

SEROLOGICAL CHARACTERIZATION OF GENOTYPICALLY DISTINCT ENTERIC  
AND RESPIRATORY BOVINE CORONAVIRUSES

by

ALEXA UKENA

B.A., Kansas State University 2013

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Diagnostic Medicine and Pathobiology  
College of Veterinary Medicine

KANSAS STATE UNIVERSITY  
Manhattan, Kansas

2015

Approved by:

Major Professor  
Richard Hesse

## **Abstract**

Bovine Coronavirus (BCoV) is known to cause enteric and respiratory diseases, such as calf diarrhea, winter dysentery, calf respiratory disease, and bovine respiratory disease complex (BRD). All of these diseases are believed to be caused by the same genotype of BCoV. BCoV exhibits tissue tropism for both the gastrointestinal and respiratory tracts. This tropism is due to 9-O-acetylated sialic acid receptor on both epithelial cells in the respiratory and enteric tract. Currently, the only vaccine available for BCoV targets the enteric form of the disease. This study addresses the hypothesis that antibodies from the enteric form of the disease can cross neutralize the respiratory form of the virus. Data from surveillance studies suggest that BCoV is one of the major contributors to BRD, for which there is no currently approved vaccine for the respiratory form of the disease.

Our approach to answering this question is to sequence and analyze the complete genome of 11 respiratory and enteric coronavirus isolates using next generation sequencing (NGS). Following the NGS, viruses were selected based on phylogenetic analysis and ability to grow and be maintained in cell culture. These viruses were then be used as serum neutralization indicator viruses in SN assays. 147 bovine serums submitted to KSVDL were used to determine if there are any serological differences between the immune response to respiratory versus enteric viruses based on the antibodies produced by the animal.

The overall results show that there are few differences between the enteric and respiratory isolates at the genomic level and the serological response from the animal to these viruses. The differences between enteric and respiratory virus will need to be further addressed and analyzed to conclude if there is a noteworthy difference between the viruses with different tropisms. Other factors, such as host immune response and environment, are believed to be involved in the virus tropism to certain areas of the body.

## Table of Contents

List of Figures .....	v
List of Tables .....	vi
Acknowledgements.....	vii
Chapter 1 - Coronavirus Literature Review.....	1
Introduction.....	1
History.....	1
Family Coronaviridae .....	2
Bovine Coronavirus .....	7
Genomic Organization .....	7
Viral Proteins .....	8
Nonstructural Proteins .....	8
Hemagglutinin Esterase .....	8
Spike .....	9
Envelope and Membrane .....	9
Nucleocapsid.....	9
Replication .....	10
Transmission and Pathogenesis .....	12
Host Immune Response .....	12
Diseases.....	13
Detection and Quantification .....	14
Control and Prevention .....	15
Vaccine .....	15
Purpose of Research.....	16
Chapter 2 - Materials and Methods.....	16
Cells .....	16
Viruses used for SN Testing .....	17
Infectivity Titers.....	17
Sequencing.....	17

Serological Characterization Based on Different Viruses .....	18
Chapter 3 - Results.....	19
Phylogenetics .....	19
Virus Selection.....	25
Virus Specific Seroprevelence.....	25
Chapter 4 - Discussion and Conclusion.....	29
Sequencing.....	29
Assays .....	29
Future work.....	30
Bibliography .....	31

## List of Figures

Figure 1-1 Coronavirus Whole Genome Phylogenetic Tree.....	4
Figure 1-2 Bovine Coronavirus Genome Organization.....	8
Figure 1-3 Viral Replication.....	11
Figure 3-1 Bovine Coronavirus Whole Genome Phylogenetic Tree.....	23
Figure 3-2 Bovine Coronavirus Spike Phylogenetic Tree.....	23
Figure 3-3 Bovine Coronavirus Hemagglutinin Esterase Phylogenetic Tree.....	24
Figure 3-4 Individual Serum Sample for Serum Surveillance SN assays.....	26

## List of Tables

Table 2-1 Field Serum Categories .....	19
Table 3-1 Sequencing Table .....	20
Table 3-2 Spike Amino Acid Sequence.....	21
Table 3-3 HE Amino Acid Sequence.....	22
Table 3-4 Breakdown of Significant Difference in Serum Surveillance Assays.....	28

## **Acknowledgements**

I would like to thank my major professor Dr. Richard Hesse for his wisdom, expertise, and motivation. Thank you to my committee members, Dr. Jerome Nietfeld and Dr. Gregg Hanzlicek for their valuable input and time. Thank you to Drew Suddith, Joe Anderson, Barbara Meadors, and Heather Sloan for your support and patience. Thank you to Dr. Erin Schirtzinger for her valuable help in sequencing. Also, thank you for the major support of Dr. Anderson and the Kansas State Veterinary Diagnostic Lab.

# Chapter 1 - Coronavirus Literature Review

## Introduction

Coronaviruses comprise a diverse group of large, enveloped, positive sense single stranded RNA viruses that belong to the family Coronaviridae. Coronaviruses are known to infect a wide variety of animals that range all the way from whales down to mice. They mainly cause enteric and respiratory disease in mammals and birds (Hurst et al., 2013) but have also caused hepatic and neurological diseases as well.(Cheng et al., 2007) While coronaviruses have a relatively low mortality rate, they can cause huge economic loss by a decrease in production standard of livestock and an increase in healthcare costs. (Martínez et al., 2012) A significant outcome of the research being done is the production of a vaccine for preventing bovine coronavirus enteric infections in calves. There has been some research that supports the use of the enteric vaccine in feedlot animals to minimize respiratory disease.(Plummer et al., 2004) However, very little is known regarding the tissue tropism of bovine coronavirus and the cross protection of antibodies to the two varieties of viruses.

## History

The first coronavirus to be identified was infectious bronchitis virus (IBV) that was found in chickens in 1932 and is now classified in the gamma-coronavirus genera. (Hudson and Beaudette, 1932) This was followed by the identification of mouse hepatitis virus (MHV) in 1940 (Cheever et al., 1949) followed by transmissible gastroenteritis virus (TGEV) in pigs. (DOYLE and HUTCHINGS, 1946) In the 1960s human coronavirus 229E (HCoV 229E) and human coronavirus OC43 (HCoV-OC43) were identified in people with common cold symptoms. (Tyrrell and Bynoe, 1966), (Hamre and Procknow, 1966) In 1975, bovine coronavirus was identified as a major contributor to calf scours and was isolated and cultured to be used as a vaccine. (1975) By 2003, porcine hemagglutinating encephalomyelitis virus (PHEV), porcine epidemic diarrhea virus (PEDV), porcine respiratory coronavirus (PRCV), and feline coronavirus (FCoV) had been sequenced and their complete genomes were being studied. (Woo et al., 2009)



Within the last few years, coronaviruses have been gaining more attention due to the media coverage of the emerging human epidemics known as severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS). The SARS outbreak in 2002, was the first coronavirus to cause severe disease in humans and gain the media's attention. Investigators believe that the source came from human exposure to exotic wild-game animals in live markets. (Hilgenfeld and Peiris, 2013) The repeated exposure led to interspecies transmission of the coronavirus and then to the human outbreak itself. After the SARS epidemic, research on coronaviruses increased and more complete genomes of different species were added. In 2012, another novel coronavirus was emerging as many elderly and immunocompromised patients in the Middle East were reporting respiratory illness. (Zaki et al., 2012) While viruses detected in bats are closely related to MERS, investigators believe that the transmitter of the virus was the dromedary camel.(Hilgenfeld and Peiris, 2013)

Today research strives to understand more about the mechanisms of replication with an emphasis on the explanation of the molecular factors of virulence and tropism and how the virus interacts with the host's immune response. There is also research going to improvement of vaccine approaches and antiviral therapies for animal and human viruses. Lastly, research is looking ahead to the potential of isolation and characterization of new coronaviruses (Weiss and Navas-Martin, 2005).

## **Family Coronaviridae**

Coronaviruses are classified by the International Committee on Taxonomy of Viruses (ICTV) as part of the order *Nadovirales* and family *Coronaviridae*. The family *Coronaviridae* includes some of the larger RNA viruses with genomes ranging from 26.2 to 31.7kb and sizes ranging from 80 to 160 nm.(Belouzard et al., 2012) (Perlman and Netland, 2009) The circular viral envelope is studded with spikes giving coronaviruses their characteristic crown appearance. (Fields, et al., 2001) Coronaviruses are classified in four different genera based on serological and phylogenetic analysis: alpha-, beta-, gamma-, and delta-coronavirus. (Belouzard et al., 2012) Figure 1.1 shows the phylogenetic tree of these different genera. The diversity of these groups comes from the large genome of coronaviruses, random template switching during RNA replication, and the high error rate of the RNA-dependent RNA polymerase.

Alphacoronavirus genus is broken into two subgroups, 1a and 1b, based on phylogenetic clustering. While viruses in group 1a demonstrate genetic similarities to each other, group 1b is more diverse and are simply grouped as “non-group 1a”. Group 1a includes TGEV, PRCV, FCoV, canine enteric CoV, ferret CoV, and rabbit CoV. Group 1b includes HCoV-229E, HCoV-NL63, PEDV, BtCoV/512/2005, BtCoV-HKU2, BtCoV-HKU8, BtCoV1A, and BtCoV1B.(Woo et al., 2009) A unique characteristic of alphacoronaviruses is that they have two papain-like proteases in their genome which are used to cleave proteins.(Woo et al., 2009)

Betacoronavirus genera is the largest genera of coronaviruses and has subgroups 2a, 2b, 2c, and 2d. Bovine coronavirus is part of the 2a subgroup (Enjuanes et al., 2006) along with human coronavirus OC43(HCoV-OC43), equine coronavirus (ECoV), porcine hemagglutinating encephalomyelitis virus (PHEV), mouse hepatitis virus (MHV), human coronavirus HKU1 (HCoV-HKU1), canine respiratory CoV and rat CoV. (Decaro et al., 2008) (Bidokhti et al., 2013) The subgroup 2a is unique because the genomes contain a hemagglutinin esterase protein that aids the virus in attachment. Also like subgroup 1, 2a has two papain-like proteins for cleaving proteins(Woo et al., 2009). Subgroup 2b includes the human SARS-CoV and bat SARS CoV. The third subgroup, 2c, includes the MERS-CoV, Bt-CoV-HKU4, and Bt-CoV-HKU5. The last subgroup, 2d, only has one member and that is Bt-CoV-HKU9.4.(Graham et al., 2013) Betacoronaviruses are notorious for homologous and heterologous recombination events within the group. This has led to the generation of various genotypes and strains within the coronavirus species.(Woo et al., 2009)(Lai et al., 1985)

For 50 years the only species in Gammacoronavirus was IBV but within the last 10 years other avian and mammal viruses have been added: turkey-CoV, duck-CoV, geese-CoV, pigeon-CoV, pheasant-CoV, and beluga whale coronavirus (SW1) which has the largest genome (32kb).

