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Emerging Respiratory Viral Pathogens

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EMERGING RESPIRATORY VIRAL PATHOGENS

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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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CHAPTER 1: LITERATURE REVIEW

INTRODUCTION TO RESPIRATORY VIRAL PATHOGENS

Respiratory disease is arguably the most important health concern for the production animal industry [1-3]. Respiratory problems accounted for the highest mortality both in the swine and beef industries [1, 4]. Respiratory pathogens remain the most vital for swine and bovine research and disease monitoring [2, 5-7]. As pathogens, in particular viral pathogens, mutate, novel disease-causing viruses emerge, and there becomes an increasing concern and need for identification with control perimeters.

One frequently identified disease syndrome is Porcine Respiratory Disease Complex (PRDC). PRDC is characterized by pneumonia of mixed respiratory infections with contributions from the environment and management practices. The main pathogens associated with PRDC include viruses, such as swine influenza virus (SIV), porcine respiratory and reproductive virus (PRRSV), and porcine circovirus 2 (PCV2), and bacteria, such as *Mycoplasma hyopneumoniae, Pasteurella multocida, Streptococcus suis, Bordetella bronchiseptica,* and *Actinobacillus suis.* Viral and bacterial pathogens can be classified as primary pathogens, capable of subverting host defense mechanisms and establishing infection on their own, or opportunistic pathogens [8]. Often, co-infections and superinfections with primary and/ or opportunistic pathogens occur with PRDC.

The bovine counterpart to PRDC is Bovine Respiratory Disease (BRD). Like PRDC, BRD is a general term for a complex multi-factorial disease that encompasses

upper and lower respiratory tract diseases. BRD is caused by stress, viral infection, and/ or bacterial infection with contributions from environmental factors (in particular transportation) and host characteristics (such as age, immune status, and genetics) [2, 9]. Bacterial and viral agents that are implicated in BRD include bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), bovine herpesvirus 1 (BoHV-1), parainfluenza 3 virus (PI3V), bovine coronavirus (BCoV), *Mannheimia haemolytica*, *Mycoplasma bovis*, *Pasteurella multocida*, and *Histophilus somni* [10]. BRD is a costly disease of beef cattle with NAHMS Beef Feedlot 2011 Study reporting the direct cost of treatment for respiratory disease in feedlot cattle at $23.60 USD per case, resulting in a total cost of $54.12 million, not including production loses due to morbidity and mortality [2, 4].

With rising costs of food, the morbidity and mortality associated with respiratory diseases is economically disastrous. Many diagnostic panels are available for common respiratory pathogens for both swine and cattle. Although these panels are helpful, emerging, novel, variant, and underdiagnosed pathogens are often missed. Monitoring for these pathogens, often using metagenomic sequencing, can aid in their control and prevention. Once identified, research can begin on prevalence, pathogenesis, and control and prevention of these pathogens. This review is focused on the discovery of respiratory viral pathogens.

DISCOVERY AND CHARACTERIZATION OF ORPHAN VIRUSES

DNA sequencing, or first-generation sequencing, was developed in 1977 by Fredrick Sanger. This opened the door to genomic analysis, ultimately leading to next-generation sequencing (NGS) [11]. NGS, also known as massive parallel sequencing,

encompasses several high-throughput sequencing methods where billions of nucleic acid fragments can be simultaneously and independently sequenced [12]. NGS has made leaps in genome sequencing allowing for the cataloging of genome mutations, copy number irregularities, and somatic rearrangements of entire cancer genomes at the genomic, transcriptomic, and epigenetic levels [13]. Further, metagenomic sequencing, a next-generation sequence technique, allows for the identification and characterization of all organisms, known or unknown, in all kinds of samples, thus increasing diagnostic detection of pathogens [12]. The ability to detect new and emerging or variant pathogens remains a major advantage of NGS and a key topic of this dissertation.

Following the identification of a pathogen through metagenomic sequencing, disease characterization and pathogenesis needs to be demonstrated. Ideally, Koch's postulates can then demonstrate a relationship between the pathogen and disease. However, many viruses often prove difficult to isolate. One method to localize pathogen nucleic acid in tissue is commercially manufactured RNAscope®. RNAscope® uses *in situ* hybridization (ISH) to visualize single RNA molecules in formalin-fixed paraffin-embedded tissues by hybridizing proprietary pathogen specific probe to single RNA molecules and amplifying the signal [14]. While virus isolation followed by animal studies would be ideal for establishing pathogenesis, localizing pathogen nucleic acid in infected tissues and cells proves to be a timely and sensitive method. This dissertation will highlight the RNAscope® ISH method for localizing novel and emerging respiratory viral pathogens.

ORPHAN RESPIRATORY PATHOGENS: PORCINE PARVOVIRUS 2

Taxonomy, Genomic Organization, and Properties

Porcine Parvovirus 2 (PPV2) is a member of the *Parvoviridae* family and belongs to the *Tetraparvovirus* genus [15]. The formal name for PPV2 is *Ungulate tetraparvovirus 3* [15]. Members of the *Parvoviridae* family are small, non-enveloped, icosahedral resilient viruses that are approximately 23-28nm in diameter [15]. The genome consists of an approximately 5-6 kilobase pair linear, single stranded DNA with short hairpin terminals [16, 17]. *Parvoviridae* genome encodes two proteins, a single capsid viral protein (VP) and a viral replication initiator protein (NSP1), and uses a rolling hairpin strategy to replicate, thus needing host cells to be in S-phase, and due to the properties of the polymerase results in high mutation and recombination rates [15]. The capsid consists of 60 VP proteins to form the icosahedral shape, but lack lipids or glycosylated proteins [15]. Parvovirus capsid protein interacts with a wide variety of cell surface receptor molecules to become internalized through the endocytic pathway [18]. Once internalized, the intact virion can enter the nucleus where the genome is exposed and replication can begin [19, 20]. Due to the resilient nature of the virus, members of *Parvoviridae*, including PPV2, may remain infectious for months or years in the environment [15].

Host Range and Response

Parvoviridae comprises two subfamilies, *Densovirinae* and *Parvovirinae* [15]. Each member is assigned to a subfamily based on the host they infect [21]. Members of *Densovirinae* infect invertebrate hosts, including arthropods and crustaceans, and will not

be discussed further [21]. *Parvovirinae* are categorized into eight genus groups, *Amdoparvovirus*, *Aveparvovirus*, *Bocaparvovirus*, *Copiparvovirus*, *Dependoparvovirus*, *Erythroparvovirus*, *Protoparvovirus*, and *Tetraparvovirus*, that infect mammals, birds, and reptiles, including humans, cattle, and pigs [15]. The focus here is on porcine parvoviruses. There are seven porcine parvoviruses, porcine parvovirus 1 (PPV or PPV1), porcine parvovirus 2 (PPV2), porcine parvovirus 3 (PPV3, PHoV, or PARV4), porcine parvovirus 4 (PPV4), porcine parvovirus 5 (PPV5), porcine parvovirus 6 (PPV6), and porcine parvovirus 7 (PPV7), falling under multiple genus groups. The clinical outcomes of infection for porcine parvoviruses includes systemic disease, reproductive failure, and respiratory distress, however some parvoviruses have been detected in serum, hearts, and rectal swabs [21-23]. While PPV2 pathogenesis is limited, PPV2 has been detected in respiratory tract samples [17, 22-28].

Epidemiology

PPV2 is prevalent, at varying rates, worldwide in both healthy and sick pigs. PPV2 has been detected in clinically healthy pig respiratory tract systems at a high prevalence in Poland (48.7%, oral fluids), Germany (70.5%, tonsils), Japan (58%, tonsils), and Thailand (83%, tonsils) [22, 24-26]. In sick pigs, PPV2 was detected in China (8.8% swine serum samples in a 2006 outbreak of “High Fever” in swine with porcine respiratory and reproductive syndrome (PRRSV) and/ or postweaning multisystemic wasting syndrome (PWMS)), Japan (100% sick domestic pig tonsils submitted for diagnostic testing in 2010), Hungary (10.5% of lung samples from Hungarian swine herds positive for porcine circovirus 2 (PCV2) from 2006-2011, and the United States (20.7% of randomly selected lung tissues from routine diagnostic cases of

varying disease in 2011-2012) [17, 25, 27, 28]. One European retrospective study of biobank archival clinically diseased pig samples identified PPV2 in samples as early as 1998 [29]. In the same study, PPV2 was most frequently detected in the fattener age group (>10 weeks) but was also identified in all age groups (foetus, 0 weeks; pre-weaners, <4 weeks; and weaners, >4 weeks to <10 weeks). Another study of lungs from clinically diseased pigs conducted in the United States, identified PPV2 again in multiple age groups, with grow-finish pigs (8-25 weeks) showing the highest prevalence [28].

Diseases and Co-Infections

Many studies on PPV2 identified virus in the respiratory tract, suggesting a possible tropism for respiratory tissue. One retrospective histopathology study found that PPV2 was significantly associated with respiratory pathogenesis and disease, resulting in bronchointerstitial pneumonia (BIP) and interstitial pneumonia (IP) [29]. Using *in situ*-PCR and *in situ* hybridization (ISH), PPV2 was identified in cells morphologically like macrophages and lymphocytes in lesions of systemic perivascular inflammation lung tissues of 2- to 3-month-old dead pigs with poor growth [16, 30].

PPV1, the porcine parvovirus prototype, is a well-established pathogen that causes reproductive failure [21]. Porcine parvoviruses are frequently identified as co-infections with other bacterial and viral pathogens in diseases such as porcine respiratory disease complex (PRDC) and porcine circovirus-associated disease (PCVAD) [31, 32]. Postweaning multisystemic wasting syndrome (PMWS) is characterized histopathologically by widespread granulomatous inflammation and large, multiple intracytoplasmic inclusion bodies in the cytoplasm of macrophages and multinucleated giant cells [32, 33]. To some extent, PPV1 replicates in circulating peripheral monocytes,

contributing to the widespread viral distribution, viremia, and histopathological lesions [32]. Porcine parvoviruses are frequently identified as co-infections with other bacterial and viral pathogens in PMWS, as well as other diseases such as porcine respiratory disease complex (PRDC), and porcine circovirus-associated disease (PCVAD). Co-infection of PPV1 and porcine circovirus 2 (PCV2) has a well-established association with PCVAD and PMWS [31, 32]

Porcine respiratory disease complex (PRDC) has multiple etiological agents including viral and/ or bacterial co-infections, environmental conditions, and poor management practices. Porcine reproductive and respiratory disease virus (PRRSV), influenza A virus (IAV), and PCV2 are well-established pathogens in PRDC and recognized as the main causative agents [34]. One European study showed concurrent infections of PPV2 and PCV2 were identified in 90 out of 695 (12.9%) tissue samples, with PPV2 identified as a co-factor with PCV2 in PRDC and PCVAD by latent class analysis [29].

While PPV2 infection pathology is limited, the similarities with PPV1, in particular co-infection and tissue distribution, warrant future studies.

Detection and Identification

The current detection method for PPV2 includes quantitative PCR (qPCR) and *in situ* PCR [35]. Additionally, PPV2 nucleic acids can be identified histologically by using the commercially available Advanced Cell Diagnostics (ACD) RNAscope® *in situ* hybridization (ISH). Methods to isolate PPV2 have proven unsuccessful, and there are no further histological staining methods for detection and identification.

ORPHAN RESPIRATORY PATHOGEN: ROTAVIRUS A

Taxonomy, Genomic Organization, and Properties

Rotaviruses are a member of the *Reoviridae* family and belongs to the *Rotavirus* genus [36]. There are nine species of *Rotavirus* (Rotavirus A, B, C, D, F, G, H, I, and J) and an atypical rotavirus E that was only identified in swine [37, 38]. The *Rotavirus* genome consists of 11 linear double-stranded RNA (dsRNA) segments that code for six structural proteins (VP1-4, 6, and 7) and six non-structural proteins (NSP 1-6) [36]. Rotavirus group A (RVA) is the best studied of the 10 RV groups and infects a wide array of species including humans, pigs, cattle, and poultry [39]. RVA has a triple-layered capsid enclosing its 11 ds-RNA segments, RNA-dependent RNA polymerase (RdRp) VP1, and VP3, and is about 100nm in diameter when mature having a wheel-like appearance under electron microscopy (EM) [36]. The innermost layer is composed of 60 asymmetric VP2 dimers (T=1) followed by the intermediate capsid composed of 760 copies of VP6 (T=13), which is known as the double-layered particle (DLP) [36]. The outermost layer of the mature virion is composed of VP4 and VP7 (T=13; 260 trimers of VP7 and 60 VP4 trimeric spikes) and are required for infectivity [36]. VP4 and VP7, which are glycoproteins, also designate subtypes of Rotavirus A. In order for VP8 and VP5 to be generated and prime the virion for infection, VP4 (the viral attachment protein) must be cleaved by trypsin thus enhancing viral infectivity and stabilizing the spike conformation [36]. However, the cellular receptor for VP4 has not yet been identified. VP7 and VP4 induce neutralizing antibodies *in vivo* and serve as the dual-typing classification system, the G and P serotypes, respectively [40].

Once VP4 has attached to the cell, the outer VP4 and VP7 layer is lost and the DLP is released into the cytoplasm [36]. The attachment of VP4 is to sialoglycoprotein and integrin receptors of mature differentiated enterocytes found at the tips of the villi in the small intestine [41]. 5'-capped, non-polyadenylated mRNAs are synthesized using the minus strand of each genome segment as a template serving 2 functions: templates for translation and templates for minus-strand synthesis, dsRNA genome segments [36]. Virus assembly is mediated by NSP2 and NSP5 in cytoplasmic inclusions where the 11 different viral mRNAs interact with one another and VP1 and VP3 followed by VP2, which triggers minus-strand synthesis resulting in the formation of cores containing the 11 dsRNA segments [36]. VP6 is then added to the core to form the DLP, which is the recruited to the endoplasmic reticulum (ER) by transmembrane glycoprotein NSP4 and buds through the ER resulting in the acquisition of a lipid envelope and the addition of VP4 and VP7 [36]. The final product is a mature RVA virion.

Host Range and Response

Rotaviruses are a major cause of acute gastroenteritis in a wide range of young hosts, including humans, piglets, and calves [36, 39]. Rotavirus has an incubation period of 1-2 days with symptoms of vomiting, and watery diarrhea that persists for 3-8 days [40]. Rotavirus disease is generally mild in adults. RVs are mainly transmitted through the fecal-oral route. Contaminated hands, environmental surfaces and objects, and water and food under poor hygienic conditions are the source for viral spread [41]. Pathogenesis of rotavirus diseases is multifactorial, including the age of the host, and is fairly well-understood [42]. The infectious dose of rotavirus infection is estimated to be 100-1000 viral particles [43].

Following entry into small intestinal enterocytes through cellular attachment of VP4, virus is uncoated, nucleic acids are transcribed, viral proteins are translated and either formation of viroplasms and release of virus occurs or NSP4 induces an age-and dose-dependent diarrheal response [41, 44]. NSP4 acts as an enterotoxin and secretory agonist to 1) stimulate Ca^{2+}-dependent cell permeability and 2) alter integrity of epithelial barriers [44]. Ca^{2+} disrupts the cytoskeleton and tight junctions, increases paracellular permeability, and stimulates the enteric nervous system (ENS) resulting in secretory diarrhea and increased intestinal mobility [38, 41]. After cleavage, the product, VP8, also disrupts the barrier integrity of tight junctions by altering the localization of tight junction components claudin-3, ZO-1, and occludin [42, 45-47]. Progeny virus is released in large numbers into the intestinal lumen 10-12 hours later, infecting neighboring cells, which can lead to the high viral load often seen [41]. The damage results in shortened villi with sparse irregular microvilli and mononuclear cell infiltration of lamina propria [44]. Diarrhea ultimately occurs from malabsorption due to destruction of enterocytes, villus ischemia, and neuro-regulatory release of vasoactive agent from infected epithelial cells [38, 48]. Viral load decreases rapidly with the decrease of symptoms, but viral nucleic acid can be detected for several weeks at low levels [49].

While RV infection was thought to be limited to intestinal epithelial cells, several studies have detected RV in serum and multiple visceral organs, suggesting viremia [50-56]. RV infection has been recently demonstrated to have systemic effects, although clinical relevance remains unclear. Several recent studies of extraintestinal RV antigenemia that reported children with a variety of clinical signs, including respiratory

disease may be the link to the extraintestinal clinical manifestations associated with RV infection [41, 57, 58].

The host will have an innate and adaptive immune response to rotavirus infection. Briefly for the innate immune response, host will sense dsRNA via receptors that activate adaptor proteins to recruit specific transcription factors, such as interferon (IFN) [59]. IFN ultimately activates the transcription of a large number of interferon-stimulating genes that are responsible for the amplification of the antiviral response and restrict the viral life cycle [59]. NSP1, VP3, NSP2, and NSP3 from RV are innate immune antagonists for viral defense [59].

Neutralizing antibodies directed against VP4 and VP7 from the outer layer of a mature viral particle provide host protection against RV infection [41, 60]. These antibodies are either IgG or IgA and may be acquired via suckling or actively following vaccination and correlate with protection from RV disease [60].

Epidemiology

Rotaviruses are prevalent and found worldwide. Epidemiology studies on rotaviruses from an array of hosts have mainly been conducted on gastrointestinal samples. The presence of rotavirus in respiratory samples is sparsely studied.

In 2013, >200,000 fatalities in children less than 5 were associated with rotavirus worldwide [61]. Children hospitalized with diarrhea due to rotavirus infection is similar worldwide (~30-50%), however greater than 90% of children with fatal rotavirus infection live in low-income countries [61]. An important note found by one study was that rotavirus disease was more frequently caused by uncommon rotavirus strains further

emphasizing the significance of disease monitoring and surveillance through metagenomic sequencing [62]. Human RVAs have been classified into 14 G type and 17 P type serotypes with combinations of G1P[8], G2P[4], G3P[8], G9P[8], and G12P[8] the most commonly responsible for rotavirus disease [48].

The most well characterized mammalian rotavirus epidemiology studies are those affecting cattle, swine, horse, and partly goats, sheep, and camels, with a focus on porcine here [48]. These studies have been conducted on gastrointestinal samples.

Porcine rotavirus A, B, C, and H have been confirmed at varying prevalence worldwide, as well as an atypical porcine rotavirus E that was only identified in UK swine [38]. Porcine rotavirus A (RVA) prevalence varies from 3.3%-67.3% and farm-level prevalence reaching up to 61%-74% [38]. Twelve G genotypes (G1-G6, G8-G12, and G26) and sixteen P genotypes (P[1]-P[8], P[11], P[13], P[23], P[26], P[27], P[32], and P[34]), with G3, G4, G5, G9, and G11 and P[5], P[6], P[7], P[13], and P[28], are considered the most common associated with pigs [38]. Genotypic variation also depends on the geographic location of the swine. In the Americas, the most prevalent G type of porcine RVA was G5 (71.4%) and P type of porcine RVA was P[7] (77.2%) [38].

Like porcine rotaviruses, bovine rotaviruses are globally distributed. Bovine RVAs have been classified into 12 G types and 11 P types with G6, G8, and G10, and P[1], P[5], and P[11] the most prevalent [48].

Although epidemiological studies have not established a role for rotavirus and respiratory tract disease, rotaviruses have been detected in nasopharyngeal secretions in infants with respiratory illness, as well as neonatal piglets in experimental settings [51,

56, 57]. In one study, piglets orally inoculated with porcine RVA subtypes G9P[23] and G9P[7] also displayed interstitial pneumonia [63]. Other animal studies where pigs were inoculated with either reassortment bovine RV or human RVA strain Wa, RV was detected in serum, lungs, and other organs by PCR and immunofluorescence assays or developed viremia, shedding virus both nasally and rectally, respectively [51, 64]. Prevalence of RV respiratory associated infection studies are necessary to more fully understand the epidemiology of RVs.

Diseases and Co-Infections

Although rotaviruses have been detected in nasopharyngeal secretions and in serum, the main rotaviral disease is gastroenteritis in young children and animals [50-57]. Experimentally, piglets orally inoculated with porcine RVA subtypes G9P[23] and G9P[7] displayed both enteritis and interstitial pneumonia [63]. Additionally, neonatal germ-free piglets that were inoculated either orally or intranasally with the human RVA strain Wa developed diarrhea and viremia and shed virus both nasally and rectally [51]. Evidence is growing for gastrointestinal diseases corresponding to respiratory disease in the same animal.

