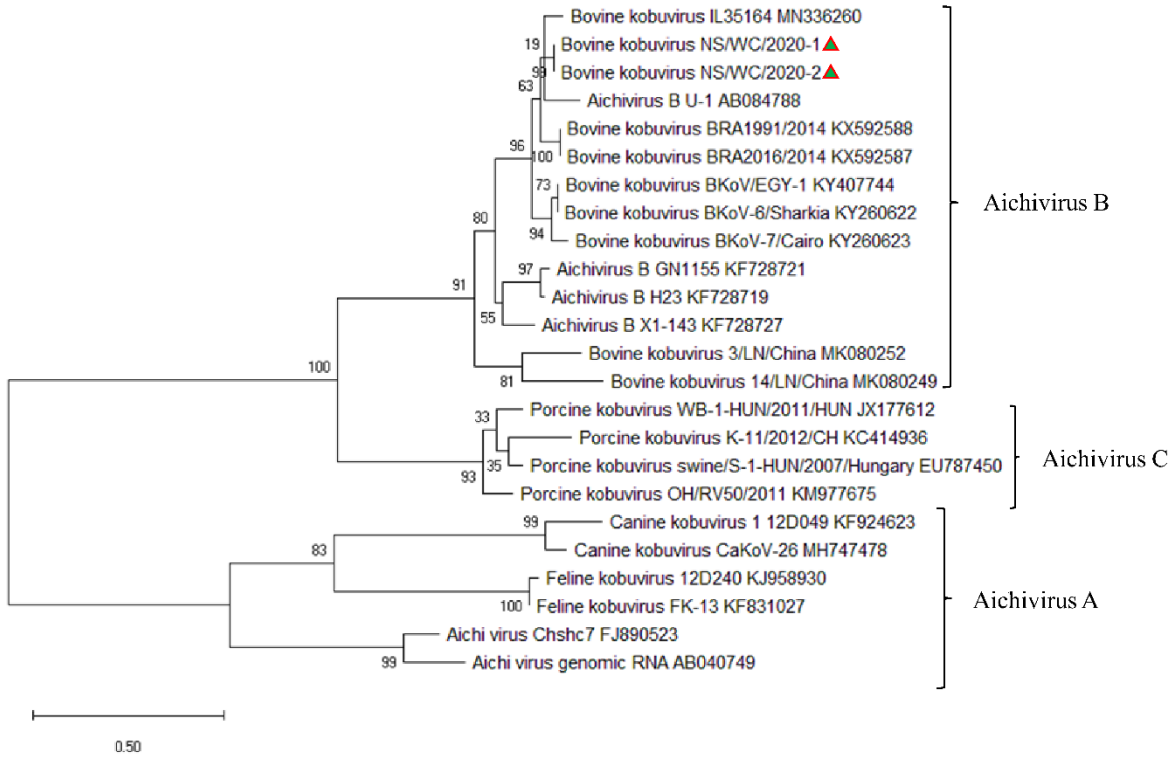

Figure 5. 3. Phylogenetic tree of bovine kobuvirus (BKV) based on 261 bp of the viral protein 1 (VP1) gene. Phylogenetic tree was constructed using the Maximum-Likelihood method with General Time Reversible model in MEGAx. The scale bar represent the number of nucleotide substitutions per site. Bootstraping of 500 replicates was performed and bootstrap (percent) values are indicated for each node. Triangles indicate BKV identified in this study.

5.5. Discussion

Based on its ability to generate long reads and portability, Nanopore sequencing has been applied globally to identify infectious agents (Loose et al., 2016), including agents of human viral pandemic diseases, such as Zika, Ebola and COVID-19 (Quick et al., 2017; Quick et al., 2016; Zhu et al., 2020). Its application in veterinary medicine, particularly for detection of respiratory pathogens is still rare (Zhang et al., 2020c). In this study, we applied Nanopore sequencing to 310

deep nasal swabs collected from cattle upon arrival at feedlot to study the composition of the virome and its association with BRD development. Transportation and commingling of animals from mixed sources makes it highly likely that individuals will be exposed to viruses from other cattle in the same vehicle and on arrival at feedlots. Given the important role of viruses in initiating the development of BRD, we speculated that the composition of the virome at arrival might provide clues as to the likelihood of BRD development during the feeding period.

