SEROLOGIC AND GENOTYPIC STUDIES OF THE SPIKE GLYCOPROTEIN OF TURKEY CORONAVIRUS

by

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(Under the Direction of Mark Jackwood)

ABSTRACT

The serologic and genotypic relationship of different turkey coronavirus isolates (TCoV) was studied. Recent analysis of available sequence data suggest the presence a hypervariable region (HVR) within the S1 subunit of the spike gene in TCoV. The effect of this region on serotype is unknown. We examined the mutation rate of the putative HVR by studying its sequence from serial passages of each isolate in SPF embryonating turkey eggs. Mutations in one strain were found to be localized to this region and another region upstream, rather than randomly placed throughout the 906 bp sequence. To study the serologic relationships, a novel means of virus neutralization was also developed. Antisera was raised against different TCoV isolates and used in homologous and heterologous neutralization reactions. Neutralization was determined by presence of virus quantified by real-time RT-PCR. Results indicate that a

serological difference exists between strains, suggesting that serotypes exist within TCoV isolates.

INDEX WORDS: Turkey Coronavirus, Mutation Rate, Virus Neutralization, Serotype, Genotype, S1 Gene, Spike Glycoprotein, real-time RT-PCR

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DEDICATION

This this is dedicated to a very special person. You know who you are. Thank you for always being there when I needed you, and always answering when called upon. Your friendship has meant more to me than anything I can imagine. And remember, no matter how far away, you'll always be my Scully.

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I would like to acknowledge the following:

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INTRODUCTION AND OBJECTIVES

Between the years 1950 and 1970, a contagious disease emerged that affected the major turkey producers in the United States, causing severe economic losses. Characterized by diarrhea, inappetance, ruffled feathers, decreased body weight, and an altogether loss in production, this disease became known as acute enteritis of turkeys, and while mortality varied greatly, the morbidity was high. In 1973, two separate groups of researchers determined that the causative agent was a coronavirus, now known as turkey coronavirus.

Turkey coronavirus is a positive sense RNA virus in the family *Coronaviridae*, who along with *Arteriviridae* make up the order *Nidovirales*. Coronaviruses are further classified into three groups, based on immunochemistry and sequence similarity. Human coronaviruses fall under Group I, and others that contain a hemagglutinin esterase gene such as bovine coronavirus belong to Group II. Group III, the final group, contains only infectious bronchitis virus, which infects chickens, and turkey coronavirus.

Infectious bronchitis virus is the closest relative to turkey coronavirus, and is known to have many serotypes, which has constantly posed a problem in creating suitable vaccines

against it. A structural gene called the spike gene encodes a protein that is responsible for the attachment and entry of the virus into host cells. The spike protein is also thought to be responsible for different serotypes, as it contains two distinct hypervariable regions.

Recent data now suggests that a hypervariable region exists in the spike protein of turkey coronavirus as well. The purpose of this thesis is to study the impact of this region on the virus, as well as to find a better means of propagating the virus *in vitro*.

The objectives of this project are as follows: 1)To determine the rate at which the proposed hypervariable region mutates, 2) Determine the impact of the hypervariable region on serotype, and 3)Adapt turkey coronavirus to an *in vitro* laboratory system for propagation.

CHAPTER I: LITERATURE REVIEW

Part 1. Transmissible Coronaviral Enteritis of Turkeys.

Transmissible coronaviral enteritis (TCE) is an acute, contagious disease infecting the intestinal tract of turkeys causing catarrhal enteritis. TCE is synonymous with mud fever, Bluecomb disease of turkeys, transmissible enteritis of turkeys, and TCV enteritis of turkeys.

TCE is characterized by diarrhea, inappetance, ruffled feathers, and decreased body weight.[35] Lesions are seen primarily in the intestines, but can be observed in the bursa of fabricius as well.[33] Once introduced into areas highly populated with turkeys, TCE is not easily eliminated and frequently isolated in young poults.[74] Age plays an important role in the severity of the disease[43], with young birds being more susceptible, though birds of all ages can be infected and develop life-long immune protection.[73]

While flocks infected with TCE exhibit morbidity ranges as high as 100%[74,43], mortality among birds varies significantly due to environmental and host factors. Secondary infections of other viruses and bacteria also contribute greatly to mortality changes. One side-effect of TCE is a dramatic decrease in the

ability of the host to clear bacterial infections, which contributes to the severity of the disease.[40]

The major concern of TCE is economic losses. Besides decreased weight and stunted growth, the disease also causes a decrease in egg production and a lower quality in those that are produced.[35] In its first year in 1951, TCE cost the industry roughly one million dollars in losses[74], and was considered the most costly disease affecting the industry for twenty more years.[53]

History of TCE.

In 1951, reports emerged of a new disease in Minnesota, a major turkey growing area in North America, locally known as mud fever.[74] Over a few years, the disease spread from Minnesota to other major turkey producing areas in the Midwest, as well as to areas in Canada.[28] Clinical reports were similar to a disease in chickens known as Bluecomb disease, and the name mud fever gave way to Bluecomb[74,78].