Recently, a novel genus, Deltacoronavirus, has been added and three coronaviruses moved from Gammacoronavirus into this genus: Bulbul coronavirus (BuCoV-HKU11), Thrush coronavirus (ThCoV- HKU12) and Munia coronavirus (MuCoV-HKU13) which have the smallest coronavirus genome (around 27 kb). (Woo et al., 2009) (Woo et al., 2012)

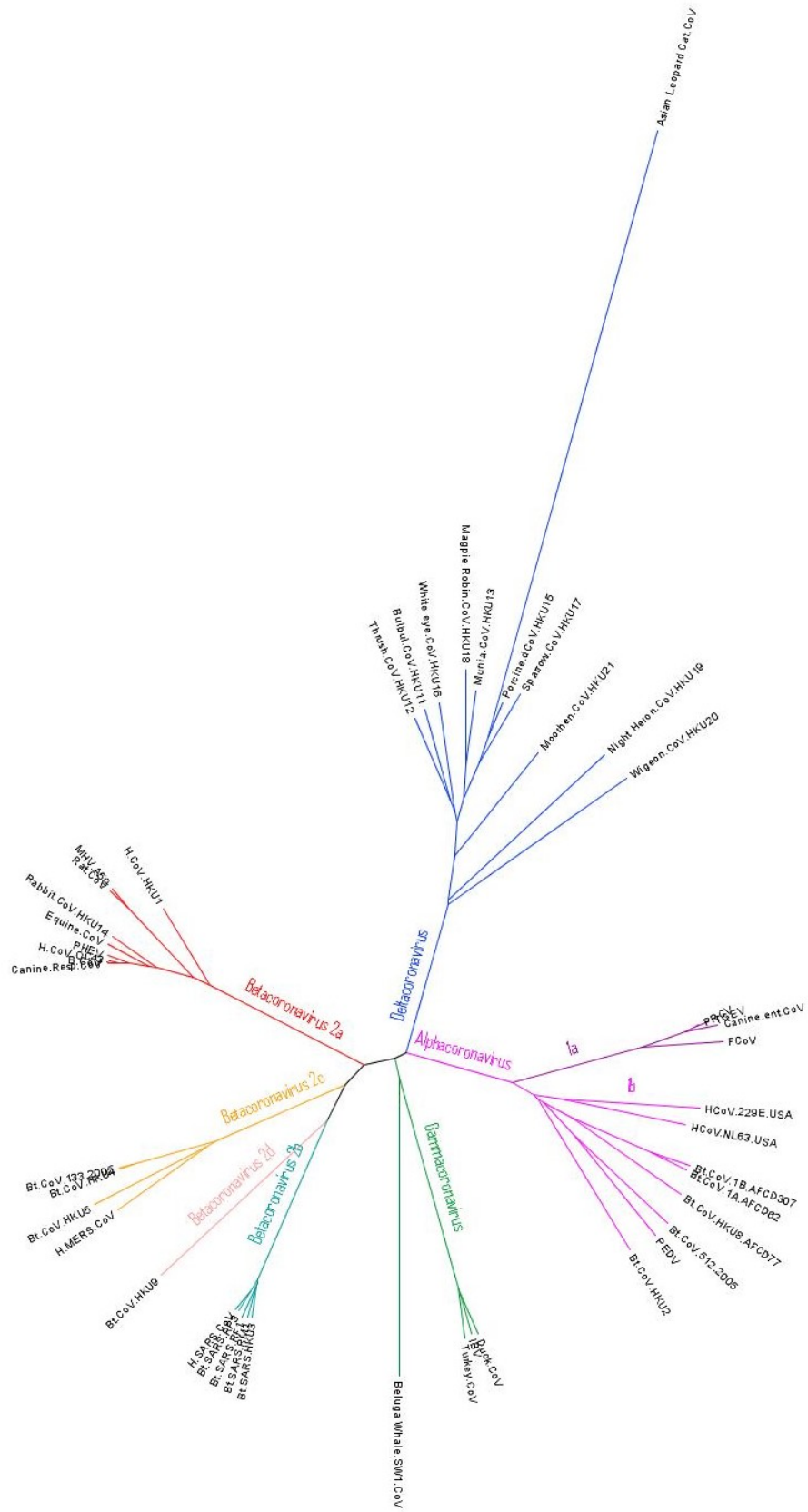
Coronaviruses newly added to this genus include the night heron-CoV, magpie robin-CoV, common moorhen-CoV, sparrow-CoV, wigeon-CoV, and white eye-CoV. They also have mammals in this genera that include porcine-CoV and asian leopard cat-CoV.(Woo et al., 2012)

### Figure 1-1 Coronavirus Whole Genome Phylogenetic Tree

The full genomic sequences of 46 coronaviruses were aligned and phylogenetically compared, based on the table below. Four distinct phylogenetic groups are shown: alphacoronavirus, betacoronavirus, gammacoronavirus, and deltacoronavirus. Subgroups for alphacoronavirus are indicated by 1a and 1b. Subgroups for Betacoronavirus are indicated by 2a, 2b, 2c, and 2d. This tree was generated using maximum likelihood in CLC Main workbench.

Genera	Abbreviation	Name
<b>Alphacoronavirus 1a</b>	FCoV	Feline coronavirus
	TGEV	Transmissible gastroenteritis virus
	PRCV	Porcine respiratory coronavirus
	Canine.ent.CoV	Enteric canine coronavirus
<b>Alphacoronavirus 1b</b>	Bt.CoV.HKU2	Bat coronavirus HKU2
	H.CoV.NL63	Human coronavirus NL63
	H.CoV.229E	Human coronavirus 229E
	PED	Porcine epidemic diarrhea virus
	Bt.CoV.512.2005	Bat coronavirus 512
	Bt.CoV.HKU8.AFCD77	Bat coronavirus HKU8
	Bt.CoV.1A.AFCD62	Bat coronavirus 1A.AFCD62
	Bt.CoV.1B.AFCD307	Bat coronavirus 1B.AFCD307
	<b>Betacoronavirus 2a</b>	H.CoV.HKU1
MHV		Mouse hepatitis virus
PHEV		Porcine hemagglutinating encephalomyelitis virus
H.CoV.OC43		Human coronavirus OC43
BCoV		Bovine coronavirus
Rat.Cov		Rat coronavirus
Equine.CoV		Equine coronavirus
Canine.resp.CoV		Respiratory canine coronavirus
Rabbit.CoV		Rabbit coronavirus
<b>Betacoroanvirus 2b</b>	H.SARS.CoV	Human SARS coronavirus
	Bt.SARS.CoV.HKU3	Bat SARS coronavirus HKU3
	Bt.SARS.CoV.RP3	Bat SARS coronavirus RP3

<b>Betacoronavirus 2c</b>	Bt.SARS.CoV.RF1	Bat SARS coronavirus RF1
	Bt.SARS.CoV.RM1	Bat SARS coronavirus RM1
	Bt.CoV.HKU5	Bat coronavirus HKU15
	Bt.CoV.133.2005	Bat coronavirus 133 2005
	Bt.CoV.HKU4	Bat coronavirus HKU4
<b>Betacoronavirus 2d</b>	H.MERS.CoV	Human MERS coronavirus
	Bt.CoV.HKU9	Bat coronavirus HKU9
<b>Gammacoronavirus</b>	IBV	Avian infectious bronchitis virus
	Turkey.CoV	Turkey coronavirus
	Duck.CoV	Duck coronavirus
	SW1.CoV	Beluga whale coronavirus
<b>Deltacoronavirus</b>	Bulbul.CoV	Bulbul coronavirus
	Thrush.CoV	Thrush coronavirus
	Munia.CoV	Munia coronavirus
	AsianLepordCat.CoV	Asian lepard cat coronavirus
	NightHeron.CoV	Night heron coronavirus
	Magpie.CoV	Magpie coronavirus
	Moorhen.CoV	Moorhen coronavirus
	Sparrow.CoV	Sparrow coronavirus
	Wigeon.CoV	Wigeon coronavirus
	Whiteeye.CoV	Whiteeye coronavirus



## **Bovine Coronavirus**

Bovine coronavirus is wide spread throughout cattle in the United States. Infection by BCoV can result in a loss of body condition of the animal which can then lead to decreased milk production and death in young animals. (Saif, 2010) Consequently, this severely impacts the economics of the cattle industry. BCoV is a pneumoenteric virus that infects both the upper and lower respiratory tracts as well as the intestine, and is therefore, shed in feces and nasal secretions. Bovine Coronavirus causes 3 distinct clinical syndromes in cattle: calf diarrhea, winter dysentery with hemorrhagic diarrhea in adults, and respiratory infections in cattle of various ages and is also included the bovine respiratory disease complex, or shipping fever, that is seen in feedlot cattle. The clinical appearance of the disease is not solely related to the virus itself but also to the host and environmental factors. No consistent antigenic or genetic markers have been identified to discriminate BCoVs isolated from animals presenting the different clinical syndromes. At present, there are no BCoV vaccines to prevent respiratory BCoV infections in cattle, and the correlates of immunity to respiratory BCoV infections are unknown. (Saif, 2010)

### ***Genomic Organization***

The coronavirus genome is a long, flexible, positive-stranded RNA that is encapsulated by the nucleocapsid protein which gives the genome its structure.(Fields, et al., 2001) The genome is composed of six open reading frames (ORFs). The first ORF comprises two-thirds of the genome and encodes the replicase proteins. The last third of the genome encodes the structural proteins in a fixed order: (HE)-S-E-M-N. HE is the hemagglutinin esterase which is responsible for neuraminidase O-acetyl-esterase activity and is only found in members of betacoronavirus subgroup 2a. The virion envelope contains three additional viral proteins, the spike protein (S), the membrane protein (M) and the envelope protein (E). The M and E proteins are involved in virus assembly, while the spike protein is the leading mediator of viral entry and accounts for a lot of the diversity in bovine coronavirus. The nucleoprotein (N) is the last of the structural proteins.(Belouzard et al., 2012b) Figure 1.2 shows the layout of the bovine coronavirus genome.

## Figure 1-2 Bovine Coronavirus Genome Organization

Genome organization of Bovine coronavirus (NC\_003045). PL1, papain-like protease 1; PL2, papain-like protease 2; 3CL, chymotrypsin-like protease; Pol, RNA-dependent RNA polymerase; Hel, helicase; HE, hemagglutinin esterase; S, spike; E, envelope; M, membrane; N, nucleocapsid.