Previous studies by our research group and others have shown that BAdV3, IDV, BRBV, BRAV and BCV have significant associations with BRD; however, in these studies, the samples were collected at the time cattle were showing clinical signs of BRD (Mitra et al., 2016; Ng et al., 2015; Zhang et al., 2019). Our previous study demonstrated that BRSV and BCV were more prevalent in tracheal washes than nasal swabs, and tracheal washes were more useful samples than nasal swabs for detection of these two viruses (Zhang et al., 2019); however, collection of tracheal washes is not practical in the feedlot environment. Taken together, these results suggest that the sampling location (deep nasal swab vs. tracheal wash) and timing of sampling (arrival on feedlot vs. cattle with clinical BRD) are critical factors affecting the results of virus detection.

BCV was by far the most frequently detected virus in on-arrival deep nasal swabs from both the BRD and control groups in this study. BCV belongs to subgroup 2a in the family *Coronaviridae* of order *Nidovirales*, together with canine respiratory coronavirus and human HCoV-OC43 and HKU1 (Saif, 2010). BCV is the cause of calf diarrhea (CD), winter dysentery (WD) in adults, and respiratory infections (RI) in cattle of various ages (Saif, 2010; Fulton et al., 2013). In addition, BCV-like human enteric coronavirus (HCoV-4408/USA/1994) has been identified in a child with acute diarrhea (Zhang et al., 1994), raising public health concerns over transmission between ruminants and human (Suzuki et al., 2020). Recently, one study demonstrated that enteric and

respiratory isolates of BCV can be classified into two antigenically distinct clades (BCV1 and BCV2) based on spike protein gene sequences and isolates in these clades (Fulton et al., 2013). However, this study was inconsistent with findings from other studies that showed no genetic or antigenic markers identified for discrimination of enteric or respiratory BCV and all BCV isolates identified to date belong to a single serotype (Saif, 2010; Park et al., 2006). Nearly all animal experiments attempting to demonstrate that BCV can cause respiratory disease led to observations of diarrhea regardless of the respiratory strain, WD strain and/or CD strain used (Ellis, 2019). The vaccines currently available to control BCV enteric disease are based on enteric strains (BCV1) (Fulton et al., 2013). The equal prevalence of BCV in 22.3% (69/155) and 22.9% (71/155) of cattle in the BRD and control groups in this study (Table 5.2) does not necessarily mean it is not a respiratory pathogen. From the viewpoint of naturally occurring disease, this observation might be the result, at least in part, of the timing of sampling. Furthermore, the high prevalence of BCV is noteworthy because of its mutability due to an error-prone replication scheme, and high rates of recombination between strains (Ellis, 2019). The high prevalence of BCV in this study is consistent with a previous study where nasal shedding of BCV was detected in 61% - 74% of cattle at the buyer-order before shipping to feedlots (Storz et al., 1996). A limitation of our study was that we did not perform quantification of viruses, which might help differentiate asymptomatic shedding from active infection (Graf et al., 2016).

Since its first report from ill pigs in USA, IDV has been found to have a global distribution, and has been identified in other species including humans and cattle. Interestingly, cattle were never considered to be susceptible to influenza infection but are now proposed as the natural host of IDV (Hause et al., 2014; Hause et al., 2013; Su et al., 2017). Even though it is unknown whether IDV is transmitted from person to person, the high seropositivity rate (~95%) in cattle-exposed

individuals suggests zoonosis (White et al., 2016). The prevalence of IDV in this study was 7.1% (22/310) with no difference between animals that developed BRD (3.55%, 11/310) and those that remained healthy (3.55%, 11/310) (Table 5.2). Our previous metagenomic studies showed a 6.9% (16/232) prevalence of IDV in nasal swabs and tracheal washes of beef cattle and 0.77% (1/130) prevalence in lungs submitted to a diagnostic laboratory in western Canada (Zhang et al., 2019; Zhang et al., 2020b). IDV can be efficiently transmitted among cattle by direct contact, but causes only mild upper respiratory symptoms in experimental inoculations (Ferguson et al., 2016). These observations suggest that although its presence alone is not an indication of future development of BRD, IDV may have a facilitator role in BRD through causing an upper respiratory tract infection (Ferguson et al., 2016; Ng et al., 2015; Zhang et al., 2019). Research into pathogenesis of IDV, its zoonotic potential, and its role in BRD continues.