By the mid-1960's, it was demonstrated that a filterable agent was responsible for TCE. First thought to be a vibrio isolate[86], pure cultures failed to reproduce the disease when inoculated into poults[1], indicating the presence of another agent that contaminated the initial vibrio cultures. These studies determined that the etiological agent could pass through as low as .22 μm filter and still retain infectivity.[85]

At the beginning of the seventies, TCE had become much more prevalent and affected most of the major turkey producers in the U.S., as well as in Canada.[25,35] The etiological agent was still not known. In 1973 Ritchie et al. finally determined the etiology to be a virus with similar morphological characteristics to coronaviruses[75,] which had just recently been discovered in 1968[4], that responded to hyperimmune anti-TCE serum, but not negative turkey sera. These findings led to the classification of the virus as simply turkey coronavirus (TCoV)[35], and with it, a new name for the disease. Bluecomb became TCE, though some reports will still use the old nomenclature.

Throughout the next twenty years, controversy erupted over the antigenic relationship of the virus. At first it was thought to be a group II coronavirus, though it has now been determined to be a member of the group III coronaviruses[34], and a close relative to infectious bronchitis virus of chickens.

Disease Hosts.

TCE infects the intestines and bursa of fabricius in turkeys of all ages, but is clinically observed mainly in young poults. No other tissues are known to be infected, including trachea, lung, and kidney.[75] Turkeys are the only known host to experience the disease and present clinical signs. Quail, sea gulls, and hamsters have been shown to all be negative for

infection.[35] Chickens are considered to be refractory when presented with TCoV, but recent literature suggests that it does infect and replicate, but no clinical signs are observed and the virus is cleared easily by the host immune system.[36] SPF chicks at one day of age seroconvert and virus can be detected in intestines and bursa of fabricius 2-8 days after inoculation.

Transmission and Vectors.

Horizontal transmission of TCE occurs when infected feces are consumed by uninfected turkeys. Vertical transmission has not been reported to occur. Turkeys continue to shed the virus in feces for up to 4 weeks after clinical signs disappear, and TCoV can be detected up to 7 weeks after inoculation.[8]

Another important means of transmission occurs mechanically. Domestic houseflies have been proven to serve as a vector for passing TCoV from bird to bird.[12] When fed infectious materials, concentrations as low as one fly per bird result in infection of experimental flocks. The means of this transmission is not known, however it is proven to be mechanical as no TCoV can be recovered from surface-sterilized flies. Transmission most likely occurs from consumption of the flies, but could also be due to their contaminating food and water and equipment in the controlled environment.

Another mechanical vector proven for TCE is the darkling beetle.[91] In an experiment almost identical to the houseflies,

beetles were fed contaminated feed, and whole beetle homogenates then fed to the turkeys. Surface sterilization of the beetles and inoculations with beetle intestinal homogenates effectively neutralize transmission. It is clear that mechanical transmission occurs, which also applies to contaminated fomites and personnel.[35]

Incubation and Symptomatic Period.

The incubation period of TCE can vary from 1-5 days, but is typically in the 2-3 day range.[35] This is supported by the calculated propagation time in turkey embryos of 3 days.[76] Once clinical signs manifest themselves, the course of the disease can run from 10-14 days. Some birds do not regain weight lost until several weeks later. Toms may remain very thin and emaciated, and some birds may never regain weight satisfactory for marketing.[74]

Clinical Signs and Lesions.

In poults, the first signs of TCE are usually listlessness and an apparent loss in weight. Constant chirping has also been observed.[78] Droppings appear watery in consistency and vary from greenish to yellowish brown, and may be frothy and full of urates and mucus.[74] Ruffled feathers and inappetance also occurs after the onset of diarrhea.[35]

An acute catarrhal inflammation of all or most of the intestinal tract mucosal membranes is observed upon necropsy.[79]

The duodenum, cecum, and free portion of the small intestine are observed to be gaseous and full of frothy liquid. Further, bulbous areas indicative of gas can often be seen along the intestinal tract.[74] All other tissues and areas are unaffected and appear normal.

In older flocks, the appearance of TCE is sudden, and birds that appear healthy can shift overnight to inappetance and lethargy. The head and skin of adult birds can darken during disease progression, and flesh will appear dehydrated due to diarrhea. Droppings will appear the same as in poults, as will intestinal contents. The dehydration can also affect other abdominal organs.

Microscopically, a decrease in villous length, increase in crypt depth, and decreased intestinal diameter are all observed.[33,35] Epithelial damage occurs by desquamation of absorptive epithelium and exposure of the lamina propria. Microvilli cannot be seen in more severe cases of TCE. Epithelial cells lose their high columnar appearance and become low cuboidal.[33] These effects begin two days post-inoculation (PI), and last until six days PI, when the recovery phase commences.[5]

The bursa of fabricius experiences similar histopathological changes, shifting from a tall columnar structure to squamous epithelium. Bursas also undergo necrosis

of follicular and interfollicular epithelium along with the presence of heterophils in epithelium and adjacent lamina propria.[35,38] Lymphocyte depletion is observed mildly in TCoV infections, but increases dramatically with bacterial coinfections.[38]

Virulence of TCE.