### *Viral Proteins*

#### *Nonstructural Proteins*

The nonstructural proteins of BCoV are highly conserved and make up 2/3 of the genome. The first nonstructural protein that is synthesized is the polymerase precursor polyprotein 1a and 1b. It is directly produced from the viral genome because its function is in viral RNA synthesis. The precursor polyprotein is processed to make RNA-dependent RNA polymerases (Pol) which will transcribe the genome to the subgenomic mRNAs. The rest of the nonstructural proteins are synthesized from 7 subgenomic mRNAs. The next nonstructural proteins come from ORF 1a and are papain-like protease (PLP) and chymotrypsin 3C-like protease (3CL). Alpha-coronavirus and beta-coronavirus in subgroup A, including bovine coronavirus, have two PLPs. Their function is to cleave the polyprotein into multiple smaller proteins. Following Pol is the helicase protein that unwinds the genomic RNA for transcription. (Fields, et al., 2001) (Woo et al., 2010)

#### *Hemagglutinin Esterase*

Hemagglutinin esterase is a disulfide linked dimer that forms the short spikes on the virus. (Fields, et al., 2001) It is believed to come from the influenza C-like HE fusion protein through lateral gene transfer, therefore, has a similar function that involves receptor binding and detachment. (Hasoksuz et al., 2002a) Like the spike protein, HE binds to 9-O-acetylated neuraminic acid residues. (Fields, et al., 2001) HE is only found in the members of betacoronavirus subgroup A which includes human, murine, and bovine coronavirus. (Hasoksuz et al., 2002a) (Woo et al., 2010) While it is not necessary for viral replication, it is considered a luxury protein that may alter the viral pathogenicity. (Fields, et al., 2001)

### ***Spike***

The variations in host range and tissue tropism comes from the spike protein. The S protein is mainly used for receptor binding and viral entry into the cell.(Woo et al., 2010) This type 1 glycoprotein is 1363 amino acids long and assembles into trimmers on the surface giving the virus a “corona”, or crown-like, appearance. The spike protein is comprised of three structural domains: a large terminal domain that is further broken into the S1 and S2 domains, and a transmembrane domain. The S1 and S2 domains are cleaved during virus maturation by the cellular protease. The cleaved subunits remain noncovalently associated in the viral spikes. (Fields, et al., 2001) The S1 is responsible for receptor binding and its sequence is more variable than S2. The S1 has two subdomains, N-terminal and C-terminal, which are both capable of binding to proteins or sugars. (Belouzard et al., 2012a) (Bidokhti et al., 2013) Mutations in the S1 region have been associated with changes in pathogenicity and antigenicity. (Martínez et al., 2012) The S2 subunit is responsible for fusion of the viral and cellular membranes and is the more conserved region of the protein. (Belouzard et al., 2012a)

### ***Envelope and Membrane***

The E and M proteins are small transmembrane proteins that aid in viral morphogenesis, assembly, and are essential for budding of virion. They are also highly conserved throughout all coronaviruses.(Woo et al., 2010)(Fields, et al., 2001)

There is evidence that the M protein has two conformations: an elongated form and a compact form. The elongated form ( $M_{LONG}$ ) has a ridged structure with very little curvature and has a higher density of spike proteins. Conversely, the compact form ( $M_{COMPACT}$ ) is more flexible and allows for more curvature of the virus and has fewer clusters of the spike proteins. These two different forms combined give the coronavirus its circular shape. (Neuman et al., 2011)

### ***Nucleocapsid***

The nucleocapsid protein is a highly conserved region found on the interior of the viral envelope and is responsible for wrapping the genomic RNA into its structure. It is shown that the nucleocapsid protein plays a key role in RNA synthesis by stabilizing and giving structure to the viral genome and may have involvement in cell signaling pathways. (Hurst et al., 2013)



## *Replication*

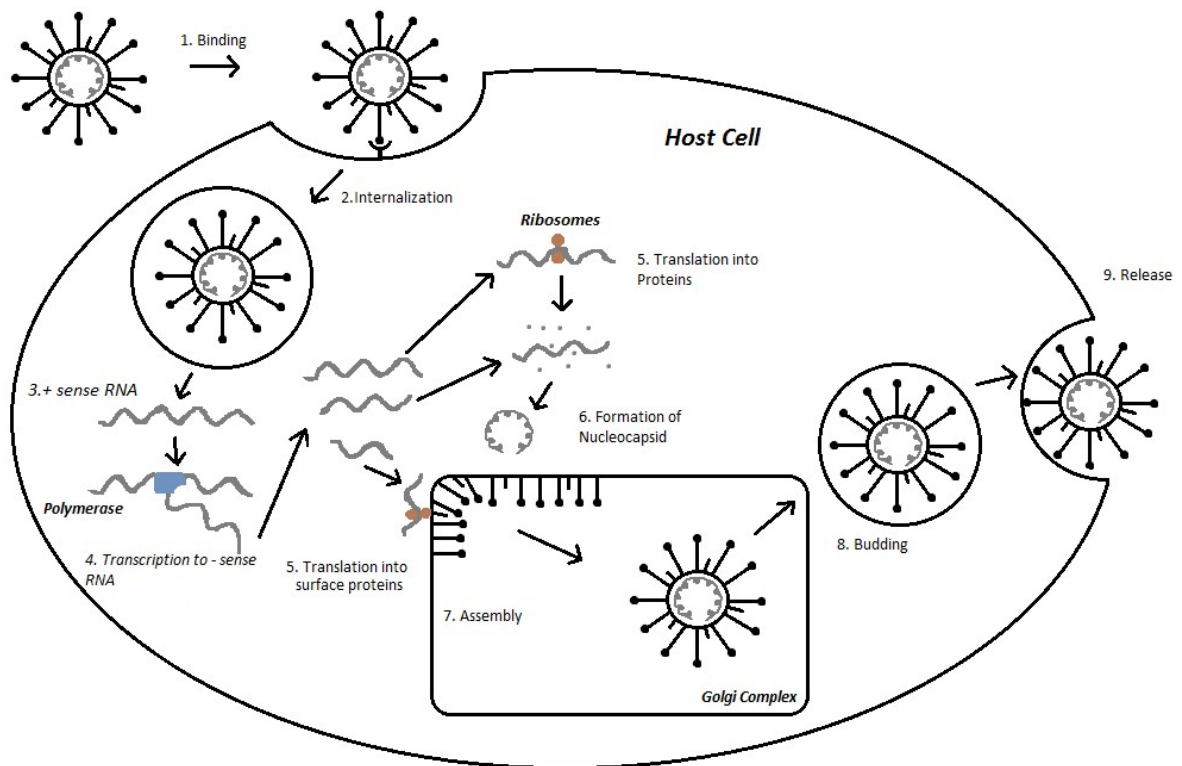
Bovine coronavirus uses the S2 subunit of the spike protein to interact with the 9-O-acetylated sialic acid receptor which is located on a wide range of mammalian tissues. (Klein et al., 1994) This particular receptor is also used by influenza C and by HCoV-OC43 which is a human strain of coronavirus that caused upper respiratory infections before the SARS epidemic. (Perlman and Netland, 2009) (Herrler et al., 1991) Once it attaches to the receptor the virus is incorporated via endosome that is acidified by proton pumps. It is still unclear how the virus uncoats itself. (Fields, et al., 2001) When a coronavirus first infects a cell, it releases RNA that is structurally comparable to eukaryotic RNA. Researchers believe that this helps the virus take control over host cell machinery to translate the viral RNA.

First, the RNA polymerase precursor polyprotein 1a and 1b are synthesized from the viral genome because they are needed for synthesis of the remaining viral RNA. The rest of the viral proteins will be translated from subgenomic mRNA. These subgenomic mRNAs will correspond with one or more proteins. The viral replication complex aids in the transcription replication of the mRNA to proteins. The other proteins that are in ORF1a and 1b are PLP (papain-like cysteine protease), 3CL (chymotrypsin 3C-like protease), Pol (predicted polymerase), Hel (helicase), and MP (membrane-binding domain). The first step of virus assembly occurs when synthesized N proteins begin to form its core structure. Once the nucleocapsid is made, it interacts with the M protein at the endoplasmic reticulum or the Golgi complex. This interaction is believed to make the round internal core that surrounds the nucleocapsid. The N protein acts as a chaperone protein to aid in template switching. This slows host protein synthesis and increases viral protein synthesis. (Enjuanes et al., 2006) The major protein that aids in the formation of virus particles is the E protein. Without the E protein, the virus would have an altered virus morphology and could become noninfectious. The interaction of the E and M protein triggers virus budding. Budding usually occurs in the budding compartment that is located where the M protein is anchored. This is where the virus coats itself in the host's membrane. Virologists believe that the E protein is then responsible for pinching off the budding virion in the budding compartment and can also cause apoptosis to certain cells (Enjuanes et al., 2006). The HE and S proteins are incorporated into the virion via interactions with the M protein. Once the virus has finished assembling, it will fuse with the plasma membrane to release the virus. The release of mature virus particles is restricted to a specific part

of the cell membrane but why there is site restriction is unknown. (Fields, et al., 2001)(Lai and Cavanagh, 1997)(Stadler et al., 2003) Figure 1.3 shows a diagram of the coronavirus replication within the host cell.

### Figure 1-3 Viral Replication

Bovine coronavirus infects multiple cell types, the most common being epithelial cells. The steps in the virus life cycle are as follows. 1) Attachment of the virus to the 9-O-acetylated sialic acid receptor on the host cell surface. 2) Incorporation into the cell occurs via the endosome and then is acidified by proton pumps. 3) It is unclear how the virus uncoats itself but the positive sense single stranded RNA genome is released and RNA-dependent RNA polymerase is synthesized. 4) The RNA-dependent RNA polymerase transcribes the positive sense RNA to negative sense RNA copies. 5) The negative sense RNA gets translated into proteins. 6) The formed nucleocapsid interacts with the M protein that will then trigger the virus surrounding itself in the host cell membrane 7) The virus begins to assemble in the Golgi Complex. 8) Once the virus has finished its assembly, it will fuse with the plasma membrane to 9) release the virus.



## ***Transmission and Pathogenesis***

Transmission of BCoV is horizontal with nose to nose interaction or through the oral-fecal route. To a lesser extent it may also be passed by respiratory routes via aerosol. There is also some data to support that dogs may play a role in spreading BCoV because canine respiratory coronavirus is genetically similar to BCoV. (Kaneshima et al., 2007) Since coronaviruses are enveloped, they are less stable in the environment than non-enveloped proteins and cannot remain infectious unless protected. However, if protected by organic material, the virus will remain infectious for a brief period of time. It is believed that the virus begins replicating in the epithelial cells of the upper respiratory tract and then moves to the enterocytes of the gastrointestinal tract.(Boileau and Kapil, 2010) Bovine coronavirus is able to survive the gastrointestinal tract because it is very resistant to the host's proteases. When growing BCoV *in vitro* you add trypsin to enhance virus infectivity. (Thomas et al., 2006) An infection with BCoV will induce fusion of the cells of infected tissues and eventually the lysis of cells.(Fields, et al., 2001) Lysis of the cells initiates an immune response that is responsible for the symptoms that we observe in these infected animals.

## ***Host Immune Response***

Most of what is known about the host's response to coronavirus infection has been determined by studies involving the mouse hepatitis virus (MHV) and SARS coronavirus. The innate immune response of the host is crucial to determining the initial virus replication and the viral load the host must overcome. Once the virus enters and infects the host cell, it will take control of the cell's machinery and begins replication. This will trigger a number of responses within the host. One of the responses is stimulation of both apoptotic and antiapoptotic molecules (Tang et al., 2005). The antiapoptotic stimulation allows for the cell's survival while rapid viral replication takes place before the cell lysis occurs (Leung et al., 2003). The apoptotic stimulation is cell specific, such as T cells, and may be responsible for the pathology of infection.(Chau et al., 2004) Lysis of the host's cells initiate a cascade of immunological responses that include inflammation and increased regulation of cytokines such as interleukin 8 (IL-8) and interferon alpha/ beta ( $INF\alpha/\beta$ ). The release of IL-8 induces chemotaxis and phagocytosis to the infected area.  $INF\alpha/\beta$ , named for its ability to interfere with viral replication, activates immune cells and increases the major histocompatibility complex (MHC) expression on

cells. Recruitment of the adaptive immune system is also critical for virus clearance. Dendritic cells and other antigen-presenting cells (APCs) present pieces of destroyed virus to the B-cells in circulation and in the lymph nodes. B-cells that have a strong affinity for an epitope of the virus will begin producing antibodies with the help of CD4 T-cells. CD8 T-cells will also be activated and search to induce apoptosis to any abnormal cells. The production of neutralizing antibodies has been shown to correlate with protection and severity of disease symptoms (Thomas et al., 2006)(Cho et al., 2001). The adaptive immune response takes time to react after initial infection which allows the virus more time to replicate. As the virus replicates and the number of infectious particles increases, and excretion of the virus particles begins. This is called virus shedding and is shown to be the highest around day 4 after infection. (Hasoksuz et al., 2002) In BCoV, virus is shed primarily through the nasal secretions and fecal matter. In healthy animals the immune system will be activated and begin clearing the virus and will recover normally. Memory B-cells will also be generated in case the host comes in contact with the virus again so the adaptive immune response can react more rapidly. In immunocompromised or stressed animals, the immune system won't be able to keep up with the viral load and the severity of the disease can increase.