Prevalence of BRAV and BRBV did not differ between the BRD and control groups in the current study. The overall prevalence of BRAV (4.8%, 15/310) and particularly BRBV (21.9%, 68/310) detected in cattle on-arrival was higher than in feedlot cattle in western Canada sampled later in the feeding period (BRAV (3.9%, 9/232), BRBV (9.5%, 22/232)) (Zhang et al., 2019). Bovine rhinitis A and B viruses (BRAV and BRBV) are in the genus *Aphthovirus*, family *Picornaviridae*, together with equine rhinitis virus and foot and mouth disease virus (Hause et al., 2015). Although mild respiratory symptoms were recorded following experimental BRAV inoculation in 1979, relatively little research has been done on its role in BRD pathogenesis since that time (Hussain and Mohanty, 1979; Hause et al., 2015). Our research group and others have shown that BRAV and/or BRBV have significant associations with clinical BRD (Ng et al., 2015; Zhang et al., 2019). Our current results show that both of these viruses are common in western Canadian cattle regardless of sampling time.

Enterovirus E (EVE) was the third most prevalent virus (19.4%, 60/310) (Table 5.2) in this study. EVE is in the genus *enterovirus*, the family *Picornaviridae* and a new strain HY12 of EVE was found to be associated with an outbreak in cattle showing severe respiratory and enteric disease (Zhu et al., 2014b). The causal role of bovine enterovirus (including EVE and enterovirus F (EVF)) in this outbreak has remained uncertain due to detection of the virus in the contaminated water of a cattle herd nearby, and failure to reproduce clinical signs in experimentally infecting calves (Ley et al., 2002; Zhu et al., 2014b). However, more and more detection of bovine enterovirus from cattle with fatal enteric and respiratory disease suggests bovine enterovirus as a causative agent of disease in cattle (Blas-Machado et al., 2007; Blas-Machado et al., 2011; Zhu et al., 2014b).

Surprisingly, Aichivirus B, also known as bovine kobuvirus, was identified in 2.6% (8/310) of animals sampled (Table 2). Aichivirus B has been detected using a real-time PCR method in in 1 - 34.5% of fecal samples from diarrheic cattle; it has also been detected in domestic sheep and goats (Khamrin et al., 2014; Reuter et al., 2011). Kobuviruses are in the genus *Kobuvirus*, family *Picornaviridae*, and include Aichivirus A (Aichi virus 1 in humans, canine kobuvirus 1, and murine kobuvirus 1), Aichivirus B, and Aichivirus C (also known as porcine kobuvirus) (Khamrin et al., 2014; Reuter et al., 2011). Based on analysis of partial VP1 gene sequences, the viruses detected in this study cluster with Aichivirus B strains from USA and Japan (Figure 5.3). We speculate that the detection of Aichivirus B in deep nasal swabs is the result of cross-contamination with feces from animals shedding virus. Fecal contamination among animals in close contact may also explain the identification of bovine torovirus (BoTV) (Table 5.1). BoTV diarrhea has been described in 3-50 day old colostrum-deprived and gnotobiotic calves inoculated orally or intranasally (Hoet and Saif, 2004). Neither BKV nor BoTV was detected in previous studies of nasal swabs or tracheal washes (Mitra et al., 2016; Ng et al., 2015; Zhang et al., 2019).