Although often identified as a monovalent viral infection, RVs have been detected with norovirus, parvovirus, and porcine epidemic diarrhea virus [65-67]. *Salmonella* spp. has also been shown to co-infect bovine with rotavirus [68]. Rotavirus infection itself causes a substantial impact on the diseased animal making a significant co-infection unnecessary to cause disease. Additional studies are needed to address the impact of rotaviruses in extraintestinal disease and the role it plays in co-infections.

Detection and Identification

Detection methods for RVs include electron microscopy, polyacrylamide gel electrophoresis, antigen detection assays, reverse transcription polymerase chain reaction (RT-PCR), and virus isolation [41]. Per the Center for Disease Control and Prevention (CDC), rapid diagnosis is conducted using PCR assays, either RV alone or on a multipathogen panel, or by antigen-detection immunoassays. To additionally characterize strains for surveillance or research purposes, nucleic acid sequencing may be performed. Correlation of immunohistochemical (IHC) staining, alongside hematoxylin and eosin (H&E), may also be useful in pathogenesis and diagnosis of deceased animals [69]. RVA, Rotavirus B (RVB), and Rotavirus C (RVC) nucleic acids can be identified histologically by using the commercially available Advanced Cell Diagnostics (ACD) RNAscope® *in situ* hybridization (ISH) [35].

Rotavirus A is readily detectable due to the immense selection of testing methods for diagnosis. The intent of the physician, pathologist, or researcher will be the guide in deciding which method is most appropriate.

Prevention and Control

Rotaviruses are ubiquitous and infect several host species, including human, bovine, and porcine. Because of the consequences, including death and hospitalization, from RV infections, species specific vaccines are deemed necessary.

The CDC recommends one of two oral rotavirus vaccines during the first year of a child's life: Rotateq® or Rotarix®. These vaccines provide 85-98% protection against

severe rotavirus illness and hospitalization, according to the CDC. They also state that the vaccines provide 74-87% protection against any severity of rotavirus illness.

In pigs, ProSystem® Rota is the first USDA-licensed modified live vaccine providing protection to two serotypes of serogroup A rotavirus (G5 (A1) and G4(A2)) given orally and intramuscularly claiming to provide 100% protection from mortality from rotavirus in a challenge of vaccinated piglets.

For cattle, Scourguard® 4K (Zoetis Inc., Kalamazoo, MI) is available for healthy, pregnant cows and heifers against bovine rotavirus (serotypes G6 and G10), bovine coronavirus, and enterotoxigenic strains of *Escherichia coli* having the K99 pili adherence factor. Zoetis claims to show significant reductions in abnormal fecal, appetite, attitude, and dehydration scores compared to colostrum-consuming control cows post-ScourGuard® 4K vaccination. BOVIS® GUARDIAN® (Merek & Co, Rahway, NJ) is an additional vaccine for cattle covering major bacterial and viral causes, including rotavirus (serotypes G6 and G10), coronavirus types 1 & 3, *Clostridium perfringens* types C & D, and *Escherichia coli* type K99. Scour Bos® 9 (Elanco, Greenfield, IN), another bovine vaccine against diarrhea that can be administered to pregnant cows up to 4 months pre-calving, provides protection against 9 pathogens, including rotavirus (serotypes G6, G8, & G10), coronavirus, 4 isolates of *E. coli*, and *C. perfringens* Type C.

While promoting good hygiene and sanitation may benefit human prevention and control of RV, animals do not live under the same conditions. Rotaviruses have maintained their prevalent status, despite readily available and seemingly efficacious vaccines. Variant genotypes also play a role in the abundant diversity and prevalence of

RVs, and the detection of RVs in alternate tissues compounds their infectivity. Continuous monitoring and evolving control strategies are necessary to contain the virus.

ORPHAN RESPIRATORY PATHOGEN: BOVINE ASTROVIRUS

Taxonomy, Genomic Organization, and Properties

Astroviruses (AstVs) have a complicated taxonomic organization. The family *Astroviridae* is split into *Avastrovirus* and *Mamastrovirus*. Members of *Avastrovirus* infect birds and will not be discussed further. Members of *Mamastrovirus* infect mammals including bovine, human, porcine, caprine, ovine, and several others. Classification of species is currently being redefined and is based on isolation from hosts that they infect [70].

AstV is a positive-sense single-stranded RNA virus with a 6.4-7.7kb genome arranged in three open reading frames (ORFs): ORF1a, ORF1b, and ORF2 [70]. Located at the 5’ end of the genome, ORF1a and ORF1b encode for the non-coding proteins, while ORF2, located at the 3’ end, encodes for structural proteins that are necessary for cell entry, including the capsid protein and is the most variable region of the genome [71]. A frame shift mechanism, at sequence AAAAAAC, occurs between ORF1a and ORF1b allowing for expression of ORF1ab that encodes RNA-dependent RNA Polymerase and protease [72].

Virions are non-enveloped, approximately 40-45nm in diameter, and have T=3 icosahedral symmetry with spike-like projections, giving them a star-like appearance [72]. Little is known about the pathogenesis of AstV and appears to vary by genus. Studies on various AstVs show astrovirus can induce epithelial permeability

independently of viral replication and is modulated by the capsid protein, which can induce an apoptotic response and activate caspases, and can bind complement proteins C1q and mannan-binding lectin (MBL) with the capsid protein thus inhibiting the classical and lectin (human) complement systems, respectively [73-75]. Species classification of genetic and host analysis will promote the direction of AstV pathogenesis studies.

Like other AstVs, the pathogenesis of bovine astrovirus (BAstV) has yet to be determined. The need for a cell culture system suitable for most AstVs contributes to the lack of understanding of AstV pathogenesis.

Host Range and Response

Astroviruses infect a wide range of hosts and cause a variety of disease states. As mentioned, *Avastroviruses* infect birds including turkey, duck, and chicken. *Mamastroviruses* have been isolated from many mammals including human, bovine, porcine, sea lion, feline, caprine, ovine, water buffalo, wild boar, yak, canine, porcupine, deer, camel, bat, mink, fox, and marmot. Most often, AstVs are isolated in the stool or from gastrointestinal tract of animals exhibiting diarrhea. However, AstVs have been identified in many other tissues, including, but not limited to, liver, kidney, brain, lung or nasopharyngeal (NP) swabs, and serum [76-83]. Bovine astrovirus (BAstV) BSRI1 (GenBank accession number KP264970) was identified in a metagenomics case-control study to identify viruses associated with bovine respiratory disease (BRD) [78]. In this study, four bovines showing symptoms of BRD had positive NP swabs, while asymptomatic animals had negative NP swabs [78]. The results showed no statistical significance. Additional studies identified and sequenced BAstV in cattle showing

symptoms of encephalitis [81, 84-86] This supports the growing evidence of diverse tissue tropism of astroviruses.

Not only have AstVs been detected in multiple tissue types, but there is evidence of intra-and cross-species transmission. Fecal-oral transmission of AstVs is the primary route of transmission. The non-enveloped property of AstVs allows them to be stable, persisting for long periods of time in the environment and allowing for easier agricultural and commercial, terrestrial, and aquatic transmission amongst species [87-89]. Living conditions of farm animals and wild animals promote intra-species, and potentially, cross-species exposure to the feces of other animals [90]. Supporting the hypothesis of cross-species transmission, ovine with encephalitis that were infected with AstV were genetically almost identical to AstV identified in neurologically diseased cattle [91]. Additionally, AstVs detected in farmed guinea fowl had 84.6-100% ORF2 amino acid similarity to turkey astrovirus type 2 [90, 92]. Recombination events have been hypothesized to play a large role in cross- and intra-species transmission [71, 93, 94].

Astroviruses have a multitude of host species and evolving tissue tropism. While the focus here will be on bovine astrovirus, it is important to note the link of BAstV to other *Mamastroviruses* through cross-species transmission.

Epidemiology

Astroviruses were first discovered in 1975 in the stool of children exhibiting signs of diarrhea and vomiting in the United Kingdom [95, 96]. Since the discovery in 1975, AstVs have been detected in over 80 different avian and mammalian hosts, including bovine astrovirus that was first detected in 1975 from fecal samples of British diarrheic

calves [70, 89, 97]. Not only is there diversity in the array of hosts, but also in the geography. BAstV has been identified worldwide in a variety of tissue types (previously discussed). Countries where BAstV has been identified with varying prevalence include United Kingdom, United States, China, Korea, Japan, Brazil, Egypt, Turkey, Switzerland, Italy, Germany, Canada, and Uruguay [81, 82, 85, 98-107]. Several of these countries reported BAstV detection both in fecal samples and in brain tissues, as well as the United States reporting BAstV in nasal swabs of cattle showing respiratory symptoms.

Animals are not routinely screened for AstV thus prevalence information is limited to surveillance studies. A recent study showed overall prevalence of BAstV in diarrheic calves was 55.17% and 36.36% in asymptomatic calves [108]. Additionally, BAstV is more common in younger cattle compared to adult cattle [101, 109].

Generally, AstVs are found in a multitude of countries at varying prevalence and recovered from a vast array of animals and tissue type.

Diseases and Co-Infections

Due to the diversity of AstVs, the disease states range, but are not limited to, from encephalitis to gastroenteritis to respiratory distress. The focus of this dissertation is respiratory disease, thus respiratory disease states associated with AstVs, BAstV, will be discussed further. To date, *Mamastroviruses* have been identified in NP swabs from camels, along with humans, pigs, and cattle where respiratory symptoms were also associated [77, 78, 110, 111]. One case-control metagenomic study of bovine respiratory disease (BRD) found four cattle positive for astrovirus with respiratory symptoms where all 50 asymptomatic animals were negative [78].

The case-control metagenomic study that identified BAstV in cattle with BRD also identified alongside common bovine respiratory pathogens, including Bovine Adenovirus 3 (Bad V-3), Bovine Rhinitis A virus (BRAV), Bovine Rhinitis B virus (BRBV), and Bovine Picornavirus (BoPV) [78]. BAstV infections often include multisystem infections of the gastrointestinal tract and nervous system. [101, 112, 113]. Evidence shows co-infection of the same host with multiple different astrovirus strains, which is hypothesized to play a role in recombination events resulting in novel or variant AstV strains [90, 114].

The diversity of astroviruses allows for a variety of diseases in infected animals. With the ease of respiratory transmission, it will be important to further the knowledge of AstVs and respiratory disease.

Detection and Identification

Multiple PCR methods are available for detection of BAstV including RT-PCR, multiplex PCR, nested RT-PCR, and qPCR [97, 108, 109, 115]. Electron microscopy (EM), immunohistochemical (IHC) staining and fluorescence *in situ* hybridization (ISH) have also been used for the diagnosis and detection of BAstV [81, 98, 116]. Recently, the most popular methods for discovery of novel viruses involve sequencing, and Italian researchers established a nanopore technology based on a macrogenome to detect astroviruses [82]. The diverse tissue tropism and disease pathogenesis of AstVs indicates how important multiple methods of detection are, in particular sequencing methods, that allow for the discovery and detection of novel or variant pathogens.

RATIONALE

The Severe Acute Respiratory Coronavirus 2 (SARS-CoV-2) outbreak in 2019 resulting in a pandemic shed light on the importance of respiratory viruses. To develop prevention and control measures for new infections we need to understand transmission routes and be able to quickly detect and identify novel and/or mutant viruses and develop vaccines. We can use the lessons learned from the SARS-CoV-2 outbreak and apply it to animal respiratory pathogens.

First, the dominant mode of respiratory pathogen transmission is through exposure of respiratory droplets and close contact of infected individuals. Airborne transmission and fomites, such as droplet-contaminated surfaces, are alternate routes for respiratory pathogen spread, and however the virus leaves an individual, the respiratory tract is the most common portal of entry for viruses [117].

Second, early detection and identification of novel and/ or mutant viruses with metagenomic sequencing are key to slowing the spread of respiratory pathogens. Metagenomic sequencing can identify viral diversity, known or unknown, that would not be detected in standard testing methods such as PCR or immunoassays. Analysis of sequenced pathogens can allow for trace back to when and where infection and/ or mutations originated. This information can lead to improvement of health management and biosecurity systems. Continuous surveillance of respiratory diseased animals is a useful tool to proactively monitor the evolution and discovery of respiratory pathogens that contribute to the loss of animals.

Third, identifying an entire genome of a novel respiratory pathogen is useful for the development of both diagnostic assays and vaccines. Vaccinomics, a systems biology approach to vaccine research, is based on the use of cutting edge, novel bioinformatics for the development of next-generation vaccines, thus expanding the capabilities of individualized medicine [118, 119]. Pasteur's original rules of "isolate, inactivate, and inject" led to the elimination of many devastating infectious diseases, however vaccinomics will promote the movement beyond this approach, allowing for an enhanced understanding of biological processes and overcoming the obstacles to the creation of effective vaccines against pathogens with the greatest public health impact [118].

Through routine metagenomic sequencing, novel or variant pathogens that are not on a typical respiratory panel can be identified. Once identified and sequenced, prevalence and disease correlation of the pathogen in tissue homogenate can be defined through PCR, and pathogenesis studies can be conducted. Pathogenesis studies may include histopathology through immunohistochemical (IHC) staining and/or *in situ* hybridization (ISH) or viral isolation followed by animal studies. If the pathogen is identified as prevalent and emerging, the goal for a respiratory pathogen would be to create an assay for diagnosis and a vaccine for future prevention.

This dissertation will discuss the relevance of each virus in the following chapters. Chapter 2 examines the role of porcine parvovirus 2 in porcine respiratory disease complex and was published by Nelsen, et.al (See Appendix 1) [120]. In chapter 3, porcine rotavirus A was found in association with porcine respiratory disease. This chapter was published by Nelsen et.al in Frontiers in Veterinary Science (See Appendix 2) [121]. The third virus, a novel bovine astrovirus most like a caprine astrovirus, was

identified in a calf with respiratory symptoms and will be characterized in Chapter 4. The final chapter, Chapter 5, will include a comprehensive discussion on the importance of monitoring and surveilling respiratory samples for novel and/ or variant pathogens.

CHAPTER 2: PORCINE PARVOVIRUS 2 IS PREDOMINATELY ASSOCIATED WITH MACROPHAGES IN PORCINE RESPIRATORY DISEASE COMPLEX

The work presented in this chapter was published in Frontiers in Veterinary Science.

ABSTRACT

Porcine respiratory disease complex (PRDC) is a significant source of morbidity and mortality, manifested by pneumonia of multiple etiology where a variety of pathogens, environment, and management practices play a role in disease. Porcine reproductive and respiratory disease virus (PRRSV), influenza A virus (IAV), and porcine circovirus 2 (PCV2) are well-established pathogens in PRDC. Porcine parvovirus 2 (PPV2) has been identified in both healthy and clinically diseased pigs at a high prevalence worldwide. Despite widespread circulation, the significance of PPV2 infection in PRDC and the association with other co-infections are unclear. Here, PPV2 was detected in lung tissue in 39 of 100 (39%) PRDC-affected pigs by quantitative polymerase chain reaction (qPCR). Using in situ hybridization (ISH) in conjunction with tissue microarrays (TMA), PPV2 infection was co-localized in alveolar macrophages and other cells in lungs with interstitial pneumonia in 28 of 99 (28.2%) samples. Viral load tended to correlate with the number of macrophages in the lungs. Assessment of frequency, viral titers and tissue distributions showed no association between the infection of PPV2 and other major viral respiratory pathogens. In one-third of qPCR PPV2-positive samples, no other known viruses were identified by metagenomic sequencing. Genome sequences of PPV2 were 99.7% identical to reference genomes. Although intensive intra-nuclear and intra-cytoplasmic signals of PPV2 were mainly detected in alveolar macrophages by ISH, no obvious virus replication was noted *in vitro*

cell culture. Together, these results suggest PPV2 may play a primary role in PRDC, warranting the control and prevention of this underdiagnosed virus.

INTRODUCTION

Porcine parvovirus 2 (PPV2), formally *Ungulate tetraparvovirus 3*, belongs to the *Tetraparvovirus* genus, *Parvoviridae* family [16]. PPV2 is a small, non-enveloped, icosahedral virus consisting of an approximately 5-6 kilobase linear, single stranded DNA genome [16, 17]. The prototype of porcine parvovirus, porcine parvovirus 1 (PPV1), is a well-known pathogen that causes reproductive failure [21]. Porcine parvoviruses are frequently identified as co-infections with other bacterial and viral pathogens in diseases such as porcine respiratory disease complex (PRDC) and porcine circovirus-associated disease (PCVAD) [31, 32]. The clinical significance of PPV1 has been well described, while for emerging parvoviruses, such as PPV2, pathological consequences of infection are poorly understood [21]. PPV2 was first identified in Myanmar in 2001, however, a retrospective study of samples collected from several European countries detected PPV2 as early as 1998 in archived porcine tissues [27, 29].

Epidemiological studies of PPV2 have reported varying prevalence worldwide. In clinically healthy pigs, a high prevalence of PPV2 was detected in oral fluids (48.7%) and serum (53.9%) in Poland between 2015 and 2017 [26]. Likewise, PPV2 was detected in 78.0% and 70.5% of tonsils and hearts in Germany [22]. Tonsils of healthy slaughterhouse pigs collected in Japan (2010) and Thailand (2011) identified 69 out of 120 (58%) and 66 out of 80 (83.0%) positive for PPV2, respectively [24, 25]. In sick pigs, PPV2 was detected in 8.8% swine serum samples in a 2006 outbreak of “High Fever” observed in swine with porcine respiratory and reproductive syndrome (PRRSV)

and/ or postweaning multisystemic wasting syndrome (PWMS) in China [27]. In Japan, PPV2 was identified in 69 out of 69 (100%) sick domestic pig tonsils submitted for diagnostic testing in 2010 [25]. A study in the United States detected PPV2 in 100 out of 483 (20.7%) randomly selected lung tissues from routine diagnostic cases of varying disease [28]. A retrospective study of Hungarian swine herds from 2006-2011, all positive for porcine circovirus 2 (PCV2), identified PPV2 in 4 out of 38 (10.5%) lung samples [17].

A more recent retrospective study using histopathology scoring showed PPV2 was significantly associated with respiratory pathogenesis and disease, in particular interstitial pneumonia (IP) and bronchointerstitial pneumonia (BIP) [29]. Concurrent infections of PPV2 and PCV2 were identified in 90 out of 695 (12.9%) tissue samples, with PPV2 identified as a co-factor with PCV2 in PRDC and PCVAD by latent class analysis [29]. PPV2 has been identified in cells morphologically resembling macrophages and lymphocytes in lung tissues of 2- to 3-month-old dead pigs by *in situ*-PCR and *in situ* hybridization (ISH) in lesions of systemic perivascular inflammation in pigs with poor growth [16, 30]. To date, studies of PPV2 infection pathology is limited.

Porcine respiratory disease complex is the result of multiple viral and/ or bacterial co-infections. PCV2, PRRSV, and IAV are recognized as the main causative agents [34]. While PPV2 is an emerging virus reportedly highly prevalent in a variety of porcine tissues from both healthy and sick pigs, it has not yet been included in regular diagnostic panels for PRDC. As part of a routine diagnostic investigation, metagenomic sequencing was performed on a swine lung tissue with interstitial pneumonia that was PCR negative for IAV, PRRSV, PCV2, and *Mycoplasma hyopneumoniae* in our diagnostic laboratory.

PPV2 was identified in the absence of other swine viruses and clinically significant bacteria leading us to investigate the role of PPV2 in PRDC.

MATERIALS AND METHODS

Sample Selection

All porcine respiratory cases submitted to the Animal Disease Research and Diagnostic Laboratory at South Dakota State University (ADRDL-SDSU) from 2019-2020 were screened for archived formalin-fixed paraffin-embedded (FFPE) lung tissue. Samples included lung tissues (n=124) and variably contained tissue from liver, kidney, lymph node, or spleen, embedded in the same FFPE block.

Retained frozen tissue samples submitted to ADRDL-SDSU from May 2020 and September 2020-January 2021 were selected from porcine respiratory diagnostic submissions that included lung tissue. Tissue (n=100) was homogenized and stored at -80°C.