As previously stated, the severity of TCE depends on environmental and host factors. Weather, age, concurrent infections, and host stress all play a part.[35,74] As with most diseases, immunosuppresion increases severity and susceptibility to TCE.[55]

Scanning electron microscopy of birds with TCE reveals that in negative samples the villi in the intestine of younger birds appears finger-shaped, and in older poults takes on a tongue shape instead.[33] While there is no direct evidence linking this natural effect to the virulence of TCE, it is thought to play some part in the fact that the disease presents itself worse in young poults.

Concurrent infection with enteropathogenic *Escherichia coli* (EPEC) also increases the severity of the disease. The virus and the bacteria exhibit a synergistic relationship with each other, increasing the severity of both, which would be otherwise be mild separately.[38,68] EPEC, in its normal hosts, induces attaching and effacing (AE) lesions in the intestines.

Experimental inoculation of EPEC alone in turkeys conferred no clinical signs or AE characteristic of the disease.[68] In these same experiments, only mild growth depression was seen with TCoV alone.[38] When infected together AE occurred and sever necrosis was seen at the sight of bacterial attachment. Severe enteritis was observed, as well as mortality rates of 79%.[38] In addition, a significant increase in the shedding of EPEC and TCoV was noticed in concurrent infections.

Severity of TCE can also be affected by weather. The very first outbreak of TCE in 1951 was aggravated by bad weather conditions during the period when the disease was most prevalent. In areas where TCE appeared, almost every flock in the area became infected within 2-4 weeks. During the second out break in 1952, conditions were milder and prevailed throughout the outbreak. The resulting losses seen were much less severe than the first cases, both economically and in mortality.[74]

Immunity to TCoV.

Because of the low mortality rate caused by transmissible coronaviral enteritis of turkeys, many studies have been done involving immunity to the disease. Recovering flocks are known to establish life-long immunity[35]. Age does not seem to be a factor in immunity, as poults infected at seven days of age illicit the same response to natural infection as older birds[73],

though the severity of the disease decreases as birds get older.[46] In some cases, a carrier state has been observed after infection.[46]

Innate immunity involving inflammation of cells and heavy macrophage recruitment occurs soon after infection is established in the intestine. While being the first immune response mounted, this does little to clear the infectious agent. Turkey coronavirus causes the recruited macrophages to lose much of their overall phagocytic function in a mechanism that is not fully understood.[40] This in turn decreases the cell's ability to uptake bacterial and viral antigens and clear them[40], allowing bacterial infections to occur and persist. This relationship between TCoV and bacterial infection is evident in the many cases where *E. Coli* is found as a co-agent in diseases such as poult enteritis and mortality syndrome.

IgM antibodies are evident at seven days after infection occurs, and soon undergo isotype switching to IgG. There is an apparent negative relationship between these antibodies[55,56], proving their role in the eradication of infection. These antibodies are observed in the initial phase of infection, but soon after cannot be detected[63] however, indicating they alone cannot provide the protective immunity described.

Instead, protective immunity is gained from secretory IgA immunoglobins that are produced in the intestinal mucosa.[46,54,62,63]

These antibodies also negatively correlate with TCoV in infected intestines[54], and are known to be long-lived. Unlike serum antibodies, mucosal IgA can be detected much later[63]. These antibodies are localized to the lamina propria of the intestines and are secreted there and in bile[62].

Cell mediated immunity also plays a role in overcoming disease. In T-cell activation experiments, lymphocytes from uninfected birds do not respond to antigen stimulation, while those of infected flocks undergo in vitro transformation upon exposure to purified TCoV antigen.[56] In natural infection, this activity remains high after serum antibodies disappear.[56] The intestinal lamina propria is thought to serve as one source of T lymphocytes when the host becomes infected.[64] This mechanism is plausible, since peripheral relocation would provide cell mediated immunity to any agent crossing over the mucosal surface.

Attempts to protect birds from infection by means of vaccination have proved difficult. Killed vaccines are reported to provide little to no protection[73], due to the necessity for a constant pressure of intracellular virus in the intestine. Attenuated vaccines do provide a means of protection, but end in a carrier state of the flock, and abnormal losses will still occur.[73]

Passive immunity has not been adequately shown or researched[46], though there are some reports. Subcutaneous inoculation of poults with sera from immune birds is known to not protect from challenge, which is not surprising given that secretory IgA would not be collected this way. Poults from immune and non-immune breeder hens were equally susceptible when challenged with TCoV.

Management and Prevention.

Since no licensed vaccine exists for TCE, the preferred means of controlling spread is prevention. TCoV is shed in feces for quite some time after recovery, and remain viable sources of infection for other birds. A good biosecurity program is the only way to prevent spread. Infected birds should be depopulated, followed by a complete disinfection of all premises. Turkeys or poults should not be placed for at least 3-4 weeks following clean-up.[35]

Initial publications concerning bluecomb report the use of antibiotics as viable means of alleviating TCE[74,78,79]. Treatment with antibiotics was shown to greatly reduce mortality. These initial publications most likely show the antibiotics to be useful in controlling a concurrent secondary bacterial infection that was also present in samples, as antibiotics do not affect TCoV.

Other Diseases Associated with TCE and TCoV Infection.