We were unable to predict BRD outcome based on virome composition in this study, which may be due in part to the multifactorial nature of BRD and the likely importance of mixed infections in its development. We applied an untargeted metagenomic method to analyze data solely for presence of respiratory viruses and not bacterial respiratory pathogens since most of those bacteria can be part of the normal upper respiratory tract microbiota. However, taking advantage of metagenomic sequencing technologies, perhaps the best way forward is to put more emphasis on studying bacteria and viruses simultaneously in the context of mixed infections rather than in the traditional paradigm of one agent (or type of agent), one disease (Murray et al., 2016; Zhang et al., 2019; Zhang et al., 2020b). Timing of sampling is an ongoing question that should be addressed with longitudinal studies since there may be a diagnostically informative stage between arrival at the feedlot and the onset of clinical signs of BRD. Quantification of viruses (rather than presence/absence) may be important, and although read number is likely related to virus quantity, this relationship is affected by difficult to control aspects of the sample (amount of material collected on the nasal swab, amount of host DNA present, etc.) (Zhang et al., 2020c). Finally, the possibility that particular genotypes of viruses are more or less involved in BRD development is an interesting target for future studies that will be more feasible with improved accuracy of third-generation sequencing technologies.

Acknowledgements

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CHAPTER 6. General Discussion and Conclusions

6.1. Summary and limitations of these studies

IDV, BCV, BRSV, BRAV and BRBV showed significant association with BRD and weak agreement between identification of viruses between nasal swabs and tracheal washes indicated that sampling location affects detection results.

A systemic study identified 2481 publication in electronic databases in February 2019 that described utilizing HTS to study viromes of livestock including cattle, goats, sheep, poultry and pigs (Kwok et al., 2020). The study found that pigs were the most commonly investigated livestock species, and there relatively few data on viromes of sheep and goats and respiratory viromes of cattle (Kwok et al., 2020). Prior to our study, there were actually only two studies of viromes from nasopharyngeal samples aimed at identification of unconventional viruses associated with BRD using metagenomic sequencing (Mitra et al., 2016; Ng et al., 2015). One of the studies found that BAdV3, IDV and BRAV showed significant association with BRD and the other suggested IDV as an etiologic agent of BRD despite of no statistically significant association between IDV and BRD (Mitra et al., 2016; Ng et al., 2015).

Our study in Chapter 2 further supported previous results in that unconventional viruses associated with BRD were identified (Zhang et al., 2019). IDV, BRAV, BRBV, BCV and BRSV was significantly associated with BRD; furthermore, the presence of at least one of the above viruses showed significant association with BRD as well (Zhang et al., 2019). These findings emphasize the challenge of prevention and diagnosis of BRD due to its complex and evolving etiologies (Caswell JL, 2016a; McVey, 2009). On the other hand, the weak agreement of virus detection between nasal swabs and tracheal washes imposed another challenge for diagnosis of BRD because tracheal samples were more informative for some viruses; however, it is not practical to collect

tracheal samples due to the laborious procedures (Zhang et al., 2019). In addition to identification of unconventional viruses associated with BRD, the most prevalent viruses in the upper respiratory tract were found to be parvoviruses UTPV1 (35.3%) and UBPV6 (23.5%), with no evidence of association with BRD (Zhang et al., 2019).

The calves included into this study were vaccinated on arrival of the feedlot, and one of the limitations of this study is that we could not differentiate field strains from vaccine strains due to the random amplification method used to enrich virus detection (Zhang et al., 2019). Another limitation is the cross-sectional study design since it only provides information on the virome when calves were showing BRD signs, and not the virome status prior to the appearance of respiratory signs. This question could be resolved by collecting samples from cattle on arrival at the feedlot.

M. haemolytica, *H. somni* and *P. multocida* were significantly associated with FBP, SBP and BP&BIP, respectively, indicating the usefulness of histopathology for differentiation of bacterial etiologies. None of viruses showed association with a particular type of pneumonia.

Some viruses and bacteria that are considered to be causes of BRD were also detected in cattle without any respiratory signs (Confer, 2009; Fulton, 2020). The bacteria in particular are generally components of normal microbiota of the nasal cavity (Zhang et al., 2019; Murray et al., 2016; Fulton, 2020). In diagnostic laboratories, lung is the most important organ for diagnosis of BRD and when combined with other tests such as histopathology, bacteria culture, PCR or IHC, a definitive diagnosis can be achieved (Caswell et al., 2012). However, no investigation of viromes of lungs with BRD has been conducted prior to our study.