Case data, including pig age and clinical signs, were recorded. Client requested diagnostic testing results, including PCR for IAV, PRRSV, PCV2, porcine circovirus 3, and *Mycoplasma hyopneumoniae*, and bacterial isolation, were collected as available.

Pigs were separated into six groups to determine PPV2 prevalence at different ages: fetuses <1-day, suckling pigs (1 day- 3 weeks), nursery pigs (3-8 weeks), grow-finish pigs (8-25 weeks), mature pigs (>25 weeks), and unknown.

Quantitative PCR

Nucleic acids were extracted from homogenized tissue with the QIAamp® Viral RNA Mini Spin per manufacturer's instructions. For FFPE samples, QIAamp® DNA FFPE Advanced Kit and QIAamp® DNA FFPE Advanced UNG Kit were used. A previously designed PPV2 TaqMan® assay was used [28]. A master mix consisting of TaqMan®, PPV2 primer/ probe, and RNase-free water was added to sample and ran on Applied Biosystems 7500 FAST Real-Time PCR System with an initial 20 second hot start at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

Construction of the tissue microarray (TMA) and histopathology examinations

To assess the pathology of PPV2 infection in the lungs, 100 FFPE blocks, including 27 samples where lung homogenates were positive for PPV2 by qPCR, 23 samples where lung homogenates were negative for PPV2 by qPCR, and 49 randomly selected cases with unknown PPV2 status were selected from the FFPE tissue archive. One tissue was mistakenly classified as lung and removed from the study, for a final total of 99 tissue cores used in the study. Tissue cores with a diameter of 5 mm, containing at least 7 intact bronchioles and adjacent pulmonary parenchyma, were extracted from donor lung blocks and transferred to a recipient paraffin block with a random distribution (AMSBIO LLC, Cambridge, MA). Five TMA recipient blocks, each containing 20 cores, were constructed. Serial sections of TMA blocks were made for routine HE, IHC, and ISH staining as described below. Finally, the TMA slides were read by a pathologist with case number blinded. They were also digitized using DP74 camera (Olympus) at 200x magnification. Signals of ISH and IHC were semi-quantified by Olympus Sens Cell Count & Measurement module.

Porcine parvovirus 2 *in situ* hybridization

In situ hybridization was performed using a commercialized RNAscope® system (Advanced Cell Diagnostics (ACD)) according to the manufacturer's instructions. The proprietary PPV2 probe (cat#899191) from ACD targeting viral capsid gene was used. A positive (RNAscope® Probe Ss-PPIB, cat#428591) and a negative (RNAscope® Negative control Probe-DapB, cat#310043) probe were used as internal controls. Briefly, TMA slides were incubated for 1 hour at 60°C in a HybEZ™ Oven from ACD. Slides then underwent deparaffinization with two rounds of 5 minutes in xylene followed by two rounds for 1 minute of 100% ethanol. Pretreatment for FFPE slides included 10 minutes at room temperature with RNAscope® Hydrogen Peroxide, 15 minutes in a Hamilton Beach 5.5 Quart 37530A Digital Food Steamer with RNAscope® 1X Target Retrieval Reagent, and 30 minutes at 40°C in the HybEZ™ Oven with RNAscope® Protease Plus. The following steps were completed according to the RNAscope® Assay using the RNAscope® 2.5 HD Detection Reagent-RED. Slides were incubated for 2 hours in the HybEZ™ Oven at 40°C for 2 hours with PPV2 ORF2 mRNA probe provided by ACD. Slides underwent four rotating amplification steps of 30 minutes at 40°C followed by 15 minutes at 40°C. Two additional amplification steps followed at room temperature, one for 30 minutes and one for 15 minutes. Signal was detected with Fast RED with a 10-minute room temperature incubation. Slides were counterstained with 50% Gill's Hematoxylin I and mounted with EcoMount.

Nuclease treatment

To differentiate between ISH detection of PPV2 mRNA as opposed to the genome of PPV2 in the lungs, serial sections from one case with intense PPV2 ISH staining were

treated with either Ambion™ DNase I (RNase-free) or RNase ONE™ Ribonuclease for 30 minutes at 37°C before the hybridization procedure. The working concentration of DNase I was 0.04U/µL and RNase ONE™ was 0.2U/µL.

Immunohistochemistry

Immunohistochemistry was performed on TMA slides to detect swine pathogens PRRSV (SDOW17/SR30, SDSU), IAV (HB65, SDSU), PCV2 (ISU-4, Iowa State University). Phenotyping of inflammatory cells was performed with cell markers CD3 (Dako), CD79a (Dako) and Iba-1(ThermoFisher) for T lymphocytes, B lymphocytes and macrophages, respectively, as described in a previous study [122]. Antigen retrieval was conducted by microwave heating (1100 watt) for 2 min and 10 sec in pH 6.0 Citrate Buffer. Subsequently, the slides were loaded onto Dako Autostainer-Universal Staining System. The protocol was carried out with Rabbit specific HRP/DAB Detection IHC Detection Kit-Micro-polymer (Abcam) according to the manufacturer's instructions.

Metagenomic sequencing

Metagenomic sequencing was performed on frozen tissue samples that were qPCR positive for PPV2 but negative (or data not provided) for IAV, PRRSV, PCV2, and *Mycoplasma hyopneumoniae* to identify co-infections present with PPV2 in respiratory disease submissions. Metagenomic sequencing was performed as previously described [123, 124]. Briefly, clarified tissue homogenate was digested with a cocktail of nucleases followed by extraction using the QIAamp® Viral RNA kit. First strand synthesis using SuperScript™ III First-Strand Synthesis Kit was performed on the nucleic acid extracts using barcoded oligonucleotides containing 3'-random hexamers followed by a digestion

with RNase H. Second strand synthesis using Sequenase was next performed. cDNA was purified with the GeneJET Gel Extraction and DNA Cleanup Micro Kit and subsequently amplified by PCR using primers specific to the integrated barcode. Amplified DNA was purified using the GeneJET Gel Extraction and DNA Cleanup Micro Kit. The concentration of purified DNA was measured on a Qubit spectrophotometer and diluted to 0.2 ng/µL. Sequencing libraries were prepared with a Nextera XT kit. DNA sequencing was performed with an Illumina MiSeq using paired 151 bp chemistry. Sequencing reads were trimmed with onboard software and imported into CLC Genomics Workbench 20 where they were assembled into contigs using the *de novo* assembler. Contigs were analyzed by BLASTX using the BLAST2GO plugin to identify reads similar to known swine viruses.

Genetic and phylogenetic analysis

To investigate the relationship of the PPV2 sequenced here to previously determined PPV2 genomes, twenty-eight PPV2 genomes determined from a broad geography from 2012-2018, along with representative *Parvovirinae*, were downloaded from GenBank. Whole genome phylogenetic analysis was performed using the best fitting K2+G (Kimura 2-Parameter) model of evolution with 1000 bootstrap replicates as implemented in the MEGA-X software program. Complete PPV2 coding DNA sequences determined directly from two clinical samples were submitted to GenBank under the accession numbers MW883398 and MW883399.

Virus isolation

Nine clinical samples that were positive by qPCR (Ct range of 16.9-32.8) for PPV2 were filtered through a 0.22μm membrane filter, and 100μL filtrate was used to inoculate porcine alveolar macrophages (PAMs) collected from pigs housed at ADRDL-SDSU for an unrelated study. Four of the nine clinical samples were also positive for PRRSV and/or IAV.

PAMs were seeded into a 24-well plate at 1x 10^6 cells/ well in RPMI with 10% FBS and 1% Penicillin-Streptomycin for 24 hours. Virus isolation was attempted both by adsorption of inoculum followed by washing and refeeding, as well with inoculum left in the cell culture media.

For virus isolation on PAM cells, 100 μL sample filtrate was added to the cells, incubated at 37°C, and supernatants were passed on day 5 post-infection for up to four passes and harvested for analysis by qPCR for PPV2. Cells were harvested on day 5 post-infection for analysis by ISH for PPV2. Alternately, virus isolation was performed with PPV2 inoculum removed from PAMs 24 hours following seeding. 100μL sample filtrate was adsorbed onto cells for 1 hour in a 24-well plate at 37°C. Supernatant was removed and media was replaced to incubate at 37°C for 5 days. Cells were harvested on day 5 post-infection for analysis by ISH for PPV2.

Statistical analysis

Statistical analyses were carried out with the Statistical Analysis System (SAS) program. A *P* value of <0.05 was considered statistically significant. However, a *P* value less than 0.2 was considered a tendency, given the high degree of variability associated

with nature of diagnostic submissions [125]. Pearson's Chi-Square Test was used to determine the frequencies in independent events. Peterson's correlation was applied on qPCR Ct values for PPV2, PRRSV, IAV, PCV2, *Mycoplasma hyopneumoniae*, PPV2 ISH signal count, and age of swine. Multiple linear regression analysis was performed using the load of each pathogen as variables to predict the number of inflammatory cell population in PRDC.

RESULTS

Prevalence of PPV2 and other respiratory pathogens by qPCR

Thirty-nine out of 100 (39%) lung tissue homogenates were qPCR positive for PPV2. Nine (9) of the 124 FFPE sections from porcine respiratory cases were qPCR positive for PPV2, giving a 7% positivity rate. Due to apparent decreased sensitivity of PPV2 detection in FFPE tissues by qPCR, prevalence and statistical analyses were only performed on tissue homogenates.

PPV2 was detected in each of the six age groups with the highest prevalence (63.6%) of cases in the grow-finish pigs (8–25-week) age group (Table 1.1). In addition, there was a negative correlation between the Ct value of PPV2 and the age of pigs ($r = -0.28$, $P<0.05$).

Lung tissue homogenates had a prevalence of 32.9% (27/82), 28.2% (20/71), 22.2% (6/27) and 33.3% (13/39) for PRRSV, IAV, PCV2, and *Mycoplasma hyopneumoniae*, respectively. Among the samples also tested for other pathogens, PPV2 was co-infected in 13.4% (11/82), 12.5% (9/72), 10.7% (3/28), and 22.5% (9/40) cases with PRRSV, IAV, PCV2, and *Mycoplasma*, respectively. Although co-infections were

commonly observed, chi-square analysis and Fisher's exact test found that the infection of PPV2 and other pathogens were independent events. There was no correlation between the Ct values of PPV2 and other pathogens. However, a significant positive correlation between the titers of PCV2 and *Mycoplasma hyopneumoniae* was noted ($r = 0.65$; $P < 0.05$).

Detection of PPV2 and other respiratory pathogens by ISH and IHC in tissue microarrays

Microscopically, varying intensities of PPV2 ISH signals with distributional patterns ranging from a single or few individual cells (Figure 1.1A) to multifocal, extensive patches (Figure 1.1B) to diffuse (Figure 1.1C-E), were detected in 28 of 99 tissue cores, while no signal was detected in other 71 cores on the same TMA slides (Figure 1.1F). PPV2 nucleic acid was hybridized as numerous pinpoints in the cytoplasm of round cells, morphologically compatible with macrophages and some lymphocytes (Figure 1.2A). PPV2 was also detected in pneumonocytes, vascular endothelial cells, and occasionally fibroblast-like cells. Frequently, intensive intracytoplasmic and intranuclear PPV2 ISH signals, diffusely obscuring the entire cellular details, were observed in large round cells, morphologically compatible with alveolar and infiltrating macrophages (Figure 1.1A insert, Figure 1.2 A-C). The distribution of PPV2 ISH signal was consistent with interstitial pneumonia, characterized by macrophages and T lymphocytes expanding the alveolar septa, activated alveolar macrophages and type II pneumonocytes lining the alveolar walls (Figure 1.2A), and hypertrophic vascular endothelial cells lining the vessel capillaries (Figure 1.2B).

Although pneumonia was commonly complicated by neutrophilic inflammation centering in airways (neutrophilic bronchopneumonia) in 36% of tissues cores, no PPV2

nucleic acid was detected in bronchial and bronchial epithelium (Figure 1.2C). However, intensive signals of PPV2 nucleic acid were clustered at cells morphologically resembling immunoblasts, dendritic cells, and macrophages at the germinal centers in peribronchial lymphoid tissues (PBLTs) (Figure 1.1E, 1.2D) and was also detected sporadically in some lymphocytes, macrophages, and dendritic cells (Figure 1.2C) infiltrating and scattering around the inflamed bronchi and bronchioles.

Overall, the number of PPV2 ISH signal counted by image software correlated significantly with the Ct values of PPV2 genome detected by qPCR ($r = -0.64$; $P < 0.001$). All the cases with PPV2 qPCR Ct value < 20 (n =6) showed a diffuse and intensive positive signal of PPV2 in TMA cores (Figure 1.1D and E). Except for 2 cases that had few PPV2 ISH positive signals detected in the germinal centers of PBLTs, no ISH signal was detected in cases with Ct value >30 (Figure 1.1F).

Antigens of PRRSV, IAV, and PCV2 were detected in 13, 1, and 0 tissue cores, respectively, by routine IHC in serial TMA sections. Among them, concurrent positive signals of PPV2 nucleic acid and PRRSV antigen were noted in 3 tissue cores, however, co-localization of PPV2 ISH and PRRSV IHC signals was not obviously observed in serial TMA sections.

The morphological diagnosis of pneumonia and interpretation of PPV2 ISH signal distribution was further supported by phenotyping of immune cells in the lungs. Semi-quantification of cell markers revealed a positive correlation between the number of CD3-positive T cells and IBA 1-positive macrophages ($r=0.36$, $P<0.05$), representing monocytic pneumonia. CD79a-positive lymphocytes were only in small number in each tissue core. The number of macrophages in the lungs correlated with the age of pigs

(r=0.39, P<0.05). Although statistical significance was not reached, cases with lower PPV2 Ct value tended to have higher numbers of macrophages (r=-0.23, P= 0.11) and T cells (r=-0.20, P= 0.17). With respect to the possible impact of PPV2 and other co-infected viruses on pneumonia, a stepwise multiple regression analysis was performed to determine which combination of PPV2, PRRSV, and IAV accounted for the greatest proportion of variance in macrophages. PPV2 was selected as a predictor variable with the highest contribution to the number of macrophages (P = 0.11), while IAV and PRRSV were eliminated.

Metagenomic and phylogenetic analysis

Twenty qPCR positive PPV2 samples that had no PCR history or were PCR negative for PRRSV, PCV2, and IAV, were analyzed by metagenomic sequencing. One qPCR positive PPV2 sample (No. 20804) that was PCR positive for PRRSV (Ct 25.01) and IAV (Ct 35.19) was analyzed by metagenomic sequencing as a positive internal control. Among them, PPV2 was detected in only two samples, both of which had qPCR Ct values of less than 18. Near complete PPV2 genomes 5,215 and 5,208 nucleotides in length were assembled that were >99.7% identical to other PPV2 in GenBank. The remaining 18 samples had Ct values ranging from 21.3 to 33.6, suggesting that the sensitivity of PPV2 detection for our metagenomic sequencing protocol is Ct< 18. For seven of the 20 samples sequenced, no other recognized respiratory viral pathogens were detected.

Consistent with previous studies, PPV2 sequences from our study display a high level of similarity among the PPV2 strains worldwide (Figure 1.3) [22, 28].

Characterization of PPV2 ISH signal in alveolar macrophages

Compared with non-treatment, PPV2 ISH signals were markedly reduced in DNase-treated slide (Figure 1.4). However, strong intra-nuclear and intra-cytoplasmic signals of PPV2 were retained in these cells morphologically resembling alveolar macrophages and occasionally lymphocytes (Figure 1.4). Treatment with both DNase and RNase showed a similar pattern to treatment with DNase alone. There was no obvious difference between RNase-treated and non-treated slides in ISH, which combined with the DNase treatment results suggests that most of the ISH signal is due to probe hybridization with PPV2 ssDNA as opposed to PPV2 mRNA.

Isolation of PPV2

Whether the inoculated PPV2 was removed or retained in culture medium, the virus titer consistently declined, with increasing Ct values following each passage. Strong intracytoplasmic, but not intranuclear, signal of PPV2 was detected in inoculated, unadsorbed alveolar macrophages by ISH for samples with a PPV2 qPCR Ct <21. These samples also showed concurrent infections with IAV or PRRSV.

This suggests that PPV2 does not infect PAM cells *in vitro* under the conditions assayed and that PPV2 detected by qPCR and ISH is due to phagocytosed genetic material.

DISCUSSION

Porcine parvovirus 2 is globally distributed with frequent high prevalence in pigs [21]. Despite occasional detection in clinical specimens, its etiologic significance is unknown [25, 28]. Here, we identified PPV2 as highly prevalent in U.S. pigs with

respiratory disease, with PPV2 ISH staining mainly in alveolar macrophages in lungs with interstitial pneumonia. Interestingly, we found a higher prevalence of PPV2 (39%) than established porcine respiratory viral pathogens, PRRSV (32.9%), IAV (28.2%), and PCV2 (22.2%), in lung tissue from pigs with acute respiratory disease. There was a negative correlation between the Ct value of PPV2 and the age of pigs (r= -0.28, $P<0.05$), with the highest prevalence detected in the grow-finish pigs (63.6%) with respiratory disease. Similarly, other studies noted grow-finish pigs with the highest prevalence of PPV2, suggesting weaned, but not fully mature, pigs are targeted by PPV2 [26, 28, 29]. Furthermore, the PPV2 genome sequences determined here demonstrated high similarity to PPV2 strains from geographically and temporally diverse samples originating back to 2012.

Porcine respiratory disease complex is the result of host, environmental, and pathogen factors that often includes multiple bacterial and viral co-infections, with PCV2, PRRSV, and IAV considered significant [34]. While many studies assess the clinical outcome of coinfection or superinfection, the interactions between microorganisms and their host and underlying mechanisms remain unclear [34]. It is not surprising that we found coinfections with PRRSV, IAV, PCV2, and mycoplasma in many of our cases. We hypothesized multiple coinfections can worsen the clinical outcomes due to a potential synergistic effect on viral replication. In this study, however, PRRSV or IAV titers, as inferred by the PCR Ct value, were not proportionally higher in those cases with higher titers of PPV2. In addition, reviewing the serial TMA sections showed the location of PPV2 signal rarely matched with that of PRRSV or IAV, despite previous work showing that the monocyte/ macrophage lineage is a common target of

PRRSV and PPV2 infection [16, 30, 126]. The lack of correlation of PPV2 and IAV and PRRSV Ct values, as well as the lack of co-localized viral signals by ISH and IHC, suggests natural infection of PPV2 and other respiratory pathogens can be independent events in pig farms.

Porcine parvovirus 1 is a well-known co-factor triggering the development of PCVAD, including postweaning multisystemic wasting syndrome (PWMS) and PRDC [31, 32]. Experimentally in PMWS, PPV1 DNA has been detected in tissue macrophages, including the lung [32, 127]. Similarly, co-infection of PPV2 was frequently detected in pigs with PCVAD in the field [31]. More recently, a retrospective study proposed a statistic model based on veterinary scores and latent class analysis and demonstrated a clustering co-factor association between PPV2 and PCV2, suggesting synergies in pathogenesis [29]. In the present study, however, the prevalence and titer of PCV2 were low based on qPCR. No obvious signal of PCV2 antigen was detected in 99 TMA cores. Different from previous or retrospective studies, our observations reflect that PCVAD is well-controlled, at least in the U.S. where our samples originated, since the development and widespread usage of PCV2 vaccine [128]. On the other hand, high prevalence and titer of PPV2 was detected in our samples, with diffuse and intense PPV2 ISH signals in the lungs (Figure 1.1C-E). Interestingly, these images are like IHC/ISH for PCV2 in PCVAD, in which abundant PCV2 genomes accumulated in the alveolar macrophages, as well as some lymphocytes and endothelial cells [32, 129]. The germinal center of lymphoid follicles are demonstrated early sites of PCV2 infection [122, 130]. Similarly, we observed a strong positive signal of PPV2 in lymphoid tissues in PBLT (Figure 1.1E, 1.2D). Combined with their genetic similarities in terms of both being small, single

stranded DNA viruses with tropisms for rapidly dividing cells, PCV2 and PPV2 may have similar pathogenesis. However, this hypothesis needs to be examined by further research.