Younger animals do not have a developed immune system and that's why lactogenic immunity is key to them fighting off disease. The first round of immunity from the mother is in the colostrum. The colostrum is full of antibodies that the calf will absorb. Within a few hours gut closure will take place and the calf will be unable to absorb the antibodies. The second round of immunity happens after gut closure. A few antibodies will still remain in the milk and will coat the villi of the gastrointestinal tract of the young animal and will be able to neutralize viruses before they can invade the villi epithelium.(Saif and Smith, 1985)

### ***Diseases***

Bovine coronavirus was first identified as a primary pathogen that was responsible for neonatal calf diarrhea, or calf scours.(Mebus et al., 1973) Calf diarrhea coronavirus infection of the small and large intestine in calves causes destruction of the absorptive intestinal villous epithelial cells, leading to profuse watery diarrhea and loss of absorptive and digestive capability of the intestinal tract. Clinical symptoms include a yellow to blood-stained mucus-containing diarrhea which progresses to watery diarrhea. Infected animals can become dehydrated,

depressed, weak, and hypothermic as well as develop acidosis and electrolyte abnormalities due to the loss of fluid in the feces and the fermentation of undigested nutrients which leads to bacterial overgrowth. (Ewaschuk et al., 2004) In extreme cases animals may develop pyrexia, recumbency, coma, and death if left untreated (Mebus et al., 1973).

Winter dysentery is an acute, highly contagious gastrointestinal disorder that affects housed adult dairy cattle primarily during winter. Clinical signs include dysentery, a profound drop in milk production, variable anorexia and depression, and mild respiratory signs such as coughing.(Campbell and Cookingham, 1978) The disease has a high morbidity but low mortality, and spontaneous recovery within a few days is typical. BCoV is transmitted via the fecal-oral route through ingestion of feed or water contaminated with feces from clinical cases or clinically healthy carrier animals. Transmission of disease is promoted by close confinement. Winter dysentery is highly contagious and easily introduced to barns by carrier animals and fomites. Winter dysentery is common in northern climates where animals are housed indoors for extended periods during the winter months. (Van Kruiningen et al., 1985) Destruction of epithelial cells in the colonic crypts results in transudation of extracellular fluid and blood, explaining the hemorrhagic nature of the diarrhea in some cases.

Lastly, bovine coronavirus is also linked with respiratory infections and is associated with bovine respiratory disease complex (BRDC), or shipping fever. Other microorganisms that are a part of BRDC include bovine respiratory syncytial virus, parainfluenza-3 virus, bovine herpesvirus, bovine viral diarrhea virus, as well as *Mannheimia haemolytica* serotype 1, and *Pasteurella multocida*.(Storz et al., 2000) There have been respiratory disease outbreaks where BCoV has been isolated without any detection of other respiratory viruses, bacteria, or mycoplasmas (Decaro et al., 2008). This indicates that BCoV may be a primary respiratory pathogen and active player in the BRDC. The clinical signs of infection are dyspnea, fever, coughing, weight loss, and, in more severe cases, pneumonia or even death. The respiratory disease may be more severe when the infection is combined with other factors such as environmental or host stresses or coinfections with other viruses or bacteria.

### ***Detection and Quantification***

Some of the most common methods for detecting BCoV are PCR and assays involving immunofluorescence. PCR is used to detect the amount of viral genomic information present in a

sample. (Reynolds et al., 1984) This technique is helpful for things such as cloning or sequencing. The disadvantage to this method is being unable to determine if the virus has the ability to infect and replicate properly within the cell. The other most common approach is using fluorescent antibody assays. These types of assays are very valuable because you can use them for the detection of antigen and antibody. One example is an immuno-fluorescent assay (IFA). (Reynolds et al., 1984) By using a monoclonal antibody, this test method allows for a very specific detection of the virus and the approximate amount of infectious cells are present. The problem with this type of assay is that it requires highly specific antisera and a way to read the fluorescence. In addition, false negative results can arise from the presence of blocking antibodies in the sample. (Athanasios et al., 1994)

An alternative common option is an enzyme linked immunosorbent assay (ELISA). These come in many arrangements but the principle is similar to the IFA. ELISAs can also be used for antibody detection or antigen detection. This assay uses monoclonal antibodies to attach to an epitope of the virus and then uses either fluorescence or a color enzyme tagged antibody to show a positive result. Another option for detecting BCoV is using an electron microscope. The characteristic appearance of the spike protein allows for identification of the virus. The drawbacks to this method include risk of damage to the viral surface structures due to improper collection and/or transport of the sample, the presence of other viral particles or membranous structures which can be mistaken for coronavirus, and false negative results due to the disruption of coronavirus particles (Athanasios et al., 1994).

### ***Control and Prevention***

Currently, the most effective protection from BCoV are commercial vaccines and proper management. Treatment of BCoV include supportive therapies such as reducing stress and replacing lost body fluid and electrolytes. Since a viral infection can lead to a secondary bacterial infection, antibiotic therapy can be administered. Other treatments are based on additional symptoms observed, which may include acidosis and hypoglycemia.

### ***Vaccine***

The first and only strained used to make the vaccines in the U.S. against bovine coronavirus was patented in 1975 by Dr. Charles A. Mebus. The vaccine was prepared by passing infectious coronavirus in fetal bovine kidney cells to attenuate the virus which was then

grown to  $10^2$  to  $10^6$  TCID<sub>50</sub> per dose. The production of this vaccine is still used for neonatal calf diarrhea. (1975) Today, two types of vaccines still use this 40-year-old strain of virus. The first is a modified live rotavirus/coronavirus vaccine that is administered orally to calves at birth. The other is a killed vaccine which that includes a killed *Escherichia coli* bacterin and is given to late pregnant cows to give passive immunity to their calves. (Radostits, 1991)(Boileau and Kapil, 2010) According to the National Animal Health Monitoring System (NAHMS), vaccination for coronaviruses are very low. In calves 1- 21 days old only 0.9% are vaccinated against coronavirus. In calves 22 days to weaning only 0.2% are vaccinated. Before breeding in heifers only 1.3% are vaccinated and after breeding and before calving 4.8% are vaccinated. In adult cows, 5.3% are vaccinated. (2009) There is currently no vaccine licensed specifically for use against the respiratory form of the disease, but there is some data indicating that the vaccine strain is protective against respiratory coronavirus as well. (Plummer et al., 2004)

### **Purpose of Research**

Bovine coronavirus exhibits tissue tropism for both the gastrointestinal and respiratory tracts. Currently, the only vaccine available for BCoV is derived from a 40-year-old enteric strain and targets only that form of the disease. It is unknown if this vaccine is still relevant for the enteric form of the disease and it has not been approved for respiratory coronavirus. The primary goal of this project is to establish a serological profile to the different genotypes of BCoV using virus serum neutralizations assays. Serological relationship will be established by doing surveillance analysis on samples sent to the KSVDL to look at genetic relatedness as well as the time of year, gender of the animal, age, and if they are dairy or beef animals.

## **Chapter 2 - Materials and Methods**

### **Cells**

Human rectal tumor cells (HCT-8), were obtained from ATCC. They were used to isolate and amplify the bovine coronavirus isolates used in this project. (Hofmann et al., 1990) The cells were grown in a 37°C incubator using growth media composed of Minimum Essential Media (MEM) (Hyclone) containing 7% Fetal Bovine Serum (Hyclone), 29.2 mg/mL L-glutamine

(Hyclone), 10000 µg/mL each of Penicillin/Streptomycin (Hylocne), 250µg/mL Amphotericin B (Fisher), 10 µg/mL Ciprofloxacin (Mediatech).

### **Viruses used for SN Testing**

Enteric and respiratory viral isolates were obtained through clinical samples submitted to the Kansas State Veterinary Diagnostic Lab. (KSVDL accession #13-149008, 13-149100, 10-78481, 14-007320 calf 1) Viral isolation was done on these samples and they were maintained in cell culture to be used as SN indicator viruses. The Mebus strain of bovine coronavirus was obtained from the National Veterinary Services Laboratories. The virus was carried an additional 5 passages. When infecting cells, viral infection media composed of MEM with 29.2 mg/mL L-glutamine, 10000 µg/mL each of Penicillin/Streptomycin, 250µg/mL Amphotericin B, and 10µg/mL Ciprofloxacin was used.

### ***Infectivity Titers***

96-well plates seeded with HCT-8 cells were maintained with MEM growth media for 4 days. The cells were washed twice with sterile phosphate buffered saline (PBS) then inoculated with MEM viral isolation media that contained 0.05% trypsin and virus that was diluted in 10-fold dilutions. After a 4-day incubation, the cells were fixed in 80% aqueous acetone for 10 minutes. A dilution of 1:100 primary hybridoma antibody in a 1:5 dilution of PBS was added and incubated for 30 minutes. This antibody was prepared by KSVDL to the spike protein, and was generated using the hybridoma Z3-A-5. The plates were washed extensively with PBS. A secondary polyclonal with a fluorescent tag was added and incubated for 30 minutes. (Mouse monoclonal antibody (Fluorescein (FITC)-conjugated AffiniPure Goat Anti-Mouse IgG(H+L) by Jackson ImmunoResearch Laboratories Inc.) The plates were again washed with PBS and 50% Glycerol/PBS solution was added. The plates were then viewed using an epifluorescent microscope under 10X magnification.

### **Sequencing**

In this study we completed next generation sequencing (NGS) (Neill et al., 2014) of the full bovine coronavirus genome, using sequence independent single primer amplification (SISPA) methodology, to wholly assess the differences between enteric and respiratory isolates.(Djikeng et al., 2008) (Allander et al., 2001) We started off with 25 viral isolates that



were submitted as clinical samples. 9 of these isolates could not be sustained in cell culture and were unable to be sequenced. 5 of these isolates had CT values over 28 and were also unable to be sequenced.