The study in Chapter 3 correlated histopathological findings with results from bacterial culture and virome sequencing, and results indicated that the relatively lower prevalence of viruses in pneumonic lungs compared to samples from upper respiratory tracts limits the diagnostic utility of

metagenomic sequencing in this specimen (Zhang et al., 2019). The most likely reasons for low prevalence of viruses in pneumonic lungs were the following: 1) clearance of viruses by the host when animals died or euthanized; 2) favorable infection location being variable due to virus characteristics; 3) host DNA depletion for tissues being less effective than non-tissue samples (Zhang et al., 2020b).

Although bacteria associated with BRD are generally considered as opportunistic pathogens (Caswell JL, 2016b), their contributions to BRD are mostly causing death of animals under circumstances of compromised host immunity (Caswell JL, 2016b; Griffin et al., 2010). The findings of association between different bacteria and various types of pneumonia can be helpful in differentiation of bacterial pneumonia (Zhang et al., 2020b). In addition, the combinations of bacteria and viruses detected in the analysis of pneumonic cases provides a unique opportunity to interpret the mixed and complex infections underlying the histopathological lesions. This is clearly demonstrated in the association of *P. multocida* with BP&BIP because *P. multocida* was frequently isolated in the presence of other viruses, which was consistent with the proposed theory about primary viral insults leading to secondary bacterial infections (Autio et al., 2007a; Caswell JL, 2016b). The most prevalent virus detected in BRD lungs was BPV2 (11.5%) followed by UTPV1 (8.5%), which both belonging to the parvovirus family, which was consistent with our previous findings (Zhang et al., 2019).

One limitation of this study is that the presence of host genetic materials significantly affects virus detection. Our results showed that 38.8% of sequence reads from the pneumonic lungs were host-derived, compared to only 27.7% of reads from nasal swabs and tracheal washes (Zhang et al., 2019; Zhang et al., 2020b). Although various methods have been assessed to reduce host background in various types of samples, their performance is quite variable and still largely

dependent on sample type (Hall et al., 2014; Sefers et al., 2006; Zhang et al., 2018a). Another limitation is the timing of sample collection. When an animal dies from BRD or is euthanized, primary viral infections are likely already resolved during the time taken for a few rounds of treatment without health improvement. This question could be addressed by collecting lung samples when animals start showing respiratory signs.

Both MiSeq and Nanopore sequencing are capable of detecting IDV in clinical specimens with a range of Cq values as well as other viruses

The advantages of HTS have been reviewed frequently including identification of unexpected or novel viruses, unnecessary ongoing optimization of primers or probes, routine surveillance, potential field application of Nanopore (Chiu and Miller, 2019). Unfortunately, the assessment of performance of non-targeted HTS including both SGS and TGS relative to gold standard qPCR has rarely been performed (Kafetzopoulou et al., 2018).

Results of non-targeted Illumina MiSeq and Nanopore sequencing described in Chapter 4 showed reasonable performance in detection of IDV. We suggested that targeted PCR might be preferable given its higher analytical sensitivity when testing samples with low virus loads (Cq > 31) (Zhang et al., 2020c). Targeted PCR, however, may miss the detection of the virus due to possible mutations in annealing site sequences as we speculated in our study (Zhang et al., 2020c). Our study also showed that different bioinformatics pipelines had variable performance when applied to the same dataset (Zhang et al., 2020c). Selection of the most appropriate analysis pipeline must consider the sequencing platform, as well as tolerance for false negatives and false positives, logistical considerations and the required taxonomic resolution (Zhang et al., 2020c). We have to admit that no single method is suitable for application for all pathogens or specimens types, and

each one has advantages in different circumstances. These choices represent a significant and ongoing challenge to the application of HTS in routine diagnostics.