Facing the highly complex pathogenesis of PRDC, we used high throughput TMA with image analysis software (semi-quantitative IHC/ISH) and statistics to develop a mathematical model to investigate the significance of PPV2 infection. Both simple correlation and multiple regression analysis showed higher levels of PPV2 ISH signal tended to associate with a higher number of macrophages in the lungs, suggesting a more severe pneumonia. This observation also aligned with the previous and present studies that PPV2 had been mainly detected in macrophages [16]. Traditionally, a *P* value of less than 0.05 was set to reject the null hypothesis. However, as a guidance stated by the American Statistical Association in 2016, a *P* value of less than 0.2 should be considered important in the interpretation of a large complex data set as the present study [125]. The statistic results here were addressed and further supported by pathology and metagenomic assays. Lack of statistical significance in our multiple regression model emphasized that in addition to PPV2, some unidentified factors, such as age, bacterial infection, immune state, and individual variation, had dominant contribution to the development of PRDC.

As described in a previous PCV2 study, the high throughput aspect of TMA has a clear benefit when compared to analyzing individual slides from each tissue [122]. The small cylindrical tissue sections can be a concern for adequate representation, particularly when compared with qPCR on sensitivity. However, PPV2, as well as many other pathogens, are commonly identified in both clinically healthy and diseased pigs [17, 22, 24-26]. Demonstration of a high level of PPV2 in associated lesions is therefore

important for the diagnosis of PPV2-associated pneumonia [31]. In addition, our result showed PCR is not an effective method to detect PPV2 nucleic acid in FFPE samples (data not shown), probably due to the small tissue section or quality of DNA extraction. In this study, all the cases with PPV2 Ct values less than 20 were detected in TMA cores. This observation leads us to believe that TMA could be useful for large scale retrospective and epidemiological studies.

Metagenomic sequencing provided an unbiased screening of pathogens in a small number of samples. We found no significant viral pathogens in over one-third of samples analyzed. However, the sensitivity is an intrinsic limitation of this methodology, as PPV2 was only detected on samples with a low Ct value (<20). Additionally, our metagenomic sequencing workflow is intended to enrich for viral nucleic acids, thus lowering our confidence in bacterial identification. While not demonstrative of causation, the combination of qPCR and metagenomic sequencing identified seven cases where PPV2 alone, or with some bacterial coinfection (per result of routine bacterial culture, data not shown), were present in tissues with marked pneumonia. Together, these results are suggestive for PPV2 having an etiologic role in PRDC.

Given that macrophages are the main cells responsible for the clearance of viruses in the bloodstream, the accumulation of PPV2 nucleic acid in the cytoplasm of alveolar macrophages may be from phagocytosis of viremia or active PPV2 replication. While RNAscope® probes are designed to specifically hybridize target mRNA, heat denaturation combined with the single stranded PPV2 DNA genome may lead to probe hybridization to genomic DNA. To explore these possibilities, we treated slides with DNase to remove PPV2 DNA, intending to detect only PPV2 mRNA signal. The DNase

treatment markedly reduced PPV2 ISH signal, leaving strong signal intra-nuclear and intra-cytoplasmic in alveolar macrophages and occasional lymphocytes, suggesting possible PPV2 replication in PAMs. However, the dramatic decrease of PPV2 signal following DNase treatment, combined with the minimal effect of RNase treatment, suggests that the vast majority of the PPV2 ISH signal is due to probe hybridization with genomic DNA. Other parvovirus studies show parvoviral DNA detection with ISH using the commercially available kit ACD RNAscope® further emphasizing the PPV2 signal detected in our study was mostly detecting PPV2 DNA [131, 132].

In this study, we investigated the natural infection of PPV2 in pigs with PRDC, which is affected by many known or unknown factors in the field. Ideally, experimental inoculation of PPV2 to pigs maintained in well-controlled laboratory condition can be a model to clarify the disease pathogenesis. However, given many pigs infected by PPV2 alone were asymptomatic in the field, it is likely that pigs inoculated with PPV2 alone will develop minimal to mild clinical signs and pathological lesions, similar with the results of previous experimental infection of PPV1 in weaned pigs [26, 127]. Therefore, obtaining high titer of infectious PPV2 stock and understanding the mechanism of PPV2 replication are critical for the reproduction of diseases in animals. Herein, *in vitro* cell culture and virus isolation was attempted. Intense ISH signal for PPV2 in alveolar macrophages suggested that PAM cells may support PPV2 replication. Other swine viruses, notably PPV1 and PRRSV, replicate in PAM cells, which are commonly used for PRRSV virus isolation [126, 133, 134]. Our attempts to isolate PPV2 were unsuccessful; however, the samples with PRRSV or IAV concurrent infections maintained a positive

qPCR value for PPV2. Further investigations regarding the pathogenesis of PPV2 and other co-infections in cell culture are ongoing.

In conclusion, our study confirmed the high prevalence of PPV2 in diseased pigs and provided insight into its significance in PRDC. Detection of intensive and diffuse PPV2 ISH signals interstitial pneumonia (Figure 1.1C-E), correlation between PPV2 load and the number of macrophages, and exclusion of other viral pathogens by metagenomic assay were evidence supporting PPV2 as one of the primary viral pathogens in the natural development of PRDC, particularly in weaned to finishing pigs. Further studies on the role of bacteria with PPV2 and PRDC will be important. Our initial work on viral isolation, as well as similarities of PPV2 to PCV2, call for continued work to isolate PPV2 and further investigate PPV2 in clinical disease with animal studies.

TABLES

Table 1.1 Prevalence of porcine parvovirus 2 (PPV2) detected by qPCR in different age groups

Age Group	Number of Samples (n=100)	PPV2-positive cases
Fetuses (<1 day)	10	1 (10%)
Suckling Pigs (1 day- 3 weeks)	10	2 (20%)
Nursery Pigs (3-8 weeks)	36	12 (33.3%)
Grow-Finish Pigs (8-25 weeks)	11	7 (63.6%)
Mature Pigs (>25 weeks)	4	1 (25%)
Unknown	29	16 (55.2%)

Table 1.2 Metagenomic sequencing of twenty-one samples qPCR positive for PPV2 where (x) indicates sequence detected and (–) indicates sequence not detected.

Sample	18014	19246	18435	25485	25391	22374	23243	21515	22935	22936	23626	20343	951	20804	20738	21755	21763	23207	10160	20388	22593
Porcine Parvovirus 2	-	-	-	-	-	-	-	-	x	-	-	-	-	x	-	-	-	-	-	-	-
Porcine Parvovirus 7	x	-	-	-	-	-	-	-	-	-	-	x	-	x	-	-	-	x	x	-	-
Rotavirus A	-	-	-	x	-	-	-	-	x	-	-	-	-	-	-	-	-	-	-	-	-
Rotavirus B	-	-	-	x	x	-	-	-	-	-	x	-	-	-	-	-	-	-	-	-	-
Rotavirus C	-	-	-	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Porcine Circovirus Type 2	-	-	-	-	-	-	-	-	-	x	-	-	x	x	-	-	-	-	-	-	-
Bastrovirus	-	-	-	-	-	-	-	x	-	-	-	-	-	-	-	-	-	-	-	-	-
Porcine reproductive and respiratory syndrome virus	-	-	-	-	-	-	-	-	-	-	-	-	-	x	-	-	-	-	-	-	-
Bocavirus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	x	-	-	-	-	-	-
Porcine Hemagglutinating Encephalomyelitis Virus	-	-	-	-	-	x	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

FIGURES

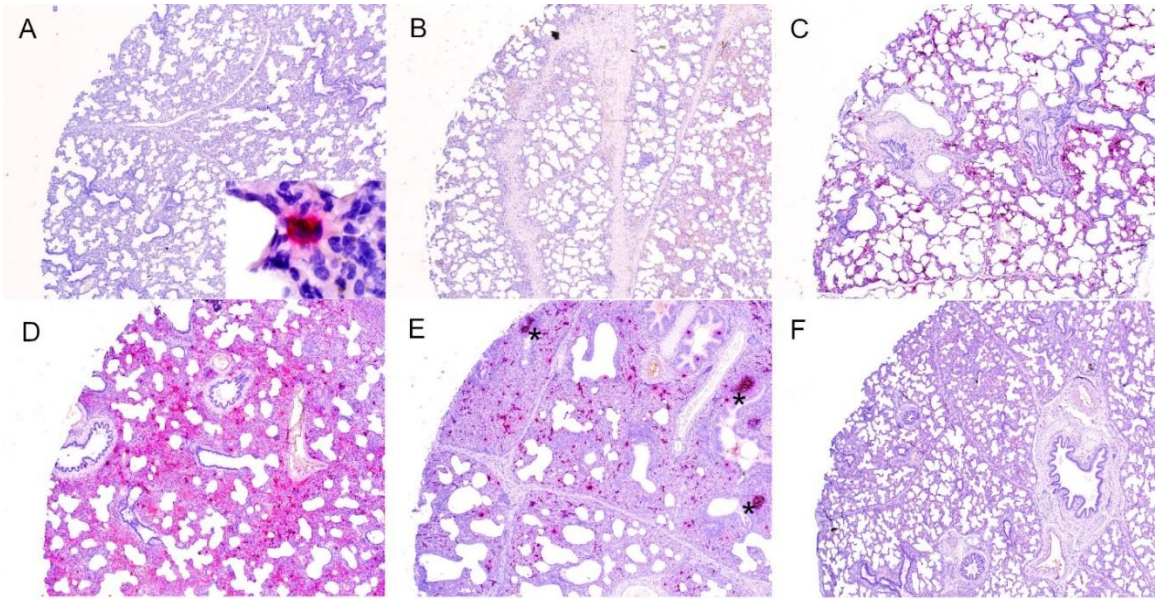


Figure 1.1 Detection of porcine parvovirus 2 (PPV2) nucleic acid in lungs obtained from porcine respiratory disease complex-affected pigs by *in situ* hybridization (ISH), 40x. Signal of PPV2 ISH ranged from a focal single individual cell (A) to multifocal (B, C) to diffuse (D, E). No signal was detected in a tissue core obtained from PPV2 qPCR negative case (F). Note the signals of PPV2 were mainly detected in the pulmonary interstitium and activated lymphoid follicles (*) in peribronchial lymphoid tissues (E).

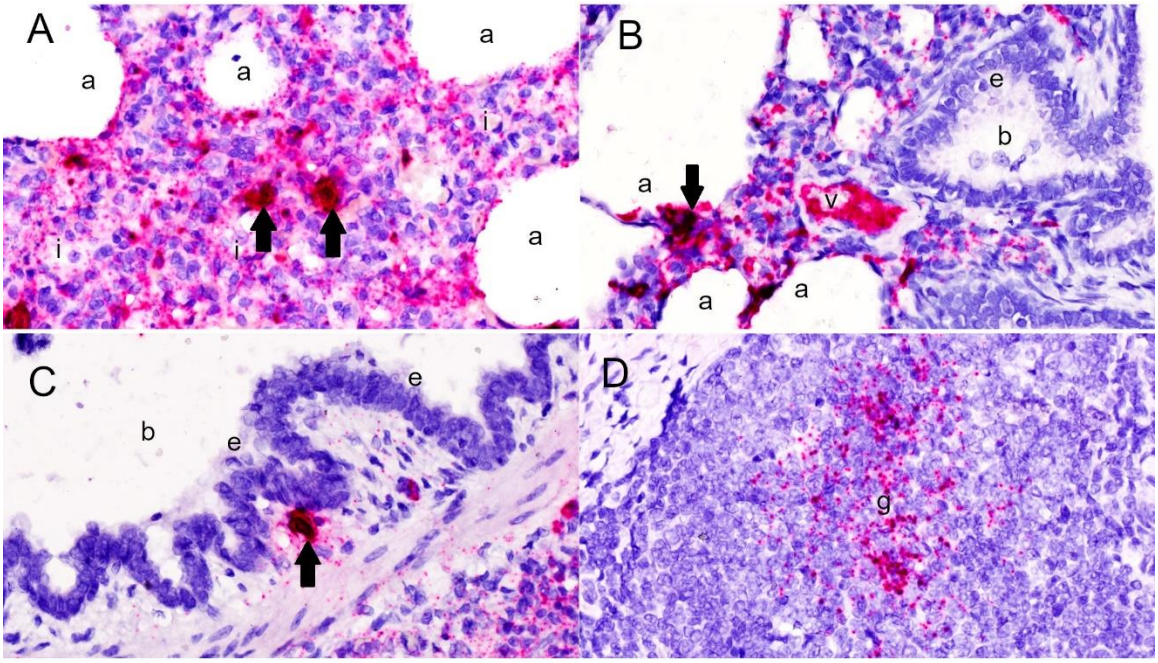


Figure 1.2 Detection of PPV2 nucleic acid in lungs by *in situ* hybridization (ISH), 600x. Signal of PPV2 ISH was detected as pinpoints in the cytoplasm of monocytic cells expanding the alveolar interstitium (i), as well as pneumonocytes and alveolar macrophages lining the alveoli (a) and endothelial cells lining the small blood vessels (v) (**A, B**). Intensive intra-cytoplasmic and intra-nuclear signals of PPV2 were detected in large cells resembling macrophages or dendritic cells (arrows). PPV2 signals were detected in round cells around the bronchus (b) but not bronchial epithelium (e) (**C**). Aggregation of PPV2 ISH signals in a germinal center (g) of the lymphoid follicle in peribronchial lymphoid tissues (**D**).

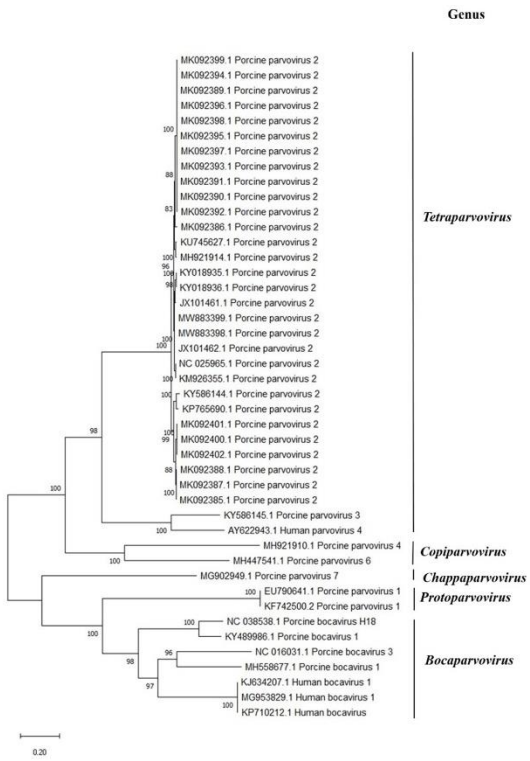


Figure 1.3 Phylogenetic analysis of members of the *Parvovirinae* subfamily in comparison to Porcine parvovirus 2 (PPV2) is shown. The analysis involved 44 nucleotide sequences of vertebrate parvovirus with their GenBank accession numbers marked in the tree. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. There were a total of 6165 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

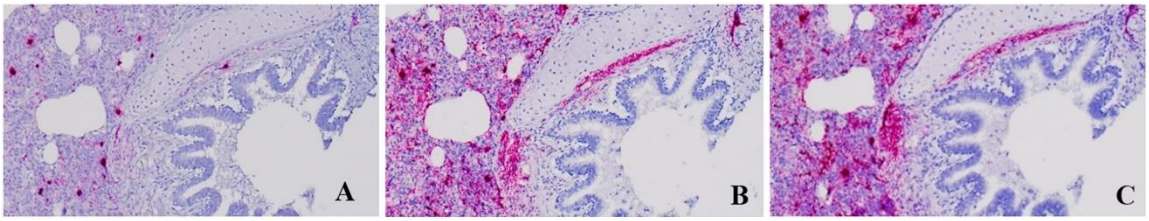


Figure 1.4 Detection of porcine parvovirus 2 (PPV2) by in situ hybridization (ISH), 200 x. PPV2 ISH signal was markedly reduced in formalin-fixed paraffin-embedded (FFPE) section treated with DNase (**A**) compared to the untreated FFPE section (**C**). There was no obvious difference between the untreated FFPE untreated (**C**) and FFPE section treated with RNase (**B**).

CHAPTER 3: IDENTIFICATION OF PULMONARY INFECTIONS WITH PORCINE ROTAVIRUS A IN PIGS WITH RESPIRATORY DISEASE

The work presented in this chapter was published in Frontiers in Veterinary Science.

ABSTRACT

While rotavirus (RV) is primarily known to cause gastroenteritis in many animals, several epidemiological studies have shown concurrent respiratory symptoms with fecal and nasal virus shedding. However, respiratory RV infections have rarely been investigated. By screening clinical samples submitted for diagnostic testing, porcine rotavirus A (RVA) was detected by quantitative reverse transcription PCR (qRT-PCR) in 28 out of 91 (30.8%) lungs obtained from conventionally reared pigs with respiratory signs. Among the positive cases, intensive RVA signals were mainly localized in alveolar macrophages (n = 3) and bronchiolar epithelial cells (n = 1) by RNAscope® in situ hybridization (ISH). The signals of RVA in bronchiolar epithelial cells were verified by ISH with different probes, immunohistochemistry, and transmission electron microscopy. Furthermore, additional cases with RVA ISH-positive signals in alveolar macrophages (n = 9) and bronchial epithelial cells (n = 1) were identified by screening 120 archived formalin-fixed and paraffin embedded lung samples using tissue microarrays. Overall, our study showed a high frequency of RVA detection in lungs from conventional pigs with respiratory disease. Further research is needed to determine if RVA infection in the respiratory epithelium correlates with nasal shedding of rotavirus and its contribution to respiratory disease.

INTRODUCTION

Rotaviruses (RVs) are members of the *Reoviridae* family with a genome comprised of 11 linear double-stranded RNA segments that code for six structural proteins (VP1-4, 6, and 7) and six non-structural proteins (NSP 1-6). The conserved structural protein VP6 is highly immunogenic and is the major diagnostic target for RVs [36]. Rotavirus is a significant global cause of acute enteritis in young children and animals, including neonatal and nursing piglets [38, 135]. While 10 groups of RVs have been described, group A RVs (RVA) are the best studied as they are found in a wide array of species including pigs, humans, cattle, and poultry [39]. It is well known that RVs infect mature enterocytes in the small intestine leading to disruption of the intestinal mucosal barrier and the development of diarrhea [50, 135]. While RV principally replicates in intestinal epithelial cells, several studies have detected RV in serum and multiple visceral organs, suggesting viremia [50-56]. In humans, evolving research shows RV infection has systemic effects, where extraintestinal RV antigenemia has been reported in numerous studies of children with a variety of clinical signs, including respiratory disease [57, 58].

Knowledge regarding respiratory infection of RVs is still limited. Rotaviruses have been detected in nasopharyngeal secretions in infants with respiratory illness, as well as neonatal piglets in experimental settings [51, 56, 57]. In addition to enteritis, piglets orally inoculated with porcine RVA subtypes G9P[23] and G9P[7] also displayed interstitial pneumonia [63]. Similarly, inoculation of pigs with a reassortment bovine RV resulted in enteric disease with RV being detected in the serum, lungs, and other organs by PCR and immunofluorescence assays [64]. In these studies, RV antigen was mainly

detected in enterocytes but also sporadically detected in the pneumocytes and lymphoid cells in the mesenteric lymph node, lungs, liver and choroid plexus. Furthermore, neonatal germ-free piglets that were inoculated either orally or intranasally with the human RVA strain Wa developed diarrhea and viremia and shed virus both nasally and rectally [51].

In our laboratory, routine metagenomic sequencing of diagnostic samples frequently detected RV in the lungs of pigs with respiratory disease. Diagnostic testing of respiratory tissues for RV is rarely performed given RV is not recognized as a primary respiratory pathogen. To date, there is no definitive tissue-based evidence demonstrating that RV can replicate in and damage lung tissues. The aims of the present study were to determine the prevalence of RV in lungs obtained from pigs with respiratory disease, and to characterize RV lung tissue distribution and its possible role in porcine respiratory disease complex (PRDC) pathogenesis.