Samples were prepared by spinning the isolates at 10000 rpm for 5 minutes. The RNA was extracted using a column system (Qiagen MinElue Virus Spin Kit, Qiagen, Hilden, Germany). First strand synthesis was performed using the Life Technologies Superscript III First strand synthesis System. Directly following first strand synthesis was second strand synthesis using Affymetrix Sequenase 2.0 DNA Polymerase. Next cDNA was synthesized with random primers and then amplified via PCR. (Djikeng et al., 2008) The resulting PCR product was prepared according to Illumina's Nextera XT DNA Sample Preparation Guide. This involved adding tags to random ends of the DNA, amplification, PCR cleanup, library normalization, and loading of the MiSeq Desktop Sequencer (Illumina). The raw data sequences that were generated with the MiSeq were aligned to BCU00735.2 in Gen Bank and processed in CLC database.

### **Serological Characterization Based on Different Viruses**

Field bovine serum samples submitted to KSVDL were divided into groups based on production, age, and gender to look at the prevalence of BCoV, age of exposure to BCoV, and potential titer differences against the unique genotypic strains of coronavirus (Table 2.1). Serum neutralization assays were used to measure antibody titers within the samples. For the assays, 96-well plates seeded with HCT-8 cells were maintained with MEM growth media for 4 days. The collected serums were diluted 1:20 in viral isolation media that contained 0.05% trypsin. The serum was then serially diluted 1:2 in sterile 96-well plates. The selected viruses were added to the diluted serum and incubated for 1 hour. The HCT-8 seeded plates were washed twice with sterile PBS then inoculated with the virus/serum media and incubated for 2 days. After 2 days the cells were washed with PBS and fixed in 80% aqueous acetone for 10 minutes. The 1:5 dilution of Z3-A-5 hybridoma mouse monoclonal antibody was added and incubated for 30 minutes. The plates were washed extensively with PBS. A dilution of 1:100 of secondary polyclonal Anti-Mouse IgG (Fluorescein (FITC)-conjugated AffiniPure Goat Anti-Mouse IgG(H+L) by Jackson ImmunoResearch Laboratories Inc.) was added and incubated for 30 minutes. The plates were again washed with PBS and 50% Glycerol/PBS solution was added. The plates were then viewed using a fluorescent microscope.

**Table 2-1 Field Serum Categories**

The bovine serums collected from KSVDL were split into groups based on age, gender, and production. Of the 147 samples, 10 samples were less than 1 year of age, 84 samples were between 1 and 2 years of age, and 53 samples were greater than 2 years of age. Of the 10 samples that were less than 1 year of age, all were female and beef animals. Of the 84 samples that were between 1 and 2 years of age, 59 were female, 25 were male, 59 were beef and 25 were dairy. Lastly, of the samples that were over the age of 2, all were female, 43 were beef and 10 were dairy.

<i>Age</i>	<i>Female</i>	<i>Male</i>	<i>Total</i>	<i>Beef</i>	<i>Dairy</i>	<i>Total</i>
<b>&lt;1 year</b>	10	0	<b>10</b>	10	0	<b>10</b>
<b>1-2 years</b>	59	25	<b>84</b>	59	25	<b>84</b>
<b>&gt;2 years</b>	53	0	<b>53</b>	43	10	<b>53</b>
<b>Total</b>	<b>122</b>	<b>25</b>	<b>147</b>	<b>112</b>	<b>35</b>	<b>147</b>

## **Chapter 3 - Results**

### **Phylogenetics**

Once NGS was completed, the reads of the nucleotide sequences of the 12 coronaviruses were processed and aligned using the Mebus strain (BCU000735) from GenBank as a reference template. Pairwise comparison of the viral sequence reads covered over 90%+ of the genome. One of the genomic reads, of virus (R7), covered less than 90% of the genome so it was not used in the analysis. From the represented sequences it was observed that the amino acids of the HE protein were conserved within the sequences and the spike protein was mostly conserved except for a few amino acid changes.

There were no nucleotide or amino acid changes that were exclusive to the enteric strains when compared with the respiratory strains. However, when aligned with a NCBI reference Mebus, several amino acid changes were apparent. The spike protein was 1363 amino acids long. Of those amino acids, 63 changes were found as illustrated in Table 3.2. Of the total amino acid changes in the spike protein, 49 were in the S1 subunit and 28 of these were significant alterations resulting in either hydrophobicity or charge modifications. The other 14 changes were located in the S2 subunit with only 6 significant alterations. This was expected since the S2 subunit is more conserved. We also looked into the HE protein (Table 3.3) for its role in binding and found that there were 10 amino acid changes. Of those 10 changes, 4 were considered significant. These significant amino acid changes could be important because they have the ability to change the conformation of the protein which could lead to a change in receptor binding or could also lead to the binding of alternate receptors.

Overall, no unanimous differences were observed between the enteric viruses and the respiratory viruses. Phylogenetic trees were constructed (bootstrapping value of 1000) using the alignments of the full genome, spike nucleotides, and HE nucleotides of the genome. During phylogenetic analysis of the full genome we can see that the Mebus strain is the most different from the other viruses sequenced. The rest of the viruses ultimately formed different clusters but the bootstrap value used in the analysis indicated no significant differences between them. When looking at the spike dendrogram and the HE dendrogram, similar results are observed. The Mebus strain is the most divergent from the set while the other viruses branch into separate groups without significant differences. (Figures 3.1, 3.2, and 3.3)

### **Table 3-1 Sequencing Table**

This sequencing table shows the viruses that were sequenced. The first column is the virus identification. In the virus ID section, if it begins with an “R” it is a respiratory isolate and if it begins with an “E” then it is an enteric isolate. E5 is the Mebus vaccine strain. The second column is the accession number associated with the case from KSVDL it was selected from and the last column is the cycle threshold value that came from RT-PCR.

Virus ID	Accession Number	CT Value
R1	12-157760-2	24.18
R2	13-149097	24.94
R3	13-149008	23.95
R4	12-155165	23.65
R5	10-78481	25.86
R8	13-149100	26.99
R11	13-138077-3	19.29
E1	14-007320 calf 1	25.59
E2	14-007320 calf 2	24.94
E4	11-129440	22.81
E5	Bovine coronavirus Mebus strain	22.22

### **Table 3-2 Spike Amino Acid Sequence**

The spike amino acid sequences were compared to a reference Mebus strain from NCBI. The first column refers to the position of the amino acid change within the spike protein. The next column to the right is the reference Mebus. The following columns refer to the virus ID that were sequenced. Any of the amino acids in white were changes in the amino acids from the reference Mebus. The significant change column refers to the type of change of the amino acid. The last column refers to the possibility that the amino acid change is a sequencing error.

Spike Amino Acid Changes														
Position	NCBI Reference Mebus	E5	R1	E1	E2	R4	R8	R3	R11	R5	R2	E4	Significant change	Potential Sequencing Error
9	L	L	L	L	L	L	L	L	L	L	L	L	no	
11	M	M	T	T	T	T	T	T	T	T	T	M	hydrophobicity	
24	V	V	V	V	V	L	V	V	V	V	V	V	no	
28	D	D	D	D	D	D	D	D	D	D	D	N	charge	
29	V	I	V	V	V	V	V	V	V	V	V	V	no	
33	A	A	V	V	V	V	V	V	V	V	V	V	no	
40	I	I	T	T	T	T	T	T	T	T	T	T	hydrophobicity	
86	L	L	L	L	L	L	L	L	L	L	L	S	hydrophobicity	yes
88	R	R	T	R	T	T	T	T	T	T	T	T	charge	
100	I	I	T	T	T	T	T	T	T	T	T	T	hydrophobicity	
115	K	K	D	D	D	G	D	D	D	D	D	D	charge	
146	N	N	I	I	I	I	I	I	I	I	I	I	hydrophobicity	
148	D	D	G	G	G	G	G	G	G	G	G	G	charge	
154	L	L	F	F	F	F	F	F	F	F	F	F	no	
169	H	H	N	N	N	N	N	N	N	N	N	N	no	
173	H	H	N	N	N	N	N	N	N	N	N	N	no	
174	P	P	S	S	S	S	S	S	S	S	S	S	hydrophobicity	
179	K	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	charge	
243	S	S	S	S	S	S	S	S	S	S	S	L	hydrophobicity	
248	L	M	M	M	M	M	M	M	M	M	M	M	no	
253	S	Y	N	N	N	N	N	N	N	N	N	N	no	
254	S	S	S	S	S	S	S	S	S	S	C	S	no	
256	M	L	L	L	L	L	L	L	L	L	L	L	no	
458	F	S	S	S	S	S	S	S	S	S	S	S	hydrophobicity	
464	P	P	P	P	P	P	P	P	P	P	P	L	no	
465	V	V	A	A	A	A	A	A	A	A	A	A	no	
470	H	H	D	D	D	D	D	D	D	D	D	D	charge	
484	S	T	T	T	T	T	T	T	T	T	T	T	no	
492	D	D	G	G	G	D	D	D	D	D	D	D	charge	
499	N	N	N	N	N	S	S	S	S	S	S	S	no	
501	P	P	P	P	P	S	S	S	S	S	S	S	hydrophobicity	
509	H	H	T	T	T	N	N	N	N	N	N	N	no	
510	T	T	T	T	T	T	T	T	T	T	T	I	hydrophobicity	
525	H	H	H	Y	Y	H	Y	H	Y	H	H	H	charge	
528	A	A	A	A	A	A	A	A	A	V	A	A	no	
531	N	D	D	D	D	D	D	D	D	H	D	D	charge	
543	S	S	A	A	A	A	A	A	A	A	A	A	hydrophobicity	
546	P	P	S	S	S	S	S	S	S	S	S	S	hydrophobicity	
571	Y	Y	H	H	H	H	H	H	H	H	H	H	hydrophobicity	
578	T	T	S	S	S	S	S	S	S	S	S	S	no	
588	S	S	S	S	S	S	S	S	S	A	S	S	hydrophobicity	
591	S	S	S	S	S	S	S	S	S	S	P	S	hydrophobicity	
607	H	H	H	H	H	H	H	H	H	H	H	Y	charge	
609	V	V	V	V	V	V	V	V	V	V	F	V	no	
668	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	no	
717	L	L	L	L	L	L	L	L	F	L	L	F	no	
729	S	S	S	S	S	S	S	S	S	S	R	R	charge	
731	L	L	L	L	L	L	L	R	L	L	L	L	charge / hydrophobicity	yes
769	A	A	S	S	S	S	S	S	S	S	S	S	hydrophobicity	
778	T	N	N	N	N	N	N	N	N	N	N	N	no	
965	V	V	E	E	E	E	E	K	E	E	E	E	charge / hydrophobicity	
979	S	S	S	S	S	S	S	S	N	S	S	S	no	
984	L	W	W	W	W	W	W	W	W	W	W	W	no	
988	V	A	A	A	A	A	A	A	A	A	A	A	no	
998	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	H	Y	no	yes
999	R	R	R	R	R	R	R	R	R	R	R	R	charge	yes
1026	D	D	G	G	G	G	G	G	G	G	G	G	charge	
1100	V	A	A	A	A	A	A	A	A	A	A	A	no	
1237	T	T	T	T	T	T	T	T	T	T	T	I	hydrophobicity	
1241	H	H	P	P	P	P	P	P	P	P	P	P	charge / hydrophobicity	
1275	D	D	D	D	D	D	Y	D	D	D	D	D	charge	
1341	I	I	K	K	K	K	K	K	K	K	K	K	charge / hydrophobicity	
1362	D	D	E	E	E	E	E	E	E	E	E	E	no	