One of the limitations of this study is that although other viruses were detected using HTS in addition to IDV, the comparison of performance for detection of the remaining viruses between HTS and qPCR was not performed. qPCR methods could be developed for all the other detected viruses to give a more comprehensive comparison. However, since the full genome sequences for some of the viruses detected in this study are not available, design of specific primers and probes will be quite challenging. Another limitation is that we did not include every available, representative and robust bioinformatic tool to evaluate the performance for each platform. Given the number of available tools and massive requirements of time, computational memory and laborious efforts, it is not practical to implement this action. However, results of such a study would be super useful to guide viral metagenomic data analysis in the future, particularly rare studies of comparison among those tools performed on viral metagenomic data when developing bioinformatic tools. For example, Guppy basecaller is trained on sequence data from *E. coli*, *Saccharomyces cerevisiae* and *Homo sapiens* without inclusion of any viruses, which could lead to errors when it is applied to data from organisms outside the training set (Rang et al., 2018; Zeng et al., 2020).

Diversity of viruses in bovine deep nasal swabs upon arrive at feedlots do not predict development of BRD

The study in Chapter 4 has addressed the capability of Nanopore sequencing for detection of viruses from clinical samples and potential future application in the field due to portability and low set-up cost (Zhang et al., 2020c). Therefore, in the study of Chapter 5, we applied Nanopore-based viral metagenomic sequencing to explore the nasal virome of cattle upon arrive at feedlot

and related the findings to the development of BRD. Unfortunately, no relationship was found between BRD development and the number of viruses detected, the presence of any specific individual virus, or combination of viruses (Zhang et al., 2020a). However, the high prevalence of BCV (45.2%) in this study combined with identification of BCV-like human enteric coronavirus (HCoV-4408/USA/1994) in an acutely diarrheic child raises public health concerns over transmission between ruminants and humans (Suzuki et al., 2020; Zhang et al., 1994). Surprisingly, Aichivirus B, also known as bovine kobuvirus, was identified in 2.6% of animals sampled without previous reports in Canada, which demonstrated the advantage of metagenomic sequencing for identification of unexpected or novel viruses (Mitra et al., 2016; Ng et al., 2015; Zhang et al., 2020a; Zhang et al., 2019).

The limitation that viromes were only identified from calves showing BRD signs raised in the Chapter 2 was addressed in Chapter 5 because all the DNS samples were collected from 310 calves upon arrival at the feedlot. In addition, knowledge of health outcomes of these animals made it possible to relate the composition of the virome of individual animals to their outcomes, and determine if the virome composition could be used to predict the likelihood of their development of BRD.

We were unable to predict BRD outcome based on on-arrival virome composition in this study, which may be due in part to the multifactorial nature of BRD and the likely importance of mixed infections in its development. One of the limitations of this study is that we did not perform quantification of viruses, which might help differentiate asymptomatic shedding from active infection (Graf et al., 2016). Another limitation is timing of sampling, which should be addressed with longitudinal studies since there may be a diagnostically informative stage between arrival at the feedlot and the onset of clinical signs of BRD.

6.2. Discussion of future prospects

The causes of BRD have been explored extensively over the past several decades with newly developed and applied antibiotics and vaccines to prevent and control the disease; however, the rates of morbidity and mortality have not been decreased despite those efforts (Loneragan et al., 2001; Smith et al., 2020; Fulton, 2009; Fulton, 2020; Griffin et al., 2010). HTS has currently been revolutionizing the genomics field and providing non-targeted approaches for detection of viruses associated with BRD without knowledge of their clinical significance (Mitra et al., 2016; Ng et al., 2015; Zhang et al., 2019; Zhang et al., 2020b). For bacterial respiratory pathogens, most of those bacteria can be part of the normal upper respiratory tract microbiota and considered as secondary infectious agents (Griffin et al., 2010; Mosier, 2014; Taylor et al., 2010). However, taking advantage of metagenomic sequencing technologies and considering the multifactorial nature of BRD and the likely importance of mixed infections in its development leads us to design studies that are more comprehensive. Perhaps the best way forward is to put more emphasis on studying bacteria and viruses simultaneously in the context of mixed infections rather than in the traditional paradigm of one agent (or type of agent), one disease (Murray et al., 2016; Zhang et al., 2019; Zhang et al., 2020b).