MATERIALS AND METHODS

Experimental Design

This study used two sampling strategies to assess the prevalence and histopathology of RVA in respiratory tissue. The first was to use frozen tissue homogenates (n = 91) retained from porcine respiratory diagnostic samples submitted to the South Dakota State University Animal Disease Research and Diagnostic Laboratory (SDSU ADRDL) between October 2020 and January 2021. These independent submissions stored at -80°C were tested for RVA by quantitative RT-PCR (qRT-PCR). Case data, including pig age, clinical signs, necropsy reports, and all client-requested

diagnostic testing results were recorded. Pig age groups were determined as fetuses <1-day, suckling pigs (1 day-3 weeks), nursery pigs (3-8 weeks), grow-finish pigs (8-25 weeks), mature pigs (>25 weeks), and unknown. Subsequently, the RVA qRT-PCR-positive cases with formalin-fixed paraffin-embedded (FFPE) lung tissue blocks were selected for histopathology. Tissue distribution of RVA genomes and antigens were detected by *in situ* hybridization (ISH) and immunohistochemistry (IHC), respectively. Transmission electron microscopy (TEM) was applied to visualize the viral particles in respiratory epithelial cells positive for RVA by histopathology.

In the second approach, to rapidly screen a large number of samples, a tissue microarray (TMA) was used for high throughput screening as described previously [136]. Retrospectively, 20 tissue blocks collected from nursing piglets with rotaviral enteritis and high titer of fecal RV shedding, as diagnosed previously in our laboratory, and 100 randomly selected PRDC-affected cases with unknown rotaviral enteritis submitted from 2015 to 2021, were used for TMA construction. Tissue cores with a diameter of 5 mm were extracted from donor lung sample blocks and transferred to a recipient paraffin block by AMSBIO LLC (Cambridge, MA). In total, 6 TMA blocks, each included 20 lung cores, were constructed and sectioned. Forty-three of the 100 randomly selected PRDC-affected cases had corresponding fresh tissues with qPCR results. Three of the 20 tissue blocks collected from nursing piglets with rotaviral enteritis had corresponding fresh tissues. TMA slides were stained by ISH and IHC for the screening of RVs as described above. Fresh lung and intestine tissues from these cases were not available.

Quantitative Reverse-Transcription PCR

Nucleic acids were extracted from homogenized lung tissue with the QIAamp® Viral RNA Mini Spin kit, per manufacturer's instructions. Quantitative RT-PCR was performed following a published protocol [137]. The primers were combined with the sample and incubated for 5 minutes at 95°C to denature dsRNA and immediately placed on ice to allow primer binding to the linear single stranded RNA. Quantitative RT-PCR was performed using Fast Virus 1-step reagents (ThermoFisher) on an Applied Biosystems 7500 FAST Real-Time PCR System with cycling conditions of 30 minutes at 50°C and an initial 30 second hot start at 95°C followed by 45 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Quantitative RT-PCR results for intestine samples were generated by the ADRDL using a proprietary method (Table 2.1).

In Situ Hybridization

In situ hybridization was performed per the manufacturer's instructions using RNAscope® (Advanced Cell Diagnostics). A RVA probe targeting the VP6 gene was obtained from Advanced Cell Diagnostics using a previous design [35]. A second RVA probe targeting the NSP3 gene was designed based on a consensus sequence derived from all swine rotavirus NSP3 sequences in GenBank. Both probes were commercialized (VP6 probe Part ID 447231, NSP3 probe Part ID1125871; Advanced Cell Diagnostics). Intestines obtained from RVA qRT-PCR-positive piglets were used as a positive control for the development, optimization, and verification of ISH and IHC assays. Intestine and lung tested negative by RVA qRT-PCR were used as negative controls.

Immunohistochemistry

The protocol was carried out with the mouse specific HRP/DAB Detection IHC Kit-Micro-polymer (Dako), per the manufacturer's instructions with minor modifications. Antigen retrieval was conducted by heating in a microwave (1,100 W) for 2 min and 10 s in pH 6.0 citrate buffer. Subsequently, the slides were loaded onto a Dako Autostainer-Universal Staining System. Commercially available anti-rotavirus capsid antibody 2B4, a monoclonal IgG_{2B} antibody against RVA VP6 (Santa Cruz Biotechnology, Dallas, TX) was used for IHC detection. Additionally, IHC was performed with the anti-dsRNA antibody, clone J2, a monoclonal IgG_{2AK} antibody that detects dsRNA (Millipore Sigma, Massachusetts, MA). Anti-dsRNA binds to dsRNA 40-bp or longer, independent of their sequence [138].

Transmission Electron Microscopy Examination

FFPE specimens positive by IHC and ISH were submitted to the National Animal Disease Center, USDA-Agricultural Research Service, for transmission electron microscopy. FFPE samples were used for EM. Identified areas of interest were taken from the FFPE block, melted and put in 2.5% glutaraldehyde in 0.1 M cacodylate buffer. They were post-fixed with 2% osmium, and then processed through graded alcohols, propylene oxide and Eponate 12 resin followed by a 48 hr polymerization. Thick sections (1 µm) were performed on select samples and a toluidine blue stain and basic fuchsin stain was applied. Polaroid photos were taken of these images, and the area of interest for thin sections was performed. A uranyl acetate and Reynold's lead stain were performed on the thin section before being examined with a

ThermoFisher FEI Tecnai G^2 BioTWIN electron microscope (FEI Co., Hillsboro, OR), and images were taken with Nanosprint12 camera (AMT Corp., Woburn, MA) [139].

RESULTS

Detection of RVA in the lungs and intestines from pigs with respiratory disease by qRT-PCR

Among the frozen lung homogenates (n = 91), 28 tested positive for RVA by qRT-PCR (Table 2.1). A high prevalence of RVA was detected in suckling (7/15, 46.7%) followed by nursery pigs (8/18, 44.4%) (Table 2.2). The cycle threshold (Ct) values of RVA detected in the lungs ranged from 19.6-31.1. Among cases with RVA-positive lung samples by qRT-PCR, 11 paired intestine diagnostic results were available. Eight out of the 11 paired samples had corresponding positive intestine samples.

Of the 28 out of 91 lung homogenates that tested positive for rotavirus A by PCR, 17 (61%) showed pneumonia by histopathology. Five of the lung homogenates had no significant lesions and one had indications of “other respiratory illness”. Five homogenates did not have histopathology performed. Of the 63 lung homogenates that were PCR negative for rotavirus A, 33 (52%) were diagnosed with pneumonia by histopathology. Eight tissues were recorded as “other respiratory illness” and six had no significant lesions. Histopathology was not performed on 16 of the PCR negative tissues.

Localization of RVA genomes in the lungs using ISH

Available (n = 22) whole tissue sections of RVA qRT-PCR-positive lungs were stained by ISH with a probe targeting the VP6 gene. Hybridization signals were observed in 4 of 22 cases. Intensive signals were detected in the cytoplasm of a few individual

round cells with abundant cytoplasm, morphologically resembling alveolar and interstitial macrophages (Figure 2.1a). Occasionally, few pinpoint signals were also observed in cells with scant cytoplasm lining the alveoli or vascular capillaries, resembling pneumocytes or endothelial cells (Figure 2.1b). The hybridization signals were also detected in cells resembling monocyte-macrophage lineage cells in small peri-bronchial lymphoid aggregates (Figure 2.1c). In one case, there was focal, intensive ISH positive signals observed in a few bronchioles (Figure 2.1d), where approximately 20% of epithelial cells displayed ISH signals as red pinpoints in the cytoplasm or diffusely obscuring the entire cells.

Detection of RVA in the lungs obtained from pigs with rotaviral enteritis or respiratory disease by ISH and TMA

In the first TMA, RVA ISH positive signals were detected in 4 of 20 lung tissue cores obtained from piglets with rotaviral enteritis. Three cases had signals detected in cells resembling alveolar macrophages and pneumonocytes as described above. One case had intensive ISH positive signals in the bronchioles. An additional 6 cases with RVA ISH positive signals detected in alveolar macrophages were noted by screening the second TMA, which included 100 lung samples submitted for PRDC without information of RVA infection in the intestine.

Validation of ISH signals detected in bronchiolar epithelial cells

By using ISH with probes targeting RVA VP6 (Figure 2.1a and 2.2a) and NSP3 (Figure 2.2b), the same pattern of positive signal was detected in the same location in serial sections from the same case. Although fewer and weaker, positive signals were also

detected in the bronchiolar epithelial cells by IHC with mAb against dsRNA and VP6 protein (Figure 2.2 c-d).

Visualization of viral particles in bronchial epithelial cells by transmission electron microscopy

Particles measuring 60-72 nm in diameter compatible with RV, were observed inside rough endoplasmic reticulum vesicles or freely nearby the apical surface of bronchiolar epithelial cells (Figure 2.3).

DISCUSSION

Concurrent enteric and respiratory diseases can lead to significant losses in neonatal piglets. Data from the Swine Disease Reporting System consistently reports rotavirus as a leading pathogen identified in gastrointestinal disease submissions [5]. Through our previous diagnostic testing of PRDC cases, unexpectedly, RVs were frequently detected in lungs using metagenomic sequencing, a diagnostic tool providing a comprehensive and unbiased identification of pathogens. This observation was supported by a high proportion of lung submissions that tested RVA positive by RT-qPCR in the present study. The positive cases are mainly, but not limited to, neonatal and nursing piglets. Given RV is not recognized as a primary respiratory pathogen in pigs, the high frequency of RVA detected in the lungs provided a justification for this investigation.

The pathogenesis of RV includes infection of non-dividing mature enterocytes, leading to cell death and malabsorptive diarrhea [140]. Disruption of the intestinal mucosal barrier allows RV to gain access to the circulatory system as evidenced by observed antigenemia and viremia [50, 51, 141-143]. An earlier study demonstrated

gnotobiotic pigs inoculated intravenously with RVA developed fecal and nasal virus shedding, suggesting RV infection might be initiated from the basolateral side of the epithelial cells via viremia [51]. In the present study, pigs with RVA qRT-PCR positive lungs had minimal or absent enteric signs based on reported clinical histories, although some diarrhea might be neglected clinically. In cases where both lung and intestine diagnostic results were available, RVA was detected in 8 out of 11 intestine samples from cases with RVA-positive lungs (Table 2.1). These results are consistent with a study in which RVA antigen was detected in 16 out of 58 cases (27.6%) of tracheal aspirates of children with a clinical diagnosis of pneumonia, only 2 of which had diarrhea with RV fecal shedding [144]. Similarly, two studies reported RV detection in nasopharyngeal secretions from 28.1% (25 of 89) of infants or 8.9% (4 of 45) of children less than five years of age hospitalized for respiratory illnesses with or without diarrhea [53, 56]. Experimentally, following oral or intranasal inoculation with attenuated human RV, 79% to 95% of gnotobiotic pigs developed nasal shedding of infectious viruses, while only 5% to 17% shed virus rectally [51]. Overall, these results suggest that respiratory and enteric RV infections can be independent events, although they frequently occurred in the same cases.

In the present study, two sample selection strategies were used to investigate the infection of RVs in the lungs. Among 22 RVA qRT-PCR positive cases submitted for PRDC diagnosis with FFPE tissues available, 4 cases were tested ISH positive in whole FFPE lung sections. Likewise, RVA-positive signals were detected in 4 of 20 TMA cores obtained from piglets with high titers of RVA detected in the intestine from archived samples. Regardless of the different criteria and strategies used for case selection, these

approaches resulted in no obvious differences in the detection rate of RVA in respiratory tissues.

In addition to mature enterocytes in the small intestine, previous studies indicated a broad cellular tropism of RV infection [50, 51]. In the present study, signals of the RVA genome and antigen were detected in macrophages and bronchiolar epithelial cells, as well as leukocytes and endothelial cells in vascular capillaries, and cells resembling the pneumonocytes in the lungs. Detection of RV antigens in alveolar macrophages has been described, however, the pathologic relevance is undetermined [54, 55]. Alveolar and interstitial macrophages in the pulmonary reticuloendothelial system function to clear pathogens from the bloodstream [145]. Intensive RVA ISH and IHC signals in the macrophages could result from phagocytosis and accumulation of rotaviral materials filtered through the bloodstream, whereas detection of RV in other cell types suggests infection. In humans, three separate fatal cases classified as pneumonitis and acute interstitial pneumonia showed rotaviral RNA localized in alveolar capillaries, macrophages, and pneumocytes by *in situ* RT-PCR [54, 55]. Likewise, inconspicuous ISH signals of RVA genomes were sporadically noted in a few individual cells lining the alveoli and vascular capillaries in the present study. Weak ISH signals reflect that localization of low levels of extra-intestinal RV infection is limited by the sensitivity of tissue-based assays as compared to qRT-PCR. Resende et al. (2019) also noted some RV PCR-positive samples that were negative by ISH possibly explained by uneven virus distribution throughout tissues or the presence of multiple RV subtype that did not cause lesions [35]. To account for genetic variability, we designed an RVA probe targeting a second RV gene, NSP3, and performed IHC with anti-dsRNA. Tissue sections stained

similarly, regardless of method, thus indicating genetic variability was not the sole cause of decreased sensitivity in ISH and IHC assays as compared to qRT-PCR.

Intensive ISH and IHC RVA signals detected in bronchiolar epithelial cells here are evidence of infection. Visualization of viral-like particles by TEM suggested the virus could complete its replication cycle. To the best of our knowledge, the infection of RV in bronchial epithelial cells has not been reported elsewhere. Rotavirus replication in the respiratory epithelium is consistent with reported nasal RV shedding [51].

An intensive ISH signal of RVA in bronchial epithelium was detected in two cases. Although the specificity of ISH was verified by different probes and supported by IHC and TEM, many questions regarding this finding remain unanswered. Unlike the typical widespread pattern of RVA infection in the small intestines, the hybridization signal of the RVA genome was only observed in a few bronchioles in a small subset of pigs in this study. This observation might be explained by the difference in anatomy and physiology between the digestive and respiratory tracts. The branching architecture of airways can limit the spread of the virus in the lung. Although RV may reach the bronchiolar epithelium by viremia, direct contact of the airway surfaces, through aspiration of RV-contaminated fecal materials or aerosols in the farm, may better explain the focal or multifocal pattern of RVA ISH signals observed under the microscope. Further research on possible aerosol transmission of rotavirus to pigs exposed to high environmental burdens is warranted. In addition, RV antigen was detected in the lung of one of 13 experimentally infected 3-week-old conventional pigs, suggesting individual variation can play a role [51].

The tropism of RVA for the respiratory system is unknown. Azevedo et al. (2005) demonstrated that gnotobiotic piglets inoculated with virulent and attenuated RVA resulted in different nasal and fecal shedding patterns. They hypothesized that lower temperature in the respiratory tract may facilitate attenuated RV infection [51]. In the present study with samples collected from the field, coinfection of pigs with several different RV strains, and multiple bacterial and viral co-infections in the same lung sample, limited our ability to assemble RVA genome sequences directly from lung specimens using metagenomic sequencing assays. Targeted sequencing of rotavirus in enteric and respiratory tissue would be useful for investigation of genetic markers of rotavirus tissue tropism. These sequences would likewise be valuable for the design of diagnostic tests with increased sensitivity. Cell damage and disruption of the respiratory mucosa are thought to promote secondary bacterial infection. Whether RVA infection can provide the initial viral insult that leads to secondary bacterial pneumonia is still unknown. The limited dataset presented here does not provide clear evidence of a correlation between rotavirus and respiratory disease. Further experimental animal studies are merited.

Awareness of extra-intestinal infection and systemic impacts of RV is increasing [50-56, 141, 146-148]. Classically known respiratory or enteric infections frequently cause multisystemic disease [149]. Here we demonstrated a high prevalence of RV detected in the lungs obtained from conventional neonatal and nursing piglets with respiratory signs, which is similar to earlier observations in infants and children [146-148]. These observations were supported by the localization of the RVA genome,

antigen, and viral particles in the bronchial epithelial cells. Further work is needed to elucidate the clinical significance of RV respiratory infections.

TABLES

Table 2.1 Summary of the results of porcine rotavirus A (RVA) qRT-PCR-positive cases.

Sample ID	Age (days)	qRT-PCR (Ct value)		ISH (cell type)	Histopathology (pneumonia)
		Lungs	Intestines		
9265	5	30.3	NEG	NEG	-
10036	NA	24.2	NA	NA	NA
10058	56	25.5	NA	NEG	+
10164	NA	28.6	NEG	NA	NA
10372	35	27.2	24.9	NEG	-
10373	21	23.4	NA	NEG	+
10560	28	22.9	26.2	NEG	+
10566	12	27.5	NA	NEG	+
10615	NA	24.1	35.9	POS (Macrophage)	+
18062	NA	22.8	23.7	POS (Macrophage)	+
18115	7	26.9	NA	POS (Macrophage)	-
18302	NA	27.7	NA	NA	-
19474	28	26.0	22.4	NEG	+
19824	NA	27.6	NA	NA	NA
21026	28	25.8	NA	POS (Bronchiolar epithelium)	+
21515	14	28.7	38.3	NEG	+
21601	NA	26.7	NA	NEG	-
21690	28	24.8	NA	NEG	+
22560	NA	29.7	NA	NA	NA
22935	NA	24.9	28.4	NEG	+
23136	NA	31.1	NA	NEG	+
23243	21	30.2	NA	NEG	-
23473	NA	27.8	NA	NEG	+
25105	28	19.6	22.7	NEG	+
25391	NA	25.0	NEG	NEG	+
25485	NA	21.1	NA	NEG	+
159	3	29.9	NA	NA	NA
951	35	27.6	NA	NEG	+

In total, 28 out of 91 porcine lung samples submitted for routine diagnosis were positive by qRT-PCR. Where available, paired intestine samples were tested. *In situ* hybridization was conducted using probes targeting the VP6 gene. The presence of pneumonia was determined in routine hematoxylin and eosin-stained lung section.

NEG, negative; POS, positive; NA, not available; -, no pneumonia noted; +, pneumonia noted.

Table 2.2 Prevalence of Rotavirus A (RVA) in lung tissue homogenates detected by qRT-PCR in different age groups.

Age Group	Number of Samples (n=91)	RVA-positive cases
Fetuses (<1 day)	9	0 (0%)
Suckling Pigs (1 day- 3 weeks)	15	7 (46.7%)
Nursery Pigs (3-8 weeks)	18	8 (44.4%)
Grow-Finish Pigs (8-25 weeks)	6	0 (0%)
Mature Pigs (>25 weeks)	1	0 (0%)
Unknown	42	13 (30.9%)

FIGURES

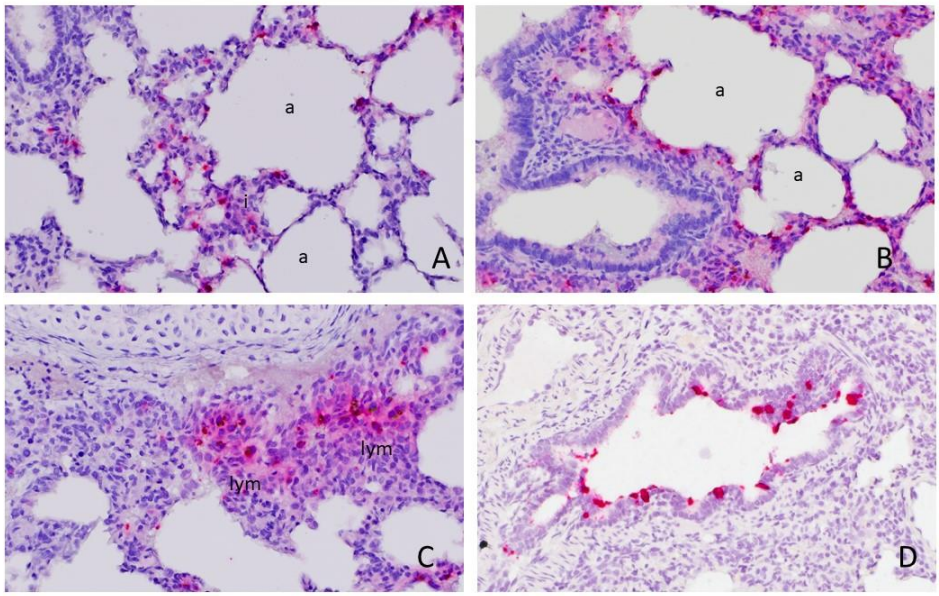


Figure 2.1 Detection of rotavirus A (RVA) nucleic acid in the lung by in situ hybridization (ISH). Positive red signals were detected in round cells resembling macrophages in the interstitium (i) or alveolar macrophages and pneumonocytes lining the alveoli (a) (A and B). Focally, they were also detected in monocyte-macrophage lineage cells in peri-bronchial lymphoid aggregates (lym) (C) and bronchiolar epithelial cells (D). Images were taken under 400x magnification.