Besides TCE, TCoV is found associated with other diseases that express similar manifestations in turkeys. The first is spiking mortality of turkeys (SMT). SMT is characterized by signs mostly identical to TCE. The distinction between them is severe bursal and thymic atrophy and a instantaneous increase in mortality in poults uncharacteristic of TCE.[10,11] The etiological agents involved in this disease are not fully understood, and isolations yield many different organisms. Statistically, however, TCoV is found more often than any other disease agent.[11] It has been suggested that SMT could be caused by a variant of TCoV, much more virulent than its counterparts.[10]

The most significant disease associated with TCoV besides TCE is poult enteritis and mortality syndrome (PEMS). PEMS is economically devastating because of high mortality. In areas where TCE is prevalent, PEMS cases are likewise frequent.[15] As with SMT, the etiology of PEMS is also not quite understood. A small round virus, TCoV, EPEC, and Rotaviruses are known to play a part.[93] TCoV alone only causes TCE, and the SRV alone produced a milder form of PEMS where mortality is not as high.[93] EPEC is also isolated in 83% percent of PEMS cases[67], and its concurrent role with TCoV has already been described. While Yu et al. found that TCoV was present in 36 flocks from 22 farms positive for PEMS[93], Carver et al. reported that TCoV is neither necessary

nor sufficient to cause PEMS.[15] This apparent contradiction has yet to be sorted out, and TCoV is still thought be a determinant in PEMS.

Part II. Turkey Coronavirus.

Turkey coronavirus is the etiological agent of TCE. Coronaviruses (CoVs) are members of the family *Coronaviridae*, and of the order *Nidovirales*. This order is composed of enveloped viruses with linear, nonsegmented, positive sense RNA genomes that replicate with a characteristic 'nested set' of mRNAs.[47] CoVs are now known to cause the majority of human cases known as the common cold, and recently become more popular due to the discovery of a new coronavirus that induces the disease severe acute respiratory syndrome (SARS).

Morphology and Biochemical properties.

Turkey coronavirus are roughly spheroid in shape, with a diameter of 50-200 nm, though they can form pleomorphic filamentous structures as well.[2,75] Upon negative staining, the envelopes bear large tubular projections ~13 nm long and ~9nm wide.[2] These projections are uniform and closely spaced, and appear club- or pear-shaped.[75] They are now known to be the major surface protein of coronaviruses, the spike protein. The structural proteins on the envelope of the virus give it a crown-like appearance, attributing to its Latin name, *corona*. In ruptured virions, negative staining reveals helical rods in

the center, a complex of nucleocapsid protein tightly bound to genomic RNA.[2,75] In a sucrose gradient, TCoV has been found to have density of 1.24 g/cm^3.[2,75]

Chemically, the virus is know to be easily destroyed by chloroform, butanol, and other lipid solvents, which disrupt the lipid envelope.[2,69] It can also be inactivated by acid pH, being stable at pH 5.0 and 7.0, but not at a pH of 3.0 or lower.[75] Heating to 50^o Celsius for 1 hour also inactivates the virus, and the addition of 1M $MgCl_2$ enhances this heat inactivation.[30] Antibiotic sensitivity has also been reported for TCoV[79], however it is generally assumed to alleviate secondary bacterial infections rather than have an affect on the disease it self.

In 1973, original studies of TCoV isolated by EM reported that no hemagglutination was seen with erythrocytes of goose, duck, turkey, chicken, mouse, guinea pug, sheep, ox, or human type O.[2] This is important to note, because in the mid-eighties reports would indicate that TCoV in fact did hemagglutinate red blood cells.[24,83] Recent studies with TCoV contain no mention of the presence of hemagglutination either. The contradictory results are usually explained as contamination with bovine coronavirus (BCV).

Genome.

The genome of TCoV consists of a single-stranded linear piece of viral RNA (vRNA). The vRNA is capped, polyadenylated

positive sense, and contains ~30,000 nucleotides.[47] Since it is positive sense, the genome itself is infectious when transfected into host cells without viral proteins.[47] The 5′ beginning of the genome contains a leader sequence of ~75 nulceotides followed by an untranslated region (UTR) of 200-400 basepairs (bp). At the 3′ end of the genome, another UTR of 300-500 bp followed by a long poly-adenosine tail is found. The sequence of UTRs have been shown to be important in RNA replication.[22,47] The genome consists of 10 open reading frames (ORFs) encoding four structural proteins, a polymerase, and several small non-structural proteins whose functions are poorly understood.

Replication and Virion Assembly.

No studies have been done in the replication of TCoV itself, but much has been learned from IBV and coronaviruses in general. The following paragraphs are a summary of the system presented in the *Coronaviridae* section of Field's Virology.[47]

The first step in the life cycle of a coronavirus is attachment to the host cell. Surface proteins on the envelope bind to specific receptors on the host cell, though the exact receptor of TCoV is not known. Once bound, the membrane of the host cell and the envelope of the virus fuse, unloading the vRNA into the host cell and exposing it to translational machinery.

Once inside, a negative template strand is synthesized from the positive-sense vRNA, and several subgenomic vRNA strands are

produced from it, as well as replication of the full genome. The generation of these subgenomic RNAs is not fully understood, but several theories exist. The predominant theory is leader-primed transcription (LPT). The leader sequence of the genomic vRNA contains a sequence homologous for intergenic sequences found throughout the rest of the genome. In LPT, it is thought that this leader sequence is transcribed from the 3′ end of the negative template, and then acts as a primer and jumps to the intergenic sequences to produce the subgenomic RNAs, much like primers function in PCR.