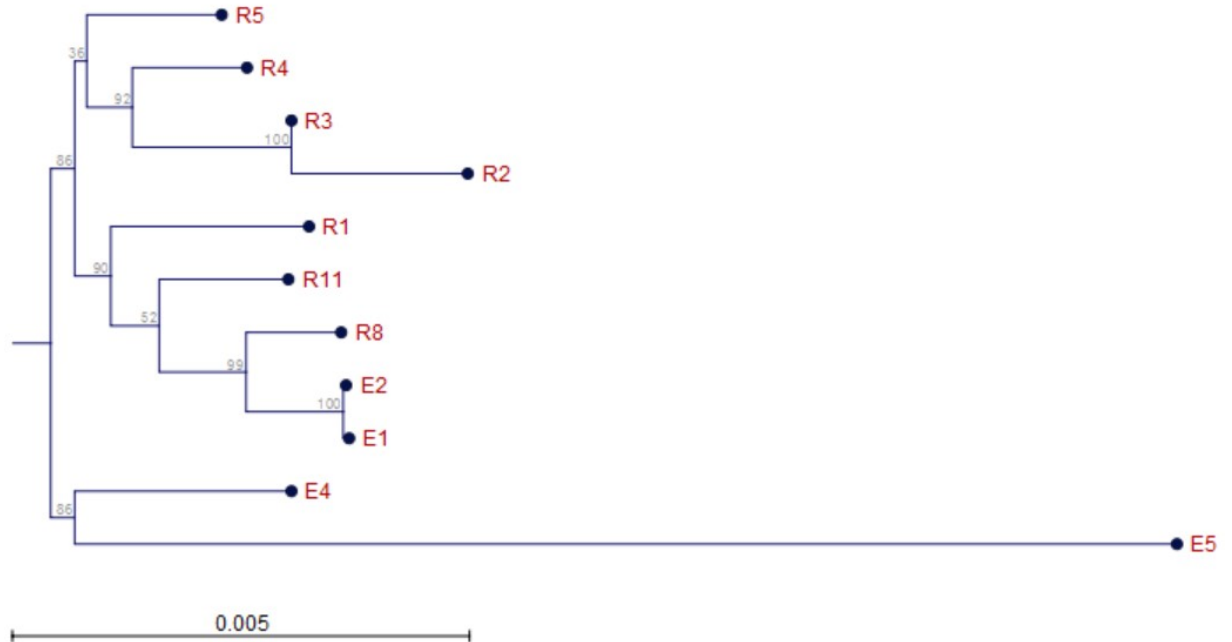
**Table 3-3 HE Amino Acid Sequence**

The HE amino acid sequences were compared to a reference Mebus strain from NCBI. The first column refers to the position of the amino acid change within the HE protein. The next column to the right is the reference Mebus. The following columns refer to the virus ID that were sequenced. Any of the amino acids in white were changes in the amino acids from the reference Mebus. The significant change column refers to the type of change of the amino acid.

HE Amino Acid Changes													
Position	NCBI Referece Mebus	E5	E1	E2	E4	R2	R3	R4	R5	R8	R11	R11	Significant change
5	L	L	P	P	P	P	P	P	P	P	P	P	no
49	N	N	T	T	T	T	T	T	T	T	T	T	no
66	D	D	G	G	G	G	G	G	G	G	G	G	charge
103	V	L	V	V	V	V	V	V	V	V	V	V	no
147	N	N	N	N	N	N	N	N	N	N	N	S	no
309	P	P	P	P	P	P	P	P	P	P	P	L	no
367	S	S	P	P	P	P	P	P	P	P	P	P	hydrophobicity
376	D	G	D	D	D	D	D	D	D	D	D	D	charge
392	L	L	I	I	I	I	I	I	I	I	I	I	no
400	G	G	G	G	G	G	G	G	G	G	G	V	hydrophobicity

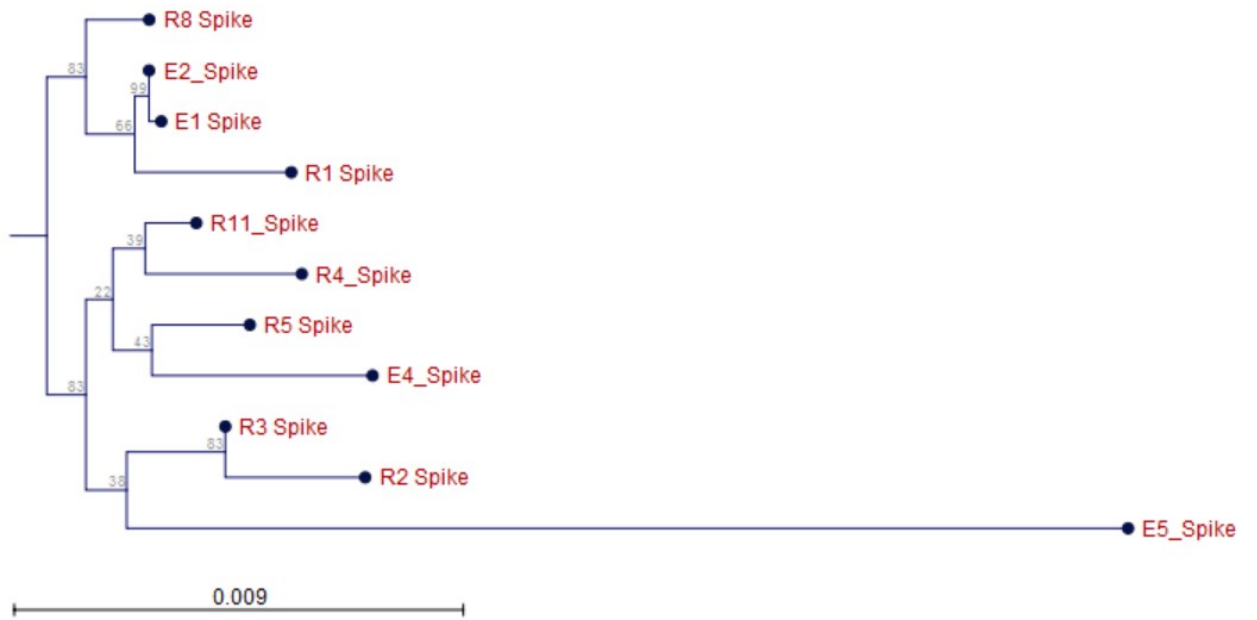
**Figure 3-1 Bovine Coronavirus Whole Genome Phylogenetic Tree**

The following dendrogram is based on the whole genome sequences with a bootstrapping value of 1000. Each sequence virus ID ties back to table 3.1



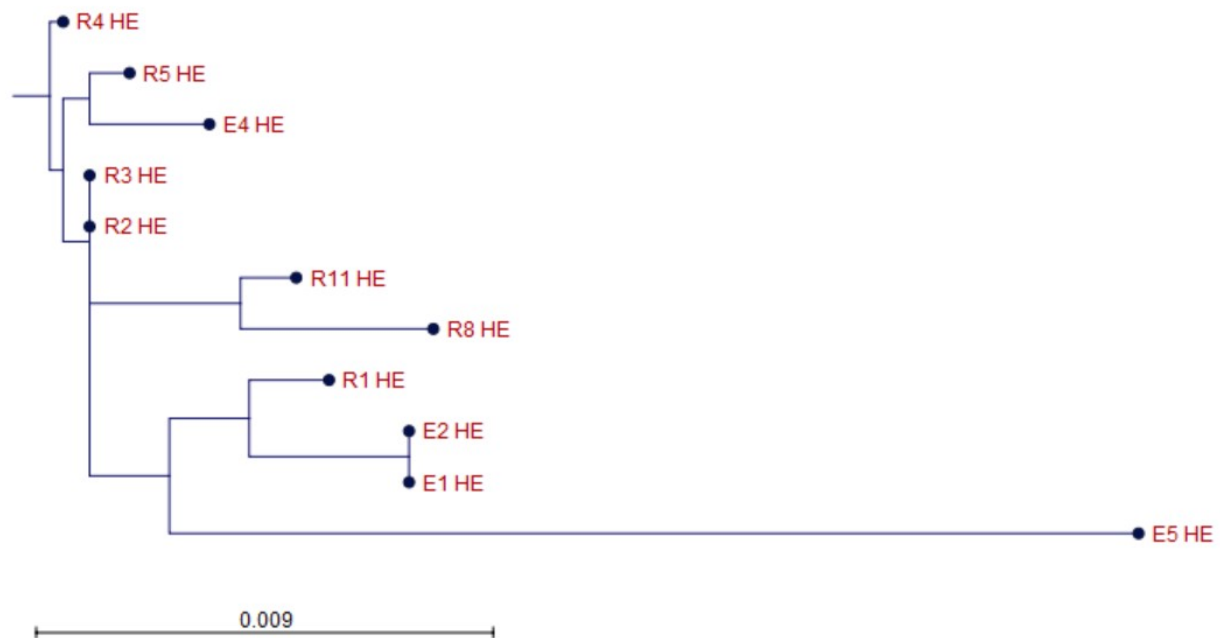
**Figure 3-2 Bovine Coronavirus Spike Phylogenetic Tree**

The following dendrogram is based on the spike protein sequences with a bootstrapping value of 1000. Each sequence virus ID ties back to table 3.1



**Figure 3-3 Bovine Coronavirus Hemagglutinin Esterase Phylogenetic Tree**

The following dendrogram is based on the HE protein sequences with a bootstrapping value of 1000. Each sequence virus ID ties back to table 3.1



## Virus Selection

The viruses were selected based on the phylogenetic analysis from sequencing. Each virus's ability to grow and be maintained in cell culture also played a role in selection. HCT-8 cells were used as the primary cell line for growth. The selected viruses contain 3 respiratory isolates (10-78481, 13-149100, and 13-149008) and 2 enteric isolates (Mebus, 14-007320 calf 1).