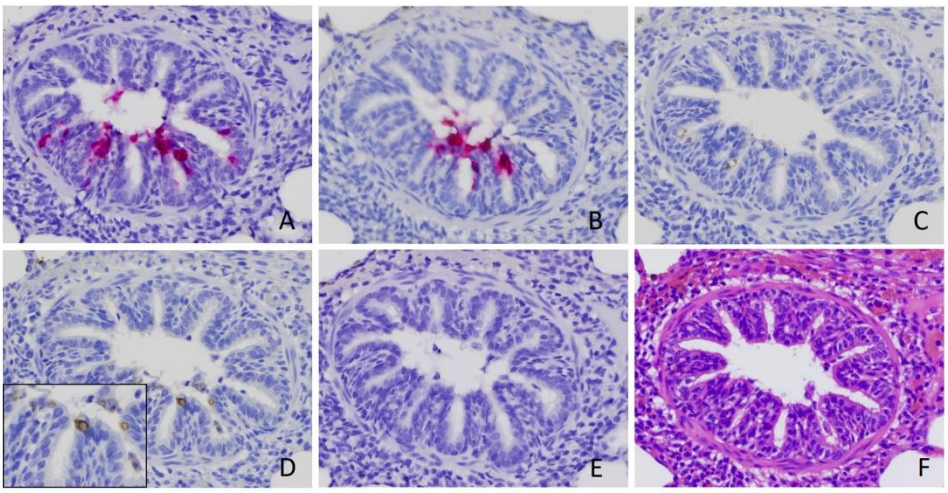


Figure 2. 2 Verification of rotavirus A (RVA) infection in bronchiolar epithelial cells. Positive signal of RVA nucleic acid was detected by in situ hybridization (ISH) using probe targeting VP6 (A) and NSP3 (B). Positive signals of immunohistochemistry staining using antibody against RVA VP6 (C) and dsRNA (D), although weaker, were also detected in the serial sections obtained from the same case. No signal was detected by ISH using a probe targeting rotavirus B (E) (18). Serial section with routine hematoxylin and eosin stain (F) was performed. Images were taken under 400x magnification.

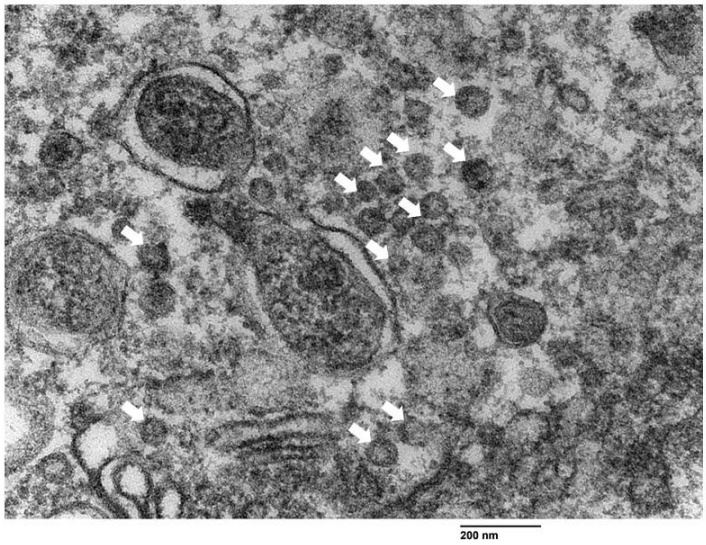


Figure 2.3 Visualization of rotavirus-like particles (VLP) in bronchial epithelial cells using transmission electron microscopy. VLP (arrows) ranging from 60 nm to 72 nm were observed near the apical border of the bronchiolar epithelial cells. Bar = 200 nm.

CHAPTER 4: IDENTIFICATION OF A NOVEL ASTROVIRUS ASSOCIATED WITH BOVINE RESPIRATORY DISEASE

ABSTRACT

Astroviruses (AstVs) cause gastrointestinal disease in mammals and avian species. Emerging evidence suggests that some AstVs have extraintestinal tissue tropism, with AstVs detected in liver, kidney, central nervous system, and the respiratory tract variably associated with disease. In cattle, AstV infection has been linked to gastroenteric or neurologic disease. Here, metagenomic sequencing of a lung from a bovine with respiratory disease identified a novel AstV with a predicted capsid-encoding ORF2 sequence with approximately 70% identity to caprine astrovirus. A quantitative reverse transcription PCR (qRT-PCR) targeting ORF2 was found in four of 49 (8%) lungs and one of 48 (2%) enteric samples obtained from bovine diagnostic submissions positive for bovine astrovirus (BAstV). In two strongly qRT-PCR-positive lung samples, intense BAstV nucleic acid signals were mainly localized in the cytoplasm of alveolar macrophages and mononuclear cells as detected by RNAscope® *in situ* hybridization (ISH). Genetic analysis of two BAstV genomes determined from qRT-PCR positive samples found high similarity for ORF1ab (91.9% and 93.45%) to BAstV, while ORF2 was most similar to caprine astroviruses (66.0% and 77.6%).

Phylogenetic analysis of the novel BAstV sequences found a close genetic relationship to the single BAstV previously identified from a bovine respiratory sample as well as bovine and caprine AstVs identified from various tissues. Further research is needed to determine the clinical significance of BAstV in respiratory disease.

INTRODUCTION

Bovine astrovirus (BAstV) is a member of the *Astroviridae* family, genus *Mamastrovirus*, which includes astroviruses using mammalian hosts [70]. A large number of astrovirus species have been identified and classified based on their host, but do not correspond to genetic phylogenies [150]. The non-enveloped virion contains a positive sense, 6.4-7.7kb, single-stranded RNA [70]. The genome contains three open reading frames (ORFs), ORF1a, ORF1b, and ORF2 [72]. A frameshift between ORF1a and ORF1b allows for expression of ORF1ab that encodes non-structural proteins, including protease and RNA-dependent RNA polymerase [72]. ORF2, at the 3' end of the genome, encodes the capsid protein [72].

Transmission of astroviruses (AstVs) is primarily via the fecal-oral route [90]. While a majority AstVs have been isolated from gastrointestinal samples, AstVs are increasingly being identified in extraintestinal tissues, often associated with clinical disease, including liver, kidney and neurologic disease [76]. Although astroviruses have been detected in respiratory tract samples, no clear association has been made between astroviruses and respiratory diseases. In cattle, a case-control study used metagenomic sequencing to identify viruses associated with bovine respiratory disease (BRD) [78]. Four symptomatic cattle were positive for BAstV, whereas all asymptomatic cattle were negative [78]. The association between BastV detection and clinical disease did not reach statistical significance however due to the small number of positive symptomatic calves.

Other mamastroviruses have also been detected in animals with respiratory disease. One study detected a novel porcine astrovirus 4 (PAstV-4) from young pigs exhibiting unexplained acute respiratory disease. Porcine AstV4 RNA levels were

significantly higher in nasal as opposed to fecal swabs, suggesting a possible respiratory tropism for PAstV-4 [77]. Another study detected a 25% overall prevalence of PAstV, including PAstV1, PAstV2, and PAstV5, in pig lung samples [151].

While performing routine metagenomic sequencing of diagnostic samples in our lab, bovine astrovirus (BAstV) was detected in a bovine lung sample submitted for testing due to respiratory disease. As limited information has been reported on astrovirus association with respiratory disease in cattle, we explored the prevalence, genetics and pathology associated with respiratory BAstV infection.

MATERIALS AND METHODS

Sample Selection

Retained frozen tissue samples submitted for diagnostic testing from December 2020 through December 2021, were randomly selected from bovine submissions that included lung tissue or enteric tissue. Lung tissue (n=49) and enteric tissue (n=48) were homogenized in phosphate buffered saline (PBS) and stored at -80°C.

Quantitative Reverse-Transcription PCR

Nucleic acids were extracted from homogenized tissue with the QIAamp® Viral RNA Mini Spin or MagMax™ Viral/ Pathogen Nucleic Acid Isolation Kit per manufacturer's instructions. Quantitative reverse-transcription PCR (qRT-PCR) was performed per the manufacturer's instructions using Fast Virus 1-step reagents (ThermoFisher). A master mix consisting of TaqMan® Fast Virus 1-step Master Mix, BAstV primers and probe, and Rnase-free water was added to sample and ran on Applied Biosystems 7500 FAST Real-Time PCR System with an initial reverse transcription step

for 5 minutes at 50°C followed by 40 cycles of 20 seconds at 95°C 15 seconds at 95°C and 1 minute at 60°C. Primers (forward primer, CTTATGCAGAACCCTCAG; reverse primer, CAGCCAAGCGTTTTATCACC) and probe (56-FAM/CCACCAACC/ZEN/TCCCTTGAACAACCA/3IABkFQ/) were designed to target ORF2 from the novel BAstV (GenBank Accession ON191568).

Metagenomic Sequencing

Metagenomic sequencing was performed on archived frozen tissue samples that were qRT-PCR positive for BAstV with a cycle threshold (Ct) value less than 25. Metagenomic sequencing was performed as previously described [123, 124].

Bovine Astrovirus Histopathology and *In Situ* Hybridization

BAstV qRT-PCR-positive cases (Ct< 25) with archived formalin-fixed paraffin-embedded (FFPE) tissue blocks were selected for histopathology.

The distribution of BAstV in fixed lung tissue was determined by *in situ* hybridization (ISH). ISH was performed using the commercial RNAscope® system (Advanced Cell Diagnostics) according to the manufacturer's instructions. The counterstain for ISH was Gill's Hematoxylin II. The proprietary BAstV probe (part ID# 1176901-C1) was designed by ACD based on the BAstV ORF2 gene sequence determined here (GenBank Accession ON191568). Manufacturer provided probes were used as controls, along with a BAstV qRT-PCR negative lung slide as a negative comparison control.

Hematoxylin and eosin staining of serial lung sections was performed at South Dakota State University Animal Disease Research and Diagnostic Laboratory (ADRDL) Histology section using the standard ADRDL protocol.

Genetic and Phylogenetic Analysis

To explore the relationship of the BAstV from respiratory tissues to previously determined mamastroviruses, reference astrovirus genomes were downloaded from GenBank. Reference genomes and protein sequences included 59 BAstV, 73 mamastroviruses from other hosts, and 4 avastroviruses (Table 3.1). Phylogenetic analyses were performed on ORF1ab and ORF2 amino acid sequences using the best fitting LG+G+I+F model of evolution with 500 bootstrap replicates as implemented in the MEGA11 software program.

RESULTS AND DISCUSSION

A lung sample from a 7-month-old bovine from South Dakota was submitted for diagnostic testing as case 20-25551. Clinically, the calf exhibited labored breathing and nasal discharge prior to being found dead. Aerobic culture isolated *Mannheimia haemolytica* and *Pasteurella multicida* from both lung and heart valve tissues. All other bovine respiratory pathogen testing, including bovine respiratory syncytial virus (BRSV), bovine coronavirus (BCV), *Mycoplasma bovis*, bovine viral diarrhea virus (BVDV), bovine herpes virus-1 (BHV-1), and influenza D virus (IDV), were negative. Histopathology noted severe subacute diffuse bronchointerstitial pneumonia with septal and pleural edema and inflammation. No cardiac lesions were observed. Metagenomic sequencing identified BAstV with a novel ORF2 gene sequence having only 70% identity

to caprine AstV, prompting us to investigate the prevalence and significance of this AstV in bovine respiratory disease.

Detection of Bovine Astrovirus by qRT-PCR

A low prevalence of the novel BAstV was detected in archived lung (4/49, 8%) and enteric tissues (1/48, 2%). Positive lung samples had Ct values of 18.5, 24.6, 33.0, and 35.3, while the sole positive enteric tissue had a Ct value of 35.9. The lung homogenate from case 20-25551 had a Ct= 18.5, which served as the positive control for qRT-PCR. A previously sequenced lung sample, negative for BAstV, served as the negative control. While prevalence was low in both tissue types, BAstV was more frequently detected in lung tissue, suggesting this strain of BAstV may have a tropism for bovine respiratory tract, however Chi-Square analysis of the qRT-PCR results found that they were not significantly different (P=0.18). Lung and enteric tissues were tested based on availability. Other tissue types were not tested to evaluate tissue tropism is a limitation of this study.

Metagenomic Sequencing, Genetic and Phylogenetic Analysis

The genomes of BAstV were determined by metagenomic sequencing of the two lung tissues with Ct values less than 25. The complete genome of strain 25551 and the near-complete genome of strain 21-24401were submitted to GenBank as accessions ON191568 and ON552247, respectively. Strain 25551 comprised 6,127 nucleotides and had the highest sequence identity (91.9%) to BAstV isolate BSRI-1 (acc.no. KP264970), which was identified by metagenomic sequencing of a nasal swab from a calf with respiratory disease [78]. Strain 21-24401 comprised 5,349 nucleotides and likewise was

most similar to BAstV BSRI-1 with 93.5% identity. No additional viruses were identified. This contrasts with the metagenomic sequencing study of a large calf ranch that identified BAstV strain BSRI-1, where bovine adenovirus 3 (BadV-3), bovine rhinitis A virus (BRAV), bovine rhinitis B virus (BRBV), and influenza D virus (IDV) were also detected and associated with BRD [78]. The ORF1ab amino acid sequences of strains 25551 and 21-24401were 96.5% and 97.5% identical, respectively, with BSRI-1. However, the capsid proteins encoded by ORF2 showed the highest sequence identity, 66.0% and 77.6%, respectively, to caprine astrovirus G2.1 (acc.no. MK404645), which came from an unpublished study of novel astroviruses in small ruminants.

Phylogenetic analyses were performed to interrogate the evolutionary history of BAstV strains 25551 and 21-24401. Sequences for ORF1ab formed nine distinct, well supported lineages which were arbitrarily labeled A-I (Figure 3.1a). A majority of the sequences in lineage A were from bovines with enteric disease or without specified symptoms. Strains 25551 and 21-24401 clustered with lineage B strains which included bovine and caprine strains. Included in lineage B was the respiratory BAstV strain BSRI-1, four caprine AstVs, one water buffalo AstV, and eight additional BAstV, two of which were identified in cattle with neurologic disease, with the remainder from enteric samples or unspecified. Lineage B sequences were most closely related to caprine and water buffalo strains in lineage C. The majority of BAstVs identified in neurologically diseased cattle clustered in clade F, where only one of the 19 BAstV sequences was identified from a bovine with enteric disease. Clade F also contained four ovine AstVs and one muskox; one of the ovine AstV was identified in a neurological case. Clade D AstV mostly originated from pigs though it contained a single bovine strain. The remaining

lineages contained AstV originating from a variety of hosts, including humans, bats, canines and felines.

Like the ORF1ab ORF, phylogenetic analysis of ORF2 found clustering of strains generally related to host and clinical disease. ORF2 is the most divergent open reading frame of AstVs and a common site of recombination [71]. The capsid protein allows for receptor binding and cell infection and consequently is the target of neutralizing antibodies [152]. Capsid mutation, recombination, and cross-species transmission allow virus to escape host humoral immunity [90]. ORF2 sequences identified in this study clustered with Clade F, which also contained six other BAstVs, including strains from respiratory and neurologic tissues, as well as two caprine AstVs, and one water buffalo AstV (Figure 3.1b). An additional nine BAstVs clustered in Clade G. Like the phylogeny based on ORF1ab, ORF2 clade A was mainly comprised of BAstV isolated from cattle neurologic disease. The majority of the remaining BAstV were found in clade H and included sequences identified in animals with enteric symptoms or no identified symptoms. Besides the 23 BAstVs in clade H, close relatives were also identified in pigs (n=4), roe deer (n=2), goats (n=2), and a porcupine, a camel, and a yak. Previous studies noted AstV interspecies transmission, with nearly identical AstV capsid proteins identified in sheep with encephalitis and cattle with neurologic disease [91]. Numerous instances of suspected interspecies transmission were evident in our phylogenetic analysis, where highly homologous clades comprised of multiple strains from a single species included a strain from a different host. Previous analysis of BAstV ORF2 sequences showed some strains clustered with porcine astrovirus type 5 and ovine astrovirus, further corroborating the findings of this study that cross-species transmission

of AstVs may occur which may result in a variety of clinical signs and diverse tissue tropism [102].

Detection and Characterization of BAstV nucleic acid by ISH in Bovine Respiratory Disease cases

In situ hybridization (ISH) for BAstV was performed on fixed tissue from two qRT-PCR-positive lungs. BAstV ISH signal distribution varied from focal (Figure 3.2A) to intensely diffuse (Figure 3.2B). Histopathology on 20-25551 showed severe diffuse bronchointerstitial pneumonia and inflammation. Probe signal was visualized as pinpoints in the cytoplasm of cells that corresponded to alveolar macrophages and other interalveolar inflammatory cells (Figure 3.2C, 3.3C). Diffuse, intense BAstV ISH signal often obscured entire cells (Figure 3.2D, 3.3D). No BAstV ISH signal was detected in bronchial epithelium (Figure 3.2E, 3.3E). Neutrophil infiltration (Figure 3.2F, 3.3F) was devoid of BAstV ISH signal, consistent with bacterial co-infection. Histopathology on case 21-24401 noted alveoli and small airways containing neutrophils or alveoli that contained necrotic cellular debris mixed with inflammatory exudate, consistent with bronchopneumonia. Bacterial infections are secondary opportunists to viral infections or other agents causing primary damage to epithelium in BRD, such as *Mycoplasma*, inhaled ammonia and/ or other gases [153]. We hypothesize that respiratory BAstV infection may serve as a primary viral insult that predisposes cattle for secondary bacterial infection, though further research is needed to confirm this hypothesis. Despite advances in the discovery of novel astroviruses and their identification in diverse tissues, little is known about their pathogenesis [76, 97, 154]. Here, we distinctly detected BAstV nucleic acid in cells corresponding to alveolar macrophages and multinucleated giant

cells and other interalveolar inflammatory cells. Studies using RNAscope® ISH have been conducted to detect viruses that are difficult to isolate or lack diagnostic tools for identification and are informative on viral pathogenesis [35, 120, 121]. However, it is unclear if the ISH signal observed here in alveolar macrophages is due to phagocytosis or active replication. Controlled inoculation with a respiratory BAstV isolate is needed to fully investigate the tissue tropism of BAstV.

ISH was also performed on brain tissue for 21-24401 and heart tissue for 20-25551, however no BAstV ISH signal was detected in either of those tissues (data not shown).

The BAstV in this study had sequence homology with BAstV recovered from BRD cases, as well as caprine astrovirus. These results contribute to the emerging evidence of diverse tissue tropism and cross-species transmission of AstVs. While qRT-PCR was only performed on lung and enteric tissues, ISH was performed on all tissues for the two diagnostic cases that were strongly positive for BAstV and failed to detect signal in brain or cardiac tissue. Further studies on the pathogenesis of BAstV are needed to ascertain its tissue tropism and etiologic significance.