Once the subgenomic RNAs are created, the open reading frames in each one are translated, and the structural proteins are packaged into the membranes of the rough endoplasmic reticulum and golgi apparatus. The nucleocapsid protein binds the genomic vRNA and is most likely recruited in the budding compartment between the RER and Golgi, forming smooth-walled vesicles containing virus. Virus is released when vesicles undergo exocytosis with the plasma membrane of the host cell.

TCoV Genes and Proteins.

The six mRNAs of TCoV are sorted based on size, 1 being the largest, and 6 the smallest. The first RNA encodes two ORFs, 1a and 1b, which eventually form the polymerase protein. The second encodes 1 ORF which is post-translationally cleaved into the two spike proteins, S1 and S2. The third contains

three ORFs, 3a, 3b and 3c.[49] 3c is now known as the envelope (E) protein. Gene 4 is translated into a transmembrane protein called the matrix (M), while gene 5 is dicistronic and the function of the two proteins it encodes, 5a and 5b, are not understood.[48] The final subgenomic RNA contains the nucleocapsid gene (N).

Polymerase Protein: The two precursor proteins 1a and 1b are the first proteins to be translated upon infection, as the final polymerase product of these genes is required to synthesize the full length genome and subgenomic RNAs. 1a and 1b both contain protease domains and are post-translationally cleaved into multiple proteins. 1b is thought to contain the domain responsible for polymerase activity. The final protein formed is a RNA-dependant RNA polymerase.[47] The polymerase of IBV is known to lack proof-reading ability, and accounts for the genetic differences seen in isolates.[14]

Spike Protein: Gene 2 is translated into one large precursor protein that is cleaved into two subunits post-translationally, S1 and S2. The protein is known to be heavily glycosylated and ranges from 150-180 kd. The S2 subunit anchors the spike protein into the membrane, while S1 form the globular head and is known to be the major antigenic determinant[13], and induces protective immunity.[41] In mouse hepatitis virus, SARS-CoV, and the human CoV OC43 the S2 subunit has been shown to contain an

aromatic domain proposed to be positioned on top of the viral envelope responsible for disrupting the target cell membrane and inducing membrane to membrane fusion.[92] Since S1 is known to be highly variable and a major antigenic determinant, it is responsible for the existence of serotypes in infectious bronchitis virus (IBV). Evidence exists that the S protein forms and oligomer on the surface of virions, most likely as a trimer.[47] Removal of glycosylation from the spike and other membrane bound proteins still results in virus particle formation, but infectivity is lost.[82]

Envelope and Membrane Proteins: The E protein, once known as the small membrane (sM) protein, and the Matrix protein are both anchored into the membrane of the virus particle. They are both required for the budding of virus to form mature virions.[72] Only a short amino-terminal domain of the M protein is exposed on the viral envelope, as the rest makes up a triple membrane spanning motif and a large carboxy-terminal end, which is located inside the virus.[47] The first membrane spanning helix was found to be responsible for targeting to the cis-golgi complex during replication.[57]

Nucleocapsid Protein: The N protein is a 50 kd phosphoprotein that binds tightly with vRNA to form the nucleocapsid core of the virus. It functions like histones, to bind and stabilize the otherwise unstable genomic RNA. It also binds to the

carboxy-terminal end of the M protein, targeting the nucleocapsid core to be incorporated into mature virions.[72]

Other Nonstructural Proteins: The functions of proteins 3a, 3b, 5a, and 5b are not fully understood. 5a is known to not be essential for IBV replication in cell cultures.[48] A truncated form of 3b was sequenced in another cell culture adapted IBV, indicating it may not be necessary either.[72] In IBV, 3a was recently discovered to be localized to a novel domain of the smooth endoplasmic reticulum. It is thought that IBV anchors its replication machinery to ER membranes, and it is possible that 3a is necessary in the formation of these complexes. Unfortunately, deletions in 3a have not been reported to be chararcterized.[72]

Propagation of TCoV.

Propagation of turkey coronavirus (TCoV) is complicated and perhaps the biggest hindrance in its research to date. For IBV-like TCoV, there have been no reports of growth in immortalized cell lines, and primary intestinal cell lines yield little results.[28,31]

The classical method for propagation of TCoV is inoculation of 18-21 day old SPF turkey embryos via the yolk-sac route. After 3-4 days the intestines are collected and homogenized and then used as further inoculum.[76] Another method along the same lines involves swabbing intestines and immersing swabs in liquid

media. In adult birds, the bursa of fabricius can be collected and ground as a source of TCoV as well.[65] While these methods are efficacious, they tend to be time-consuming and not cost-effective.

It has been reported that TCoV will replicate in the HRT-18 cell line[29,76,84], but the results are confounded by the knowledge that bovine coronavirus also replicates in HRT-18 cells.[84] It is generally thought that these isolates were in fact bovine coronavirus that infected turkeys. A few avian immortal cell lines exist[45,61], but no reports of coronaviral replication exist.