Timing of sampling is an ongoing question that should be addressed. Our studies included deep nasal swabs from calves upon arrival at feedlot, nasal swabs and tracheal wash samples from calves showing BRD signs, and pneumonic lungs from calves that had died or were euthanized due to respiratory issues. However, significant associations between viruses and BRD were only found in calves showing BRD signs (Zhang et al., 2019; Zhang et al., 2020b). Due to the relatively low prevalence of viruses in pneumonic lungs and viral infection characteristics, *post-mortem* virus detection in lung tissues is less likely to be diagnostically informative and provides low analytical

sensitivity relative to *ante-mortem* sampling of the upper respiratory tract for virus surveillance (Zhang et al., 2020b). End-stage pneumonic lungs are not recommended for virome investigations based on our study. Longitudinal studies are highly recommended since there may be a diagnostically informative stage between arrival at the feedlot and the onset of clinical signs of BRD. Quantification of viruses (rather than presence/absence) may be important because we did not develop qPCR methods for each identified virus, and although total read number is likely related to virus quantity, this relationship is affected by difficult to control aspects of the sample (amount of material collected on the nasal swab, amount of host DNA present, etc.) (Zhang et al., 2020c). Finally, the possibility that particular genotypes of viruses are more or less involved in BRD development is an interesting target for future studies that will be more feasible with improved accuracy of third-generation sequencing technologies.

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Appendix 1

Following publication of Chapter 5, we compared the performance of Minimap2 and Bowtie2 for identification of virus-like sequence reads in Nanopore sequencing data, using a batch of ten multiplexed samples selected from Chapter 5. After demultiplexing and trimming using Porechop (https://github.com/rrwick/Porechop) with quality score (Qscore) 7, high quality reads were aligned to the virus Reference Sequence (RefSeq) database using both Minimap2 and Bowtie2. Mapped reads from each sample were then *de novo* assembled using Trinity (Langmead and Salzberg, 2012; Grabherr et al., 2011). Assembled contigs were aligned to the virus RefSeq database using BLASTn and virus-like contigs with a minimum alignment length of 100 bp and an expectation (e) value $< 10^{-3}$ were further examined by BLASTx alignment to the GenBank non-redundant protein sequence database to confirm the nucleotide sequence-based identification and to remove any spurious matches (Brister et al., 2015). The total number of viral reads was determined as previously described (Zhang et al., 2019).

The results demonstrated that in most (9/10) cases more reads were mapped to the reference database by Minimap2 than Bowtie2. Minimap2 also showed generally better performance in diversity of viruses detected, with more viruses identified in every sample except for Sample 6. The lengths of viral contigs assembled from mapped reads were also consistently greater in the Minimap2 workflow (Appendix Table 1).

Based on these results, Minimap2 is recommended for future Nanopore metagenomic virome sequencing applications.

Appendix Table 1. Comparison of results of Bowtie2 and Minimap2: viruses detected, number of viral reads identified and length of assembled contigs.

Sample	Minimap2			Bowtie2		
	Viruses	No. of viral reads	Length*	Viruses	No. of viral reads	Length*
1	BCV/BRBV/UTPV1	863	1257/506/685	BCV	1,213	1046
2	BCV/BRBV	43,623	15503/487	BCV	12,445	9366
3	BCV/EVE/UTPV1	12,987	6269/520/875	BCV	4,130	6678
4	BRBV/BCV/EVE/UTPV1	12,764	6361/5998/616/994	BCV/BRBV	3,055	4228/2667
5	BCV/UTPV1	19,559	7838/1003	BCV	5,325	6590
6	BCV	38,022	19557	BCV	10,100	3050
7	BCV/UTPV1	643	1362/ 942	BCV	599	653
8	BCV/UTPV1	1,911	1105/ 813	BCV	1,732	1689
9	BCV/UTPV1/BHV6	4,008	1157/3592/238	UTPV1/BCV	1,708	3797/1213
10	BCV/UTPV1	1,103	1245/1046	BCV	1,203	1044

Note: * represents the length of the largest contig assembled. BCV – bovine coronavirus, BRBV – bovine rhinitis B virus, UTPV1 – ungulate tetraparvovirus 1, UBPV6 – ungulate bocaparvovirus 6, BHV6 – bovine herpesvirus 6, EVE – enterovirus E.