TABLES

Table 3.1 Reference Strain Information from GenBank

TABLE 1 REFERENCE STRAIN INFORMATION FROM GENBANK

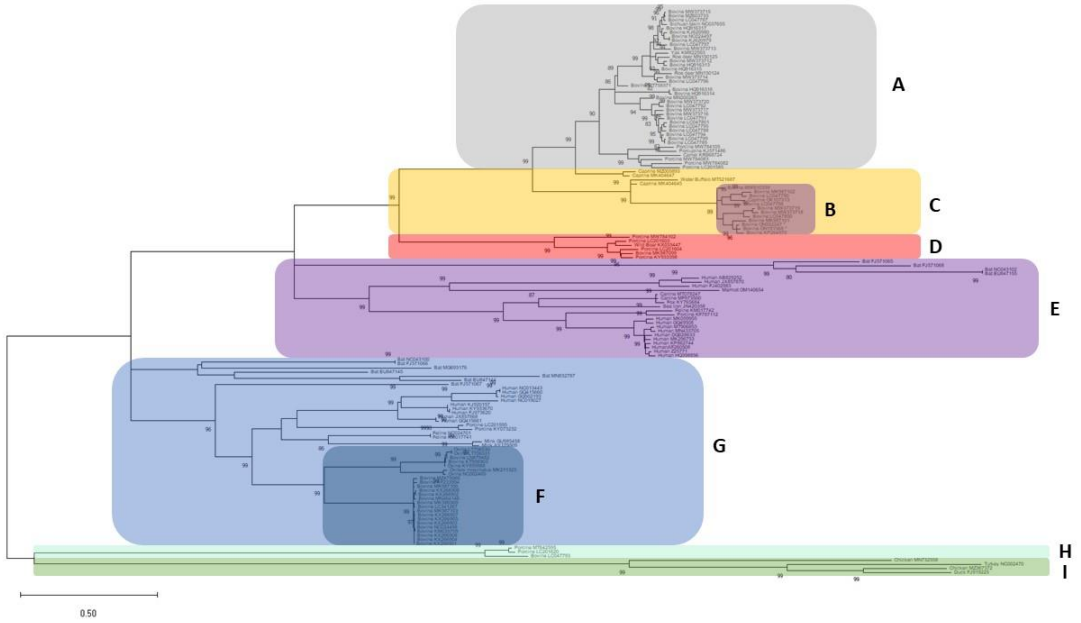
STRAIN	HOST	CLINICAL SIGNS	GENBANK ASCESSION NO.	TIME	LOCATION
BOVINE ASTROVIRUS	Bovine	Respiratory	ON191568	2020	USA
BOVINE ASTROVIRUS	Bovine	Respiratory	ON552247	2021	USA
BOVINE ASTROVIRUS B18/HK	Bovine	None	HQ916313	2011	China
BOVINE ASTROVIRUS B170/HK	Bovine	None	HQ916314	2011	China
BOVINE ASTROVIRUS B34/HK	Bovine	None	HQ916315	2011	China
BOVINE ASTROVIRUS B76/HK	Bovine	None	HQ916316	2011	China
BOVINE ASTROVIRUS B76/HK	Bovine	None	HQ916317	2011	China
BOASTV/JPN/ISHIKAWA24-6/2013	Bovine	None	LC047787	2013	Japan
BOASTV/JPN/ISHIKAWA9728/2013	Bovine	None	LC047788	2013	Japan
BOASTV/JPN/HOKKAIDO11-7/2009	Bovine	Diarrhea	LC047789	2009	Japan
BOASTV/JPN/HOKKAIDO11-55/2009	Bovine	Diarrhea	LC047790	2009	Japan
BOASTV/JPN/HOKKAIDO12-7/2009	Bovine	Diarrhea	LC047791	2009	Japan
BOASTV/JPN/HOKKAIDO12-18/2009	Bovine	Diarrhea	LC047792	2009	Japan
BOASTV/JPN/HOKKAIDO12-25/2009	Bovine	Diarrhea	LC047793	2009	Japan
BOASTV/JPN/HOKKAIDO12-27/2009	Bovine	Diarrhea	LC047794	2009	Japan
BOASTV/JPN/KAGOSHIMA1-2/2014	Bovine	None	LC047795	2014	Japan
BOASTV/JPN/KAGOSHIMA1-2/2014	Bovine	Diarrhea	LC047796	2014	Japan
BOASTV/JPN/KAGOSHIMA2-3-1/2015	Bovine	Diarrhea	LC047797	2015	Japan
BOASTV/JPN/KAGOSHIMA2-3-2/2015	Bovine	Diarrhea	LC047798	2015	Japan
BOASTV/JPN/KAGOSHIMA2-24/2015	Bovine	Diarrhea	LC047799	2015	Japan
BOASTV/JPN/KAGOSHIMA2-38/2015	Bovine	Diarrhea	LC047800	2015	Japan
BOASTV/JPN/KAGOSHIMA2-52/2015	Bovine	Diarrhea	LC047801	2015	Japan
BOASTV/JPN/KAGOSHIMASR28-462/2016	Bovine	Diarrhea	LC341267	2016	Japan
BOVINE ASTROVIRUS ISOLATE NEUROS1	Bovine	Encephalitis	KF233994	2011	USA
BOVINE ASTROVIRUS ISOLATE BSRI-1	Bovine	Respiratory	KP264970	2013	USA
BOVINE ASTROVIRUS	Bovine	Encephalitis	MN464146	2019	Italy
BOASTV/LVMS2704	Bovine	Unknown	MN200263	2016	Uruguay
BOASTV-NEURO-UY	Bovine	Encephalitis	MK386569	2018	Uruguay
BOVINE ASTROVIRUS CH13	Bovine	Encephalitis	KM035759	2012	Switzerland
BOVINE ASTROVIRUS CH13/NEUROS1 ISOLATE 23871	Bovine	Encephalitis	KX266901	2015	Switzerland
BOVINE ASTROVIRUS CH13/NEUROS1 ISOLATE 26730	Bovine	Encephalitis	KX266902	2015	Switzerland
BOVINE ASTROVIRUS CH13/NEUROS1 ISOLATE 26875	Bovine	Encephalitis	KX266903	2015	Switzerland
BOVINE ASTROVIRUS CH13/NEUROS1 ISOLATE 36716	Bovine	Encephalitis	KX266904	2015	Switzerland
BOVINE ASTROVIRUS CH13/NEUROS1 ISOLATE 23985	Bovine	Encephalitis	KX266905	2015	Switzerland
BOVINE ASTROVIRUS CH13/NEUROS1 ISOLATE 42799	Bovine	Encephalitis	KX266906	2015	Switzerland
BOVINE ASTROVIRUS CH13/NEUROS1 ISOLATE 43661	Bovine	Encephalitis	KX266907	2015	Switzerland
BOVINE ASTROVIRUS CH13/NEUROS1 ISOLATE 43660	Bovine	Encephalitis	KX266908	2015	Switzerland
BOVINE ASTROVIRUS ISOLATE CH15	Bovine	Encephalitis	KT956903	2015	Switzerland
BOASTV-VC34/338	Bovine	Encephalitis	MK987099	2016	Switzerland
BOASTV-VC34/346	Bovine	Encephalitis	MK987100	2016	Switzerland
BOASTV-VC34/375	Bovine	Encephalitis	MK987101	2016	Switzerland
BOASTV-VC65/693	Bovine	Encephalitis	MK987102	2016	Switzerland
BOASTV-VC65/698	Bovine	Encephalitis	MK987103	2016	Switzerland
BOVINE ASTROVIRUS BH89/14	Bovine	Encephalitis	LN879482	2014	Germany
BOINE ASTROVIRUS CHN/HEBEI-1/2019	Bovine	None	MW373712	2019	China
BOVINE ASTROVIRUS/CHN/HUNAN-1/2019	Bovine	Diarrhea	MW373713	2019	China
BOVINE ASTROVIRUS/ CHN/HLJ-2/2019	Bovine	Diarrhea	MW373714	2019	China
BOVINE ASTROVIRUS/CHN/HLJ-1/2019	Bovine	Diarrhea	MW373715	2019	China
BOVINE ASTROVIRUS/CHN/SD-1/2019	Bovine	None	MW373716	2019	China
BOVINE ASTROVIRUS/CHN/HUBEI-1/2019	Bovine	Diarrhea	MW373717	2019	China
BOVINE ASTROVIRUS CHN/HENAN-2/2019	Bovine	None	MW373718	2019	China
BOVINE ASTROVIRUS/CHN/HENAN-1/2019	Bovine	None	MW373719	2019	China
BOVINE ASTROVIRUS/CHN/JL-1/2019	Bovine	Diarrhea	MW373720	2019	China
BOVINE ASTROVIRUS STRAIN 51-ASTROVIRIDAE-16	Bovine	Unknown	MW810339	2018	China
BOVINE ASTROVIRUS STRAIN BASTV-GX7/CHN/2014	Bovine	Unknown	NC024297	2014	China
BOVINE ASTROVIRUS CH13	Bovine	Encephalitis	NC024498	2102	Switzerland
BOASTV-GX7/CHN/2014	Bovine	Diarrhea	KJ620979	2104	China
BOASTV-GX27/CHN/2014	Bovine	Diarrhea	KJ620980	2014	China
BOVINE ASTROVIRUS ISOLATE BOASTV/CN/HB-SJZ/2021	Bovine	Diarrhea	MZ603733	2021	China
BOVINE ASTROVIRUS ISOLATE EGY-1	Bovine	Diarrhea	MT758371	2015	Egypt
BOVINE ASTROVIRUS STRAIN 20B05	Bovine	Encephalitis	MZ475060	2020	South Korea
CAPRINE ASTROVIRUS STRAIN SWUN/F2/2019	Caprine	Unknown	OK107513	2020	China
CAPRINE ASTROVIRUS G2.1	Caprine	Unknown	MK404645	2017	Switzerland
CAPRINE ASTROVIRUS G5.1	Caprine	Unknown	MK404647	2017	Switzerland

CAPRINE ASTROVIRUS ISOLATE CHINA/ SWUN/F4/2019	Caprine	Unknown	MZ005893	2019	China
WATER BUFFALO ASTROVIRUS STRAIN BUFASTV/CN/NNA17	Water Buffalo	Diarrhea	MT521687	2019	China
ASTV UK/2014/LAMB	Ovine	Unknown	LT706530	2014	UK
OASTV/UK/2013/EWE/LIB01454	Ovine	Unknown	LT706531	2013	UK
OVINE ASTROVIRUS	Ovine	Unknown	NC002469	Unknown	Unknown
OASTV-CH16 MASTV13	Ovine	Encephalitis	KY859988	2006	Switzerland
POASTV-2/JPN/BU5-10-1/2014	Porcine	None	LC201585	2014	Japan
POASTV-3/JPN/BU2-5/2014	Porcine	None	LC201595	2014	Japan
POASTV-4/JPN/BU5-10-2/2014	Porcine	None	LC201603	2014	Japan
POASTV4/JPN/BUTA17/2014	Porcine	None	LC201604	2014	Japan
POASTV-5/JPN/ISHI-IM1-2/2015	Porcine	None	LC201620	2015	Japan
MAMASTROVIRUS 3 ISOLATE PASTV-GX1	Porcine	Unknown	KF787112	2013	Guangxi
MAMASTROVIRUS 4 ISOLATE K456	Porcine	Unknown	KY933398	2012	Kenya
XINJIANG MAMASTROVIRUS 6 ISOLATE 227-342448	Porcine	Unknown	MW784082	Unknown	China
XINJIANG MAMASTROVIRUS 7 ISOLATE 227-67505	Porcine	Unknown	MW784083	Unknown	China
SICHUAN MAMASTROVIRUS 12 ISOLATE R67-15434	Porcine	Unknown	MW784102	Unknown	China
SICHUAN MAMASTROVIRUS 11 ISOLATE R69-151373	Porcine	Unknown	MW784105	Unknown	China
PORCINE ASTROVIRUS 5 STRAIN PASTV-AH29-2014	Porcine	Swine Fever Symptoms	MT642595	2014	China
PORCINE ASTROVIRUS 3 STRAIN NI-BRAIN/386-2015/HUN	Porcine	Encephalitis	KY073232	2015	Hungry
MAMASTROVIRUS 3 STRAIN WBASTV/CH/2015	Wild boar	Diarrhea	KX033447	2015	China
TAKIN ASTROVIRUS	Sichuan takin	None	NC037655	2013	China
CANINE ASTROVIRUS STRAIN CHN/2017/44	Canine	Diarrhea	MF973500	2017	China
CANINE ASTROVIRUS ISOLATE MN1-USA/ORF/2017	Canine	Unknown	MT078247	2017	USA
FELINE ASTROVIRUS D1	Feline	None	KM017741	2013	USA
MAMASTROVIRUS 2 ISOLATE FASTV-D2	Feline	None	KM017742	2013	USA
FELINE ASTROVIRUS D1 ISOLATE FASTV-D1	Feline	None	NC024701	2013	USA
CALIFORNIA SEA LION ASTROVIRUS	Sea lion	None	JN420358	2010	USA
MOXASTV-CH18 MASTV13	Ovibos moschatus	Unknown	MK211323	1982	Switzerland
PORCUPINE ASTROVIRUS HB/LP084/GUANGXI	Porcupine	Unknown	KJ571486	2011	China
CCASTV/ROE_DEER/SLO/D5-14/2014	Roe deer	None	MN150124	2014	Slovenia
CCASTV/ROE_DEER/SLO/D12-14/2014	Roe deer	None	MN150125	2014	Slovenia
HUMAN ASTROVIRUS VA1/HMO-C	Human	None	KJ920197	2014	UK
MAMASTROVIRUS 1 ISOLATE KOR85	Human	Diarrhea	KP862744	2014	South Korea
MAMASTROVIRUS 8 ISOLATE NI-295	Human	Diarrhea	GQ415660	2007	Nigeria
ASTROVIRUS VA1	Human	None	KY933670	2017	USA
HUMAN ASTROVIRUS TYPE 1	Human	Unknown	Z25771	Unknown	UK
HUMAN ASTROVIRUS 1 STRAIN HU/NYERGESUFJALU/HUN4520/2010/HUN	Human	Diarrhea	HQ398856	2010	Hungary
HUMAN ASTROVIRUS 2 STRAIN HU/US/2014/CA-RGDS-1072	Human	Unknown	MN433705	2014	USA
HUMAN ASTROVIRUS 3 ISOLATE 17W1028	Human	Unknown	MK296753	2018	Ireland
HUMAN ASTROVIRUS 4 STRAIN HU/BRA/TO-207/2014	Human	Diarrhea	MT906853	2014	Brazil
HUMAN ASTROVIRUS 5 ISOLATE GOIANIA/GO/12/94/BRAZIL	Human	Diarrhea	DQ028633	1994	Brazil
HUMAN ASTROVIRUS 6 ISOLATE 192-BJ07-CHN	Human	Diarrhea	GQ495608	2007	China
HUMAN ASTROVIRUS 7 STRAIN OXFORD	Human	Unknown	MK059955	Unknown	USA
HUMAN ASTROVIRUS TYPE 8	Human	Unknown	AF260508	Unknown	Mexico
ASTROVIRUS VA1	Human	Diarrhea	FJ973620	2008	USA
ASTROVIRUS VA2 ISOLATE VA2/HUMAN/STL/WD0680/2009	Human	Diarrhea	GQ502193	2009	USA
ASTROVIRUS VA4 ISOLATE VA4/HUMAN/NEPAL/S5363	Human	Diarrhea	NC019027	2008	Nepal
ASTROVIRUS MLB1 STRAIN WD0016	Human	Diarrhea	FJ402983	2008	USA
ASTROVIRUS MLB2	Human	Diarrhea	AB829252	2005	Turkey
ASTROVIRUS VA3 ISOLATE VA3/HUMAN/VELLORE/28054/2005	Human	Diarrhea	JX857868	2005	India
ASTROVIRUS MLB3 ISOLATE MLB3/HUMAN/VELLORE/26564/2004	Human	Diarrhea	JX857870	2004	India
HMO ASTROVIRUS A	Human	Diarrhea	NC013443	2007	Nigeria
HMO ASTROVIRUS B ISOLATE NI-196	Human	Diarrhea	GQ415661	2007	Nigeria
DROMEDARY ASTROVIRUS	Camelus	Unknown	KR868724	2013	United Arab Emirates
BAT-ASV/P02	Bat	None	MG693176	2013	Cameroon
MAMASTROVIRUS 14 ISOLATE AFCD57	Bat	None	EU847144	2005	Hong Kong
MAMASTROVIRUS 16 ISOLATE AFCD11	Bat	None	EU847145	2005	Hong Kong
MAMASTROVIRUS 18 ISOLATE AFCD337	Bat	None	EU847155	2006	Hong Kong
BAT ASTROVIRUS TM/GUANGXI/LD77/2007	Bat	None	NC043100	2007	Guangxi
MAMASTROVIRUS 18 ISOLATE AFCD337	Bat	None	NC043102	2006	Hong Kong
BAT ASTROVIRUS TM/GUANGXI/LD38/2007	Bat	None	FJ571065	2007	Guangxi

BAT ASTROVIRUS TM/GUANGXI/LD77/2007	Bat	None	FJ571066	2007	Guangxi
BAT ASTROVIRUS TM/GUANGXI/LD71/2007	Bat	None	FJ571067	2007	Guangxi
BAT ASTROVIRUS HA/GUANGXI/LS11/2007	Bat	None	FJ571068	2007	Guangxi
BAT ASTROVIRUS ISOLATE BTASTB/13585-58/M.DAU/DK/2014	Bat	None	MN832787	2014	Denmark
MINK ASTROVIRUS	Mink	None	AY179509	2002	Unknown
MINK ASTROVIRUS ISOLATE SMS-ASTV	Mink	Shaking mink syndrome	GU985458	2000	Sweeden
YAK ASTROVIRUS ISOLATE 58	Yak	Diarrhea	KM822593	2013	China
MAMASTROVIRUS 5 STRAIN CRAB-EATING_FOX/2016/BRA	Cerdocyon thous	Central nervous system disease	KY765684	2015	Brazil
MARMOT ASTROVIRUS STRAIN MCASV/XJ4/CHN/2016	Marmot	Unknown	OM140654	2016	China
DUASTV-1_DA06_CHN	Duck	Unknown	FJ919225	2016	China
TURKEY ASTROVIRUS	Turkey	Unknown	NC002470	1997	USA
CHICKEN ASTROVIRUS ISOLATE CAV/BELGIUM/4134_001/2019	Gallus gallus	Respiratory	MZ367372	2019	Belgium
AVIAN NEPHRITIS VIRUS STRAIN ANV/CHN/BJCP10-2/2018	Gallus gallus	Unknown	MN732558	2018	China

FIGURES

a.



b.

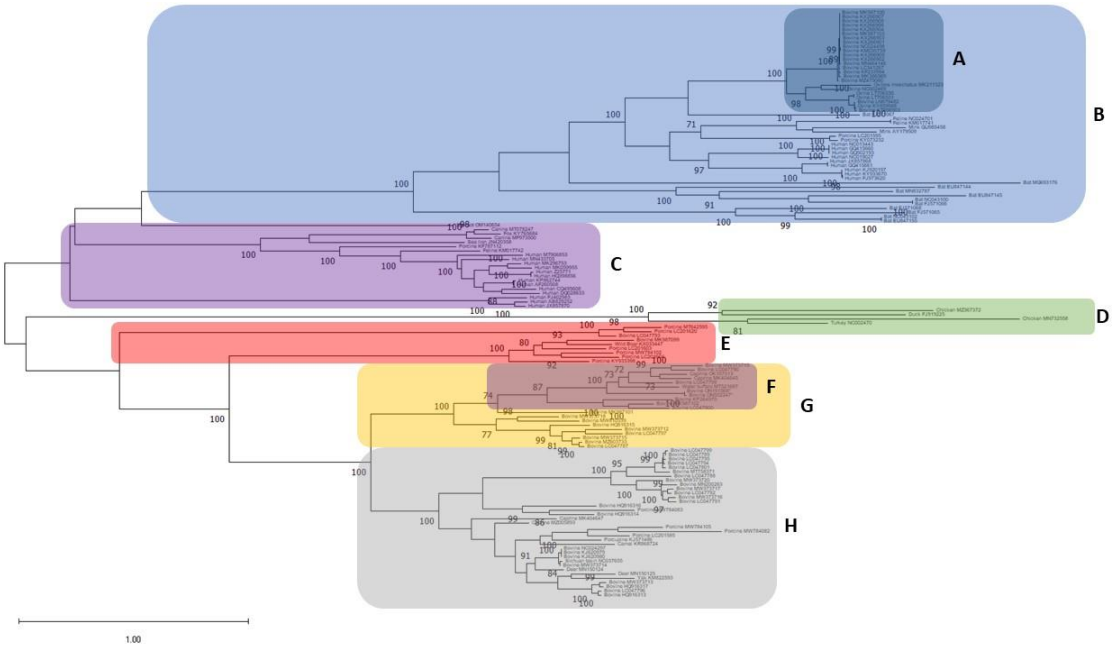


Figure 3. 1 Phylogenetic analysis of astrovirus. Phylogenetic trees were constructed by maximum likelihood analysis with 500 bootstrap replicated in MEGA11 software. Bootstrap values are shown as values above the branches. GenBank accession numbers are indicated with the species AstV was isolated from. The scale bar indicated 0.5 substitutions per site for Figure 1a and 1.0 substitutions per site for Figure 1b. ORF1ab clades are arbitrarily labeled A-I with clade B contains BAstV sequences identified in this study highlighted in yellow (a). ORF2 clades are arbitrarily labeled A-H with clade F contains BAstV sequences identified in this study highlighted in yellow (b).