The closest relative of TCoV, IBV, has been documented to grow in several cell lines, indicating that it may be possible to adapt TCoV to these as well. The Beaudette strain of IBV has been adapted to grow readily in primary chicken embryo kidney cells[21], as well as an immortalized primate line known as VERO cells.[20,32] Interestingly, another coronavirus, SARS, was found to infect Vero cells naturally with no adaption.[39] IBV also has been shown to infect feline kidney cells expressing feline aminopeptidase N (fAPN)[58]. fAPN is known to be a receptor for many group I coronaviruses.[6] IBV can also be produced via a vaccinia virus vector containing full length genomic cDNA in baby hamster kidney cells.[9]

Purification of TCoV.

TCoV can be purified using sucrose gradients to separate the virus from other proteins and contaminates found in intestinal homogenates.[66] Besides the classical sucrose gradient, TCoV can be concentrated against a 30% sucrose cushion[52], and then further purified. These methods provide problems, as ultracentrifugation is known to be fairly destructive to viral particles and their surface proteins[16], especially given the amount of glycosylation present on the surface proteins.

Size-exclusion chromatography has also been used to purify viral particles. Sephacryl S-1000 columns equilibrated with 0.02 M phosphate buffer can be used to elute homogenates and the absorbance of the fractions monitored. Fractions containing virus can then be cushioned against 60% sucrose to alleviate the effects of ultracentrifugation.[52]

Diagnosis and Detection of TCoV.

As with any disease, detection of the etiological agent is imperative. The clinical signs observed with transmissible enteritis of turkeys can be produced by other factors besides TCoV, so detection methods are essential to pinpoint coronaviral infection and rule out other means. As such, many different methods have been developed over time.

Virus Isolation

In classical virus isolation, clinical samples suspect of TCoV are collected and treated as describe in the propagation section of this review. Briefly, intestines from suspect birds are collected, and then homogenized or swabbed. The bursa of fabricius can also be used, but should always be used in conjunction with intestines. Once an inoculum is prepared from these swabs or homogenates, they must be passed through .22 μm filters to remove any bacterial contaminates, then inoculated into SPF turkey embryos or 7 day old poults.[8,23] Further tests are then needed to determine the presence of coronavirus.

Electron Microscopy

Electron microscopy (EM), a method of magnifying images 10,000x or more, was the first test that detected the presence of coronavirus particles in the intestines of flocks infected with transmissible entertis.[75] While confirmation of virions is undeniable, this method is far too labor-intensive and expensive to be used in large scale diagnostics.

Immunofluorescent Chemistry

The first means of detecting TCoV without first performing virus isolation or using EM was the direct fluorescent antibody (DFA) test.[71] Histological intestinal sections are fixed to microscope slides then incubated with antibodies directed against the virus. The slides are then incubated with anti-

turkey immunoglobins labeled with a fluorescent compound. Alternately, anti-TCoV antibodies directly labeled with fluorescent compounds can be used. Positives can then be viewed using a fluorescent microscope.

A similar test was developed to detect anti-TCoV antibodies in serum samples. Known as the indirect fluorescent antibody (IFA) test, it utilizes the same principles as DFA with a slight modification. Slides are fixed with known TCoV positive tissue, and then incubated with test serum. Fluorescent tagged anti-turkey antibodies are then bound, and visualized as with DFA.[70,71] Both tests provide a easier means of detection in large numbers of samples compared with EM, requiring less time for. Since an answer of positive or negative is desired, this test is qualitative, but not quantitative.

RT-PCR

Advances in molecular biology led to the ability for researchers to detect specific sequences of DNA in an organism's genome in a process known as the Polymerase Chain Reaction (PCR). For RNA, reverse transcriptase (RT) is needed to first synthesize DNA from the genomic RNA. In the past decade, PCR and RT-PCR have become methods of choice in most laboratories as detection tools, because of reproducibility and ease of operation.

The first RT-PCR tests reported in the literature amplified regions in the membrane and nucleocapsid genes, unfortunately these reports also report the genes as being 99% identical to reference strains of bovine coronavirus (BCoV)[88,89]. As stated elsewhere in this review, BCV infects turkeys readily and produces the same clinical signs as TCoV[42], so it is generally thought that these early publications refer to BCoV isolated from turkeys rather than TCoV.

For TCoV, RT-PCR tests have been developed for the N[87], M[8], and P[81, 87] genes as well, but most of these tests will also amplify their corresponding regions in IBV[8,87]. While IBV is not known to infect turkeys *in vivo*, these tests could still provide false positives in the event of laboratory contamination. To combat this, some researches have also included primers which are known to only amplify IBV and not TCoV as a control.[87] These tests are then compared. Studies comparing these tests to immunochemistry and VI have determined them to be much more sensitive[8], giving another advantage to RT-PCR.

A multiplex RT-PCR test has also been developed that simultaneously detects three virus types associated with poult enteritis complex (PEC), TCoV, turkey astrovirus type 2 (TAstV-2) and both turkey origin and chicken origin avian reoviruses (ARVs).[80]

While RT-PCR is qualitative, it is at best only semi-quantitative. Further advances in PCR techniques have led to a system known as real time PCR, whereby the amount of nucleic acid present can be determined after every cycle. In contrast to normal PCR, this system can be used to accurately quantitate the amount of DNA or RNA present in the initial sample.