## Virus Specific Seroprevalence

To determine if the serum antibodies would have a diverse reaction to the selected viruses, serum neutralization assays were performed. Initial serosurveillance were carried out using 147 collected serum from the KSVDL against the selected viruses. The results as listed in Figure 3.4 show the individual serum samples against each virus. Only 12% of the serums showed a 4-fold difference in the neutralizing ability to at least one of the viruses indicating that, for the most part, the serum antibodies reacted similarly among the viruses. For the viruses that did have a 4-fold difference, Table 3.4 shows the analysis between the different groups and the respiratory and enteric viruses. All of the samples that were significant were female and 83%



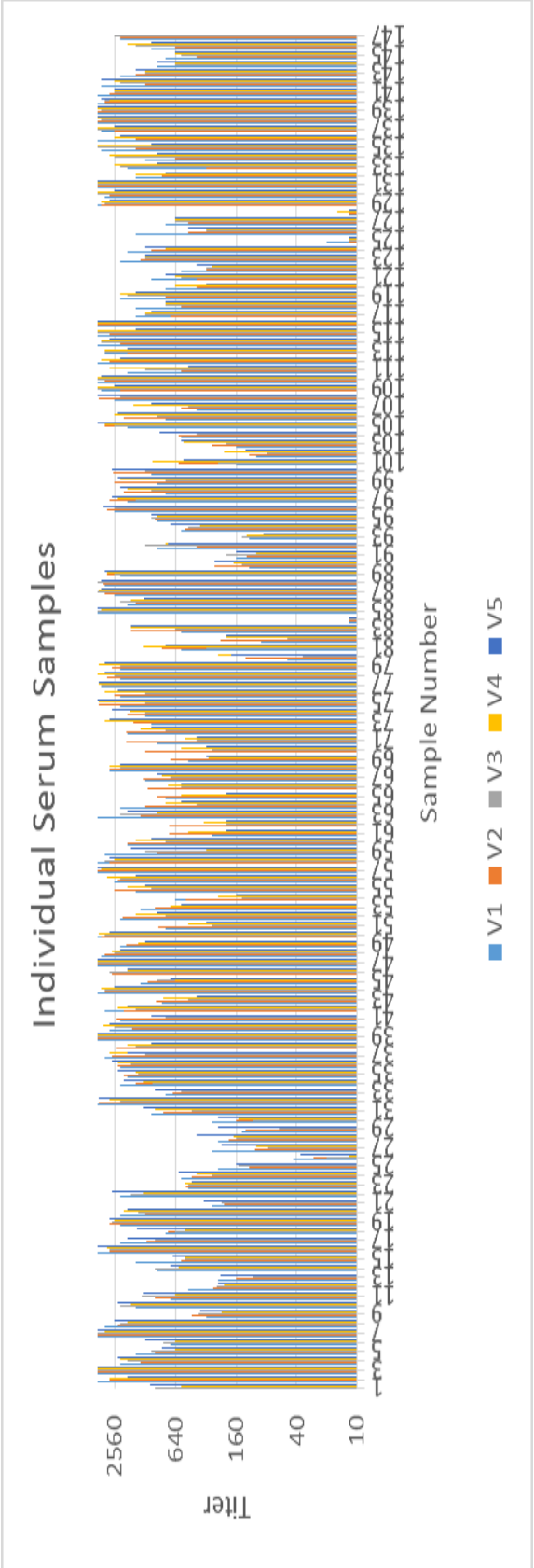
were beef animals. Looking at the virus breakdown shows that 13-149008 was high in 5 of those samples and low in 2 samples. 10-78481 was high in 7 of the samples and low in 2 samples. Interestingly, the Mebus strain was only high in 2 sample and low in 10 of the samples. The other enteric sample, 14-007320, was high in 6 samples and low in 3 samples. The last respiratory sample, 13-149100, was high in 5 of the samples and low in 5 of the samples. This concludes that there is no real pattern of certain viruses always being high or low.

When it comes to the overall group breakdown, the biggest difference was observed between age groups. Animals <1 year of age showed the lowest titers followed by 1-2 years of age and, lastly, > 2 years of age. The analysis of the male and female groups showed males to have lower titers to the viruses than females. This is not conclusive, however, because of the low number of sample numbers for the males. The difference could also be due to the low age of the males as compared to the females. Examination of dairy versus beef groups indicates that there is no significant difference between the two.

**Figure 3-4 Individual Serum Sample for Serum Surveillance SN assays**

The individual samples from the virus seroprevalence assays were graphed on a log base 10 scale. Each serum sample was tested against the 5 viruses. Each virus is color coded and ties back to the selected viruses and is arranged in the table below.

Virus ID	Accession Number
V1	13-149008
V2	10-78481
V3	BCoV Mebus strain
V4	14-007320 calf 1
V5	13-149100



**Table 3-4 Breakdown of Significant Difference in Serum Surveillance Assays**

A breakdown of the 12% of the viruses that showed a 4 fold difference in serum antibody titer to at least two different viruses. The antibody titer is based on a log 10 scale. The low antibody titer virus is shown in green and the high antibody titer virus is shown in red. The groups category shows the production type (B=Beef, D=Dairy), gender (F=female, M=male) and the age of the animal in years. The viruses are labeled with a “R” for respiratory virus or an “E” for an enteric virus, followed by the KSVDL accession number or name.

<b>Groups</b>	<b>Serum Number</b>	<b>R: 13-149008</b>	<b>R: 10-78481</b>	<b>E: BCoV Mebus</b>	<b>E: 14-007320 calf 1</b>	<b>R: 13-149100</b>
B, F, 0.75y	<b>26</b>	40	20	20	12	40
B, F, 0.75y	<b>29</b>	140	130	60	70	240
B, F, 2y	<b>54</b>	640	500	140	240	160
B, F, 5 y	<b>59</b>	3200	1280	1280	320	1760
B, F, Adult	<b>61</b>	280	740	160	480	200
B, F, Adult	<b>62</b>	640	740	200	340	200
B, F, Adult	<b>64</b>	2240	1280	400	800	560
D, F, 1.6y	<b>65</b>	800	960	320	560	200
D, F, 4.5y	<b>70</b>	640	1260	280	560	320
D, F, 4.5y	<b>71</b>	960	2000	400	520	400
B, F, 6.5y	<b>80</b>	50	130	35	240	180
B, F, 6.5y	<b>82</b>	90	230	50	200	200
B, F, 1.5y	<b>101</b>	160	600	240	1080	540
B, F, 8y	<b>107</b>	400	560	480	1680	1120
B, F, 1.5y	<b>111</b>	1920	560	1280	2880	480
B, F, 0.5y	<b>126</b>	1600	480	320	320	480
B, F, 2y	<b>133</b>	1920	320	2240	2560	960
B, F, 2y	<b>134</b>	1280	640	2560	2880	960

## **Chapter 4 - Discussion and Conclusion**

In the past BCoV was associated primarily with enteric disease in calves but more recently, it has also been isolated in adult cattle with respiratory disease. This raises the question: Are the respiratory coronavirus isolates and the enteric coronavirus isolates different and how do they compare to the vaccine strain? This study evaluates the diversity of the BCoV isolates via sequencing and serum neutralization assays.

### **Sequencing**

In this study, 11 coronavirus isolates were sequenced using Next Generation sequencing. The full genomes were analyzed with a focus on the spike and hemagglutinin esterase segments. Isolates were selected based on the tissue they were isolated from, their ability to grow in cell culture and the cycle threshold (CT) values based on RT-PCR that were determined by KSVDL. The virus isolates that were to be sequenced demonstrated that there is slight genomic difference between the different respiratory and enteric isolates based on the phylogenetic tree group clustering.

Also, looking at the amino acids of each sequence showed that there are few differences in the structural proteins. Spike had the most amino acid changes followed by HE, which is to be expected since these regions are not very conserved. By not being as conserved they have more mutations that could lead to a change in the structure and would allow the virus to bind to different receptors. For the more conserved regions, the envelope protein had two amino acid changes but neither had a significant change in charge or hydrophobicity. The membrane protein also had two amino acid changes with both being a significant change. Lastly the nucleocapsid had one amino acid change that was significant. Because these regions are more conserved we expected less change. They also will not play as big of a role in virus diversity since they don't contribute to attachment and entry of the virus.

### **Assays**

To further investigate the isolate differences, serum neutralization assays were performed. Analysis of the SN assays showed that only 12% of the viruses showed a greater than

four-fold increase between two viruses. From what we can analyze, there is not a significant difference between the neutralization of the enteric and respiratory isolates. The serum neutralization assays measure the amount of neutralizing antibody within the serum. It is hard to make a full analysis because the vaccination history and background of the animals is unknown. Also the animal might be making cross protective antibodies.

There was a plan to make a killed vaccine with the selected isolates and inject them into guinea pigs to create monospecific antisera. The antisera would then be put against the other viruses to see if the sera against the respiratory virus could neutralize the enteric virus and vice versa. However, we were unable to complete this portion based on an inability to grow the virus to a high enough titer for vaccine development.

### **Future work**

For a better understanding of the virus's receptor attachment, additional investigation needs to be done on the location of these amino acids. If these amino acids are on the surface of the protein, then there might be a difference in the conformation of the proteins. Changes in the protein conformation allows the virus to attach to different receptors on cells or to not bind at all.

Also, an additional study can be done, using an animal model to examine the neutralizing capabilities of monospecific serum to the different isolates. This study could reveal valuable information on the neutralizing capabilities of monospecific serum against the different viruses. It would also be interesting to test generate monospecific serum to the Mebus vaccine strain to see if it has the greatest neutralizing capabilities.

With coronavirus outbreaks on the rise, it is more important to remain diligent in our research to find out more about the virus. Continuing to strive to understand more about the mechanisms of replication and how different factors such as virulence and tropism play a role in how the virus interacts with the host's immune response. This will further help the research going to improvement of vaccine approaches and antiviral therapies for animal and human viruses.

## Bibliography

- Allander, T., Emerson, S.U., Engle, R.E., Purcell, R.H., and Bukh, J. (2001). A virus discovery method incorporating DNase treatment and its application to the identification of two bovine parvovirus species. *Proc. Natl. Acad. Sci. U. S. A.* *98*, 11609–11614.
- Athanassious, R., Marsolais, G., Assaf, R., Dea, S., Descôteaux, J.P., Dulude, S., and Montpetit, C. (1994). Detection of bovine coronavirus and type A rotavirus in neonatal calf diarrhea and winter dysentery of cattle in Quebec: evaluation of three diagnostic methods. *Can. Vet. J.* *35*, 163–169.
- Belouzard, S., Millet, J.K., Licitra, B.N., and Whittaker, G.R. (2012a). Mechanisms of coronavirus cell entry mediated by the viral spike protein. *Viruses* *4*, 1011–1033.
- Belouzard, S., Millet, J.K., Licitra, B.N., and Whittaker, G.R. (2012b). Mechanisms of coronavirus cell entry mediated by the viral spike protein. *Viruses* *4*, 1011–1033.
- Bidokhti, M.R.M., Trávén, M., Krishna, N.K., Munir, M., Belák, S., Alenius, S., and Cortey, M. (2013). Evolutionary dynamics of bovine coronaviruses: natural selection pattern of the spike gene implies adaptive evolution of the strains. *J. Gen. Virol.* *94*, 2036–2049.
- Boileau, M.J., and Kapil, S. (2010). Bovine coronavirus associated syndromes. *Vet. Clin. North Am. Food Anim. Pract.* *26*, 123–146, table of contents.
- Campbell, S.G., and Cookingham, C.A. (1978). The enigma of winter dysentery. *Cornell Vet.* *68*, 423–441.
- Chau, T.-N., Lee, K.-C., Yao, H., Tsang, T.-Y., Chow, T.-C., Yeung, Y.-C., Choi, K.-W., Tso, Y.-K., Lau, T., Lai, S.-T., et al. (2004). SARS-associated viral hepatitis caused by a novel coronavirus: report of three cases. *Hepatology* *39*, 302–310.
- Cheever, F.S., Daniels, J.B., Pappenheimer, A.M., and Bailey, O.T. (1949). A MURINE VIRUS (JHM) CAUSING DISSEMINATED ENCEPHALOMYELITIS WITH EXTENSIVE DESTRUCTION OF MYELIN. *J. Exp. Med.* *90*, 181–194.
- Cheng, V.C.C., Lau, S.K.P., Woo, P.C.Y., and Yuen, K.Y. (2007). Severe acute respiratory syndrome coronavirus as an agent of emerging and reemerging infection. *Clin. Microbiol. Rev.* *20*, 660–694.
- Cho, K.O., Hoet, A.E., Loerch, S.C., Wittum, T.E., and Saif, L.J. (2001). Evaluation of concurrent shedding of bovine coronavirus via the respiratory tract and enteric route in feedlot cattle. *Am. J. Vet. Res.* *62*, 1436–1441.
- Decaro, N., Martella, V., Elia, G., Campolo, M., Mari, V., Desario, C., Lucente, M.S., Lorusso, A., Greco, G., Corrente, M., et al. (2008). Biological and genetic analysis of a bovine-like coronavirus isolated from water buffalo (*Bubalus bubalis*) calves. *Virology* *370*, 213–222.