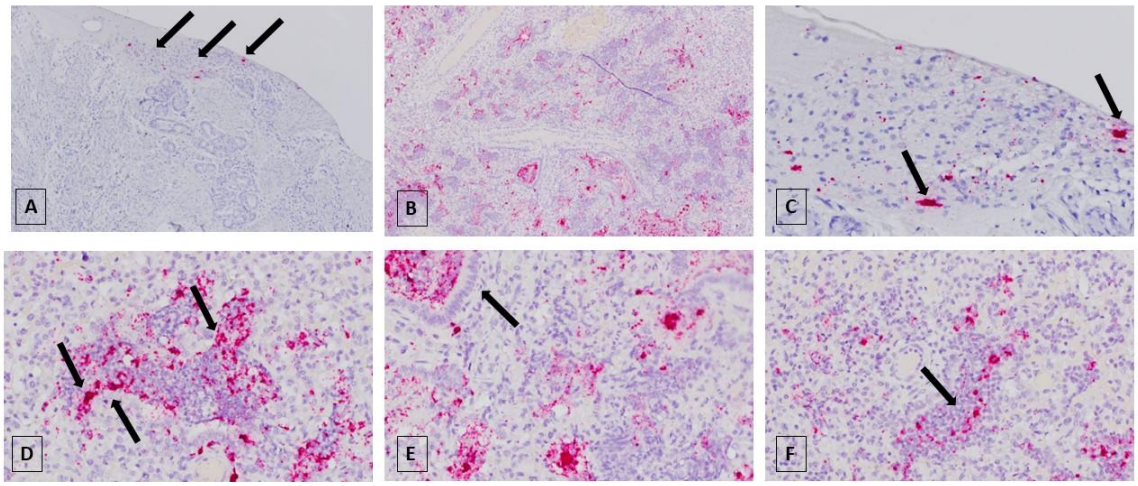


Figure 3.2 Detection of bovine astrovirus (BAstV) nucleic acid in lungs from cattle with respiratory disease by in situ hybridization (ISH). Signal of BAstV ISH ranged from a few patches, indicated by arrows (**A**) to intensely diffuse (**B**), 100X. Pinpoint signals were detected in the cytoplasm of cells that corresponded to large alveolar macrophages, as well as mononuclear cells (**C**), 400X. Diffuse, intense BAstV ISH signal obscured entire cells (**D**), 400X. No signal was detected in bronchial epithelium (**E**) or neutrophils (**F**), 400X.

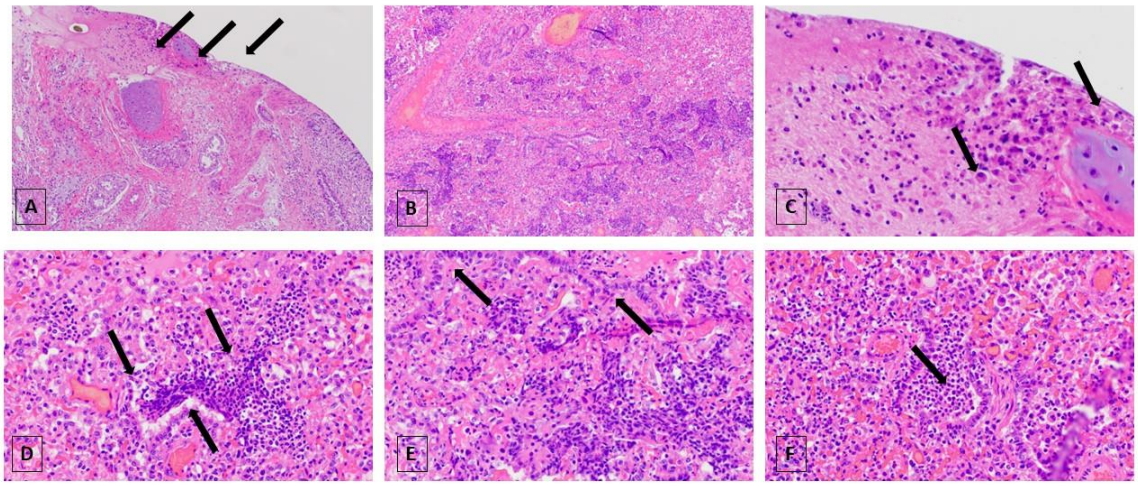


Figure 3.3 Hematoxylin and eosin staining of serial lung sections used for ISH analysis. Arrows indicate areas where BAstV ISH signal was identified in patches (**A**), 100X. Serial H&E-stained section with intense diffuse ISH BAstV signal (**B**), 100X. Large alveolar macrophages and mononuclear cells are indicated by the arrows (**C, D**), 400X. Bronchiolar epithelial cells (**E**) and neutrophils (**F**), 400X.

CHAPTER 5: GENERAL DISCUSSION

The economic impact of respiratory tract infections is substantial, regardless of the species. Costs associated with supportive care, productivity loss, and treatment costs the economy billions of dollars each year. One way to help prevent these losses is through the rapid identification and diagnosis of these respiratory pathogens. To this end, metagenomics and next-generation sequencing can provide valuable tools to improve well-being of humans and animals.

Given the global economy, pathogens previously affecting one part of the world now have gained access to other countries. This has resulted in increased potential for zoonotic, intra-, and cross-species transmission and emergence of pathogen variants. The potential for zoonotic, intra-, and cross-species transmission occurs through reassortment, recombination, and environmental factors. The interaction of farm animals and wild animals promotes intra-species, and potentially, cross-species exposure. The ability of viruses to be stable for long periods of time in the environment allows for easier terrestrial and aquatic transmission amongst species [87-90]. All these factors play a role in how pathogens evolve and evade one host to infect another host. Even though astroviruses, rotaviruses, and parvoviruses all have different genomes and replication strategies, they all have the potential for mutation events that lead to alternative host infection.

ALTERNATE HOSTS

Rotavirus (RV) has a segmented genome. Reassortment is a form of genetic exchange that may occur in segmented viruses when multiple viruses co-infect the same

cell, replicating their progeny segments in the same cytoplasm. RVs were historically believed to be host specific. Growing evidence suggests otherwise. Animal reservoirs of zoonotic RVs include porcine, bovine, ovine, pteropine, rodent, avian, and insectivore species [38, 155-159]. To date, a total of 10 G genotypes (G1-5, G9-12, and G26) and 7 P genotypes (P[4], P[6], P[8], P[13], P[14], P[19], and P[25]) RVAs of porcine origin have been identified in humans [38]. G9 and G12 VP7 specificities often observed in piglets have emerged globally in human RVs, likely because of gene reassortment [160-163]. There are numerous reports of human disease with RVs from different animal species origin [164-168].

While there are many RVs infecting a multitude of hosts, most studies that have been done are on RVA and are in animal models, in particular pigs. In one study, neonatal germ-free piglets inoculated either orally or internally with human RVA strain Wa shed virus both nasally and rectally and developed diarrhoea and viremia [51]. Another study where pigs were inoculated with reassortment bovine RV resulted in detection RV in serum, lungs, and other organs by PCR and immunofluorescence assays and developed enteric disease [64]. While reassortment of RVs can result in zoonotic and cross-species transmission, we also see alternative tissue tropism and disease. Adding to the detection of RVA in lung tissue of diseased pigs in our studies, another study of piglets orally inoculated with porcine RVA subtypes G9P [23] and G9P [7] displayed interstitial pneumonia alongside enteritis [63]. This leads to a change in tissue tropism and viruses that are pneumoenteric: respiratory disease and gastrointestinal disease caused by the same pathogen.

Parvoviruses make use of their target cell to generate these changes. Because parvoviruses need the host cell to be in S-phase during replication, they have a high mutation and recombination rates [15]. PPV2, a single-stranded DNA virus, is an emerging respiratory viral pathogen and does not currently have information on mutation and/or recombination rates. Recombination is an exchange of genetic material between different genomes by crossing over of chromosomes or artificially joining of segments of DNA, which leads to offspring having different combinations of traits from the parents. A genetic mutation is a change in the genomic sequence, often found in RNA viruses due to their lack of proofreading ability. While large double-stranded (ds-DNA) viruses show low mutation rates, some small ssDNA viruses appear to have substitution rates close to RNA viruses potentially due to superinfections or co-infections [169].

Astrovirus, a single-stranded (ss-RNA) virus, like most other RNA viruses lacks proofreading capabilities and is thus error prone leading to genetic mutations. Recombination events due to coinfection of multiple circulating lineages within the same host can also contribute to viral diversity, evolution, and emergence of novel AstVs [90]. The most predominant recombination site is at the OFR1b/ORF2 junction [90]. ORF2 is the most variable region of the genome and encodes the capsid protein that is responsible for viral entry [90]. A mutation or recombination at the ORF1b/ ORF2 location could alter the virus's ability to enter host cells thus providing the evidence for the emergence of diverse tissue tropism and intra- and cross-species transmission of AstVs. The evidence provided by the bovine astrovirus (BAstV) study emphasizes AstV cross-species transmission as well as alternate tissue tropism. Our study showed BAstV ORF2 to be most like a caprine astrovirus. The novel BAstV was identified in the lungs of

respiratory diseased animals, in contrast to BAstV historically being a gastrointestinal pathogen. By use of metagenomic sequencing, this novel BAstV was identified. *In situ* hybridization (ISH) aided in identifying the proposed tissue tropism for this BAstV by detecting nucleic acid signal in the lungs of the diseased bovine. Additional studies identified AstV in sheep with encephalitis that was almost genetically identical to AstV in neurologically diseased cattle, and AstVs in farmed guinea fowl with 84.6-100% ORF2 amino acid similarity to turkey astrovirus type 2 [90-92]. While the use of metagenomic sequencing has been useful in the identification of novel and/ or variant viruses, the phylogenetic analysis of AstVs will be key in defining the classification of AstVs. Based on the information from this study and the results of the phylogenetic analysis, it would appear that classification system should be based on host reservoir rather than the tissue in which the virus was detected.

TISSUE TROPISM AND DISEASE

As described above for RV, mutations and variants may also lead to alternative tissue tropism of viruses. This appears to be highly relevant to astrovirus, as well as rotaviruses. PPV2 is a novel virus, thus more studies on pathogenicity will need to be conducted. However, some parvoviruses have been detected in tissue other than the lungs, including feces, serum, and fetus [170, 171].

Strong evidence suggests a diverse tissue tropism for AstVs. AstVs have been detected in multiple animal tissues with varying disease states, including encephalitis, gastroenteritis, and respiratory distress. What is interesting is the emerging evidence of numerous known enteric pathogens causing respiratory symptoms, and vice versa. Pathogens with rising suspicion of both enteric and respiratory disease include influenza

virus, human immunodeficiency virus, and coronaviruses [149]. Interestingly, respiratory associated pathogens and enteric pathogens remain the most vital for swine and bovine research and disease monitoring [2, 5-7]. Genetic mutations and recombination have been discussed as a possible explanation for alternative tissue tropism and hosts. However, how and why animals of the same species react differently to pathogens is not well understood. A continuation of the presented studies to assess animal microbiome, gene expression, and coinfections with addressed pathogens will be useful in understanding the host-pathogen interactions and why one animal is diseased as opposed to another animal to appropriately diagnose and treat cases. The use of metagenomic sequencing proves useful for early detection in all specimen types and to trace lineage of when and where mutations or recombination occurred.

Following the detection of a novel or variant virus, assessment of infection by histological examination is necessary to confirm presence and infectivity of the pathogen. The historic method (Koch's postulate) of establishing a relationship between the host and pathogen requires four criteria: 1) pathogen found in diseased, but not healthy individuals; 2) pathogen must be cultured from the diseased individual; 3) inoculation of healthy individual with cultured pathogen must recapitulate disease; and 4) pathogen must be reisolated from the inoculated, diseased individual and match the original pathogen. Many pathogens are difficult to isolate, including the 3 viruses described here. To localize those viruses in infected cells, RNAscope® *in situ* hybridization (ISH) becomes an ideal method. RNAscope® is a method to visualize pathogen specific nucleic acids in formalin-fixed paraffin-embedded (FFPE) tissues. This method is timely and sensitive when compared to viral isolation followed by animal studies. Specifically

infected cells are pinpointed and are a strong indicator of viral pathogenicity. The use of tissue microarrays (TMAs) further increases testing volume, thus showing ISH with a TMA to be a timely method for identification of pathogen nucleic acids. ISH partnered with metagenomic sequencing will improve the understanding and direction of studies for many previously unculturable pathogens as well as emerging pathogens.

FUTURE DIRECTIONS

Early discovery of pathogens through metagenomic sequencing leads to better control of highly pathogenic organisms and the ultimate development of vaccines. Continuation of the work presented in this dissertation includes additional pathogenesis and immunologic studies with animals. Studies may include inoculating healthy animals with tissue samples from infected animals and recovery of pathogen in suspected infected cells or vaccinomic trials on healthy animals and assessment of immunological response (i.e., antibodies and/ or cytokines). Continuous surveillance of both healthy and sick animals by use of metagenomic sequencing is a prophylactic method for prevention and control of pathogens.

Animal studies following the discovery of a novel pathogen involves the previously discussed Koch's postulates. Novel pathogens are found in diseased animals through metagenomic sequencing. The novel pathogen then needs to be cultured from the diseased animal. It is not always possible to isolate a virus so the alternative to this approach would be to extract virus from known infected tissue. The tissue is confirmed to be infected by metagenomic sequencing or by histological staining or ISH. Tissue extract or isolated virus can then be given to a healthy animal. Animals ideally would show similar signs and symptoms to the diseased animal. Depending on those signs and

symptoms, for example respiratory distress, tissue can be removed and tested diagnostically. The pathogen would need to be confirmed through sequencing or isolation once again to correlate the pathogen with disease.

Vaccinomics, also known as personalized vaccines, is a new approach to vaccine development that tailors to an individual genetic profile. This approach has many paths. A pathogen may be discovered through metagenomic sequencing, but not isolated, and a vaccine may be developed. Pathogen evolution for immune escape, coinfection, and prior exposure to infection, may be examined following vaccinomic trials. The pathogens discussed in this dissertation infect a wide range of hosts. Knowledge of virus interactions with the genetic profile of a multitude of hosts could minimize zoonotic, intra-, and cross-species transmission. As mentioned, the science of vaccinomics is new and evolving, and it that can be applied to preventative vaccines against viral infections through animal studies with novel viruses.

In combination of routine surveillance of diseased animals with metagenomic sequencing and identification of pathogen nucleic acids in affected tissues by *in vitro* hybridization (ISH), emerging viral pathogens can be identified and placed in affected tissues. While many pathogens, including viral pathogens, are difficult to culture and isolate, the methods used in this dissertation aid in the discovery and pathogenesis of pathogens, in particular viral respiratory pathogens. In addition, alternative studies and future technology will continue to expand the field of emerging pathogens and epidemiology, ultimately leading to prophylactic prevention.

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APPENDIX 1.

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ORIGINAL RESEARCH
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Porcine Parvovirus 2 Is Predominantly Associated With Macrophages in Porcine Respiratory Disease Complex

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Porcine respiratory disease complex (PRDC) is a significant source of morbidity and mortality, manifested by pneumonia of multiple etiologies, where a variety of pathogens and environment and management practices play a role in the disease. Porcine reproductive and respiratory syndrome virus (PRRSV), influenza A virus (IAV), and porcine circovirus 2 (PCV2) are well-established pathogens in PRDC. Porcine parvovirus 2 (PPV2) has been identified in both healthy and clinically diseased pigs at a high prevalence worldwide. Despite widespread circulation, the significance of PPV2 infection in PRDC and its association with other co-infections are unclear. Here, PPV2 was detected in the lung tissue in 39 of 100 (39%) PRDC-affected pigs by quantitative polymerase chain reaction (qPCR). Using *in situ* hybridization (ISH) in conjunction with tissue microarrays (TMA), PPV2 infection was localized in alveolar macrophages and other cells in the lungs with interstitial pneumonia in 28 of 99 (28.2%) samples. Viral load tended to correlate with the number of macrophages in the lungs. Assessment of the frequency, viral titers, and tissue distributions showed no association between infection of PPV2 and other major viral respiratory pathogens. In one-third of the PPV2-positive samples by qPCR, no other known viruses were identified by metagenomic sequencing. The genome sequences of PPV2 were 99.7% identical to the reference genomes. Although intensive intranuclear and intracytoplasmic signals of PPV2 were mainly detected in alveolar macrophages by ISH, no obvious virus replication was noted in *in vitro* cell culture. Together, these results suggest that PPV2 is associated, but may not be the sole causative agent, with PRDC, warranting the control and prevention of this underdiagnosed virus.

Keywords: porcine parvovirus, porcine respiratory disease complex, tissue microarray, metagenomic sequencing, *in situ* hybridization

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INTRODUCTION

Porcine parvovirus 2 (PPV2), formally *Ungulate tetraparvovirus 3*, belongs to the *Tetraparvovirus* genus, Parvoviridae family (1). PPV2 is a small, non-enveloped, icosahedral virus consisting of an ~5–6 kb linear, single-stranded DNA (ssDNA) genome (1, 2). The prototype of porcine parvovirus, porcine parvovirus 1 (PPV1), is a well-known pathogen that causes reproductive

APPENDIX 2.

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Identification of Pulmonary Infections With Porcine Rotavirus A in Pigs With Respiratory Disease

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While rotavirus (RV) is primarily known to cause gastroenteritis in many animals, several epidemiological studies have shown concurrent respiratory symptoms with fecal and nasal virus shedding. However, respiratory RV infections have rarely been investigated. By screening clinical samples submitted for diagnostic testing, porcine rotavirus A (RVA) was detected by quantitative reverse transcription PCR (qRT-PCR) in 28 out of 91 (30.8%) lungs obtained from conventionally reared pigs with respiratory signs. Among the positive cases, intensive RVA signals were mainly localized in alveolar macrophages ($n = 3$) and bronchiolar epithelial cells ($n = 1$) by RNAscope® *in situ* hybridization (ISH). The signals of RVA in bronchiolar epithelial cells were verified by ISH with different probes, immunohistochemistry, and transmission electron microscopy. Furthermore, additional cases with RVA ISH-positive signals in alveolar macrophages ($n = 9$) and bronchial epithelial cells ($n = 1$) were identified by screening 120 archived formalin-fixed and paraffin-embedded lung samples using tissue microarrays. Overall, our study showed a high frequency of RVA detection in lungs from conventional pigs with respiratory disease. Further research is needed to determine if RVA infection in the respiratory epithelium correlates with nasal shedding of rotavirus and its contribution to respiratory disease.

Keywords: rotavirus A, porcine respiratory disease complex, interstitial pneumonia, enteritis, extraintestinal rotavirus

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INTRODUCTION

Rotaviruses (RVs) are members of the *Reoviridae* family with a genome comprised of 11 linear double-stranded RNA segments that code for six structural proteins (VP1-4, 6, and 7) and six non-structural proteins (NSP 1–6). The conserved structural protein VP6 is highly immunogenic and is the major diagnostic target for RVs (1). Rotavirus is a significant global cause of acute enteritis in young children and animals, including neonatal and nursing piglets (2, 3). While 10 groups of RVs have been described, group A RVs (RVA) are the best studied as they are found in a wide array of species including pigs, humans, cattle, and poultry (4). It is well known that RVs infect mature enterocytes in the small intestine leading to disruption of the intestinal mucosal barrier and the development of diarrhea (2, 5). While RV principally replicates in intestinal epithelial cells, several studies have detected RV in serum and multiple visceral organs, suggesting viremia (5–11). In humans, evolving research shows RV infection has systemic effects, where extraintestinal RV antigenemia has been reported in numerous studies of children with a variety of clinical signs, including respiratory disease (12, 13).