While no reports of real time RT-PCR exist for TCoV, a highly sensitive test has been developed by Callison et al. (unpublished results). This test is directed towards the 5′ untranslated region and amplifies both TCoV and IBV.

Enzyme-Linked Immunosorbent Assay (ELISA)

Like FA, the ELISA test can be used to measure both the presence of anti-TCoV antibodies[37,53] as well as the presence of virus.[23] While similar to FA, these tests rely on an enzyme linked to final antibody rather than a fluorescent tag. This enzyme, when presented with substrate, produces a color change that can be measured. Interestingly, TCoV antigen or IBV antigen can be used in the indirect ELISA method to detect anti-TCoV antibodies[37,53], another indication that IBV and TCoV are similar.

Virus Neutralization Test

Another way to quantify antibodies against TCoV is virus neutralization. There are two forms of virus neutralization, alpha and beta. Alpha involves a constant amount of serum and

variable amounts of virus, whereas beta utilizes a constant amount of virus and variable amounts of sera. The procedure then involves mixing the sera with virus, incubating, and inoculating into specific pathogen-free (SPF) turkey embryos. Generally, 3-5 days later the embryos are evaluated for clinical signs of TCoV and scored to determine the neutralization capability of the suspect sera.[76]

This technique is rarely used, due to the time frame involved and the difficulty in obtaining SPF turkey embryos. ELISA tests are far easier to use, quicker, and require no embryo or poult; most diagnostic labs are equipped to run them. Classically, virus neutralization is used more for determining serotypes than detection of antibodies, using the Archetti-Horsefall system.[5] Though the closest relative, no serotypes have been reported for TCoV, and no research has been published examining the issue.

Hypervariable Regions and Their Importance.

Serotypes are viruses that, while they are the same species, are characterized by different sets of antigens. Viruses are placed in separate serotypes based on immunological tests. If two virus both contain the same epitopes to induce immune responses, then they are the same serotype. If different epitopes exist, then they are considered separate serotypes, even if some cross-protection occurs. These variations in

serotype hinder attempts to vaccinate chickens, as monovalent vaccines do not afford complete protection against the other serotypes.

The S1 subunit of the spike protein is known to contain virus-neutralizing epitopes. Different serotypes of IBV are thought to be the result of nucleotide insertions, deletions, or point mutations that affect these epitopes[59], caused by the viral polymerase's lack of proofreading ability. The S1 subunit has been found to contain three distinct hypervariable regions (HVR) within the spike, I (amino acid residues 38-67), II (91-141), and III (274-387).[60] Genotypical differences in HVR I have been shown to be sufficient in predicting serotypical differences for IBV Isolates alone[90], though neutralization tests should still be performed.

Recently Jackwood et al. identified a region in TCoV similar to HVR II in IBV, from amino acid resides 123-158 in the spike protein. The following is the nucleotide sequence of this region in five separate isolates of TCoV (Unpublished data):

```
    1          11         21         31         41
Gh  TGGGGCATTG ----GTACTA ATG------- -CAGATAAAA AATCCAATGA
Gl  TGGGGCATTG AAAT----TA ATG------- -CGGGTGAAG TAACCGTGGA
Hs1 TGGGGCATTG AGACGTACTC ATGGGTGTCA TCGGGTAAAC AACCCAATGA
R   TGGGGCATTA A----TACTA AAGG------ ---------- ----CAATGA
Tx  TGGGGCATTG ----GTACTA ATG------- -CAGATAAAA AATCCAATGA
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    51          61          71          81          91
Gh  TCCCATTTTT AATTTAACAT GGGGCAACTT TTTCCTTAAT T---------
Gl  TCCTACTTTT AATTTAACAT GGGGCAACTT TTTCTTTAAT TCTAAGAATT
Hs1 TCCCACTTTT AATTTAACAT GGGGCAACTT TTTCCTTAAT TCTAAGAATT
R   TCCTATTTTT AATTTAACAT GGGGCAACTT TTTCTTTAAT TCTAAGAATT
Tx  TCCCATTTTT AATTTAACAT GGGGCAACTT TTTCCTTAAT TCTAAGAATT

    101
Gh  TTACA
Gl  TCACA
Hs1 TTACA
R   TTACA
Tx  TTACA
```

The implications of this proposed hypervariable region are hard to ignore. While the presence of this region alone is not enough to confirm that serotypes of TCoV exist, evidence relating HVRs to serotypes in the spike gene of IBV does suggest that different TCoV serotypes might exist.[60] It is clear different genotypes do occur in TCoV.

Part III. Antigenic relationship of TCoV.

Coronaviruses are subcategorized into three antigenically distinct groups, as well as the genetic similarities of the viruses. Group I includes some human coronaviruses, feline coronavirus, and canine coronavirus. Group II includes bovine coronavirus (BCV), mouse hepatitis virus, and other human

coronaviruses. Group II contain an extra hemagglutinin-esterase gene that the others do not. Finally Group III contains infectious bronchitis virus. The following indicates the gene order of the respective groups[47]:

Group I 5′-1A,1B-S-3a,3b,E-M-N-3′

Group II 5′-1A,1B-HE-S-4-5a,E-M-N-3′

Group III 5′-1A,1B-S-3a,3b,E-M-5a,5b-N-3′

The 5a and 5b genes of group III are unique, and do not correspond to the 5a in group II. They are numbered based on the subgenomic RNA, not sequence or functional homology.