Djikeng, A., Halpin, R., Kuzmickas, R., Depasse, J., Feldblyum, J., Sengamalay, N., Afonso, C., Zhang, X., Anderson, N.G., Ghedin, E., et al. (2008). Viral genome sequencing by random priming methods. *BMC Genomics* 9, 5.

DOYLE, L.P., and HUTCHINGS, L.M. (1946). A transmissible gastroenteritis in pigs. *J. Am. Vet. Med. Assoc.* 108, 257–259.

Enjuanes, L., Almazán, F., Sola, I., and Zuñiga, S. (2006). Biochemical aspects of coronavirus replication and virus-host interaction. *Annu. Rev. Microbiol.* 60, 211–230.

Ewaschuk, J.B., Naylor, J.M., Palmer, R., Whiting, S.J., and Zello, G.A. (2004). D-lactate production and excretion in diarrheic calves. *J. Vet. Intern. Med. Am. Coll. Vet. Intern. Med.* 18, 744–747.

Fields, B., Bernard N. Fields, Peter M., MD Howley, Diane E., Ph.D. Griffin, Robert A., Ph.D. Lamb, Malcolm A., MD Martin, and Stephen E., MD Straus, David M., Ph.D. Knipe (2001). *Fields Virology*. 2.

Graham, R.L., Donaldson, E.F., and Baric, R.S. (2013). A decade after SARS: strategies for controlling emerging coronaviruses. *Nat. Rev. Microbiol.* 11, 836–848.

Hamre, D., and Procknow, J.J. (1966). A New Virus Isolated from the Human Respiratory Tract. *Exp. Biol. Med.* 121, 190–193.

Hasoksuz, M., Sreevatsan, S., Cho, K.O., Hoet, A.E., and Saif, L.J. (2002a). Molecular analysis of the S1 subunit of the spike glycoprotein of respiratory and enteric bovine coronavirus isolates. *Virus Res.* 84, 101–109.

Hasoksuz, M., Hoet, A.E., Loerch, S.C., Wittum, T.E., Nielsen, P.R., and Saif, L.J. (2002b). Detection of respiratory and enteric shedding of bovine coronaviruses in cattle in an Ohio feedlot. *J. Vet. Diagn. Investig. Off. Publ. Am. Assoc. Vet. Lab. Diagn. Inc* 14, 308–313.

Herrler, G., Szepanski, S., and Schultze, B. (1991). 9-O-acetylated sialic acid, a receptor determinant for influenza C virus and coronaviruses. *Behring Inst. Mitt.* 177–184.

Hilgenfeld, R., and Peiris, M. (2013). From SARS to MERS: 10 years of research on highly pathogenic human coronaviruses. *Antiviral Res.* 100, 286–295.

Hofmann, M.A., Sethna, P.B., and Brian, D.A. (1990). Bovine coronavirus mRNA replication continues throughout persistent infection in cell culture. *J. Virol.* 64, 4108–4114.

Hudson, C.B., and Beaudette, F.R. (1932). INFECTION OF THE CLOACA WITH THE VIRUS OF INFECTIOUS BRONCHITIS. *Science* 76, 34.

Hurst, K.R., Koetzner, C.A., and Masters, P.S. (2013). Characterization of a critical interaction between the coronavirus nucleocapsid protein and nonstructural protein 3 of the viral replicase-transcriptase complex. *J. Virol.* 87, 9159–9172.

- Kaneshima, T., Hohdatsu, T., Hagino, R., Hosoya, S., Nojiri, Y., Murata, M., Takano, T., Tanabe, M., Tsunemitsu, H., and Koyama, H. (2007). The infectivity and pathogenicity of a group 2 bovine coronavirus in pups. *J. Vet. Med. Sci. Jpn. Soc. Vet. Sci.* *69*, 301–303.
- Klein, A., Krishna, M., Varki, N.M., and Varki, A. (1994). 9-O-acetylated sialic acids have widespread but selective expression: analysis using a chimeric dual-function probe derived from influenza C hemagglutinin-esterase. *Proc. Natl. Acad. Sci. U. S. A.* *91*, 7782–7786.
- Van Kruiningen, H.J., Hiestand, L., Hill, D.L., Tilton, R.C., and Ryan, R.W. (1985). Winter dysentery in dairy cattle: recent findings. *Compend. Contin. Educ. Pract. Vet. USA.*
- Lai, M.M., and Cavanagh, D. (1997). The molecular biology of coronaviruses. *Adv. Virus Res.* *48*, 1–100.
- Lai, M.M., Baric, R.S., Makino, S., Keck, J.G., Egbert, J., Leibowitz, J.L., and Stohlman, S.A. (1985). Recombination between nonsegmented RNA genomes of murine coronaviruses. *J. Virol.* *56*, 449–456.
- Leung, W.K., To, K.-F., Chan, P.K.S., Chan, H.L.Y., Wu, A.K.L., Lee, N., Yuen, K.Y., and Sung, J.J.Y. (2003). Enteric involvement of severe acute respiratory syndrome-associated coronavirus infection. *Gastroenterology* *125*, 1011–1017.
- Martínez, N., Brandão, P.E., de Souza, S.P., Barrera, M., Santana, N., de Arce, H.D., and Pérez, L.J. (2012). Molecular and phylogenetic analysis of bovine coronavirus based on the spike glycoprotein gene. *Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis.* *12*, 1870–1878.
- Mebus, C.A., Stair, E.L., Rhodes, M.B., and Twiehaus, M.J. (1973). Pathology of Neonatal Calf Diarrhea Induced by a Coronavirus-Like Agent. *Vet. Pathol. Online* *10*, 45–64.
- Neill, J.D., Bayles, D.O., and Ridpath, J.F. (2014). Simultaneous rapid sequencing of multiple RNA virus genomes. *J. Virol. Methods* *201*, 68–72.
- Neuman, B.W., Kiss, G., Kunding, A.H., Bhella, D., Baksh, M.F., Connelly, S., Droese, B., Klaus, J.P., Makino, S., Sawicki, S.G., et al. (2011). A structural analysis of M protein in coronavirus assembly and morphology. *J. Struct. Biol.* *174*, 11–22.
- Perlman, S., and Netland, J. (2009). Coronaviruses post-SARS: update on replication and pathogenesis. *Nat. Rev. Microbiol.* *7*, 439–450.
- Plummer, P.J., Rohrbach, B.W., Daugherty, R.A., Daugherty, R.A., Thomas, K.V., Wilkes, R.P., Duggan, F.E., and Kennedy, M.A. (2004). Effect of intranasal vaccination against bovine enteric coronavirus on the occurrence of respiratory tract disease in a commercial backgrounding feedlot. *J. Am. Vet. Med. Assoc.* *225*, 726–731.
- Radostits, O.M. (1991). The role of management and the use of vaccines in the control of acute undifferentiated diarrhea of newborn calves. *Can. Vet. J.* *32*, 155–159.



- Reynolds, D.J., Chasey, D., Scott, A.C., and Bridger, J.C. (1984). Evaluation of ELISA and electron microscopy for the detection of coronavirus and rotavirus in bovine faeces. *Vet. Rec.* *114*, 397–401.
- Saif, L.J. (2010). Bovine respiratory coronavirus. *Vet. Clin. North Am. Food Anim. Pract.* *26*, 349–364.
- Saif, L.J., and Smith, K.L. (1985). Enteric Viral Infections of Calves and Passive Immunity<sup>1</sup>. *J. Dairy Sci.* *68*, 206–228.
- Stadler, K., Massignani, V., Eickmann, M., Becker, S., Abrignani, S., Klenk, H.-D., and Rappuoli, R. (2003). SARS — beginning to understand a new virus. *Nat. Rev. Microbiol.* *1*, 209–218.
- Storz, J., Purdy, C.W., Lin, X., Burrell, M., Truax, R.E., Briggs, R.E., Frank, G.H., and Loan, R.W. (2000). Isolation of respiratory bovine coronavirus, other cytocidal viruses, and *Pasteurella* spp from cattle involved in two natural outbreaks of shipping fever. *J. Am. Vet. Med. Assoc.* *216*, 1599–1604.
- Tang, B.S.F., Chan, K.-H., Cheng, V.C.C., Woo, P.C.Y., Lau, S.K.P., Lam, C.C.K., Chan, T.-L., Wu, A.K.L., Hung, I.F.N., Leung, S.-Y., et al. (2005). Comparative host gene transcription by microarray analysis early after infection of the Huh7 cell line by severe acute respiratory syndrome coronavirus and human coronavirus 229E. *J. Virol.* *79*, 6180–6193.
- Thomas, C.J., Hoet, A.E., Sreevatsan, S., Wittum, T.E., Briggs, R.E., Duff, G.C., and Saif, L.J. (2006). Transmission of bovine coronavirus and serologic responses in feedlot calves under field conditions. *Am. J. Vet. Res.* *67*, 1412–1420.
- Tyrrell, D.A., and Bynoe, M.L. (1966). Cultivation of viruses from a high proportion of patients with colds. *Lancet* *1*, 76–77.
- Weiss, S.R., and Navas-Martin, S. (2005). Coronavirus Pathogenesis and the Emerging Pathogen Severe Acute Respiratory Syndrome Coronavirus. *Microbiol. Mol. Biol. Rev.* *69*, 635–664.
- Woo, P.C.Y., Lau, S.K.P., Huang, Y., and Yuen, K.-Y. (2009). Coronavirus Diversity, Phylogeny and Interspecies Jumping. *Exp. Biol. Med.* *234*, 1117–1127.
- Woo, P.C.Y., Huang, Y., Lau, S.K.P., and Yuen, K.-Y. (2010). Coronavirus genomics and bioinformatics analysis. *Viruses* *2*, 1804–1820.
- Woo, P.C.Y., Lau, S.K.P., Lam, C.S.F., Lau, C.C.Y., Tsang, A.K.L., Lau, J.H.N., Bai, R., Teng, J.L.L., Tsang, C.C.C., Wang, M., et al. (2012). Discovery of seven novel mammalian and avian coronaviruses in Deltacoronavirus supports bat coronaviruses as the gene source of Alphacoronavirus and Betacoronavirus and avian coronaviruses as the gene source of Gammacoronavirus and Deltacoronavirus. *J. Virol.* *JVI.06540–11*.

Zaki, A.M., van Boheemen, S., Bestebroer, T.M., Osterhaus, A.D.M.E., and Fouchier, R.A.M. (2012). Isolation of a Novel Coronavirus from a Man with Pneumonia in Saudi Arabia. *N. Engl. J. Med.* 367, 1814–1820.

(1975). Vaccine for neonatal calf diarrhea.

(2009). Vaccination of Cattle and Calves on U.S. Beef Cow-calf Operations.