For decades, controversy has existed over which viruses TCoV shares antigenic and genotypic relationships. Publications contradicted each other, and each new finding further complicated matters. To better understand this issue, it is important to go back to the first publications involving bluecomb disease.

In the mid fifties Drs. Pomeroy and Sieburth did extensive research on transmissible coronaviral enteritis as it was just beginning to emerge. In one paper, various laboratory tests were run, and the presence of a filterable agent was observed.[78] Nowhere in these studies is hemagglutination ever mentioned. Twenty years later, some of the first researchers to observe actual coronavirus particles by EM actually documented hemagglutination experiments. Adams et al. reported that no

agglutination was observed with RBCs of 9 different species, including turkey, chicken, and several mammalian types.[2] It did exhibit a strong adherence to epithelial cells. At this point however, coronaviruses were new to researchers, and groups and classifications had not yet begun.

In 1990, antigenic research into TCoV began in the form of immunoblotting, seroneutralization tests, as well as hemagglutination (HA) and hemagglutination inhibition (HI) tests. Of 49 monoclonal antibodies (mAbs) against BCV or TCoV, only 11 differentiated between the two. Polyclonal sera were found to cross react between the four major TCoV proteins and their homologues in BCoV. Further, TCoV was reported to have HA properties identical to BCoV. Finally, these researchers reported that TCoV was found to grow readily in the HRT-18 cell line without any adaption.[26] Reports the following year showed that HRT-18 cells were also the only cell line capable of propagating BCoV.[94] The same group (Dea et al.) then went on to show that the virus was identified using BCoV-specific single-stranded cDNA probes[89], furthering the idea that TCoV and BCoV were antigenically similar. A final paper that year concluded that TCoV and BCoV were, on a molecular basis, indistinguishable. They stated that, though they infected different animal species, they should be reclassified into a single subgroup.[93]

The nucleocapsid and membrane genes of coronavirus isolated from turkeys were sequenced. Results showed that the amino acid identities of both the M and N genes were more than 99% similar to the respective genes in BCoV.[88] Other researchers examined the spike gene of many isolates of coronaviruses from turkeys and BCoV by sequencing and found them to have greater than 97% similarity, and in some cases 100%[77]. This finding was important because of the genetic variability usually seen in the spike gene. Clearly, at this point, all evidence except the initial reports pointed to this coronavirus being almost identical to BCoV.

The same year that the spike sequencing was published, another interesting study was reported as well. Guy et al. reported that, using polyclonal sera, antibodies specific for a coronavirus isolated from turkeys strongly reacted to IBV. Monoclonal antibodies specific for the IBV matrix protein also reacted very strongly against the virus as determined by fluorescent antibody (FA) and immunoperoxidase (a procedure similar to the ELISA method).[36] This publication provided evidence of a close antigenic relationship between TCoV and IBV, contradicting the past 6 years of research.

A few years later, a paper emerged describing a PCR reaction that was used to amplify coronaviruses of all three groups using 11 reference viruses. To develop this test, they

sequenced part of the 1b polymerase gene of all 11 viruses. The results allowed them to create a PCR test with primers in conserved regions, but they all showed something beneficial for the classification of TCoV. The 1b sequence of 307 amino acids showed 97% similarity between TCoV and IBV. BCoV only shared 60% similarity with TCoV[81]. Sequencing of the nucleocapsid gene of TCoV in 1999 showed sequence similarity of greater than 90% with three strains of IBV[3,7], but only ~20% similarity with that of BCoV[7]. Yet, as described, the TCoV nucleocapsid gene had been published some eight years earlier to share 99% of its sequence with BCoV. These results led to the publication of a review in 2000 by Dr. James Guy suggesting that TCoV now be put with its fellow avian counterpart, IBV, into group III coronaviruses.[34]

Soon after the proposal to reclassify TCoV, Cavanagh et al. reported the presence of gene 5 (ORFs 5a, 5b) in TCoV.[19] This was the first report of a coronavirus associated with disease in turkeys outside of North America, and indicated its gene order as that of Group III coronaviruses.[19] That same year, it was documented that BCoV could infect turkeys.[42] Reports had already existed of BCoV being found histologically in turkey intestinal tissue and being detected by FA[27], but they had stated it did not cause disease. This time, though, Ismail et al. found that BCoV did, in fact, cause a symptomatic enteritis in turkeys with

similar characteristics to TCE. The disease was transmissible and the virus had HA activity.[42]

Further research came forth all showing even more antigenic relationships between the two poultry viruses.[17,18,42,50] Yet, in 2002, a paper was published by Lin et al. that found the spike gene of TCoV had a high degree of identity with both BCoV and group II human coronaviruses.[51] The sequence showed that in 568 base pairs, only one nucleotide had changed. Another paper later that year by the same researchers repeated the existence of gene 5 in TCoV[48], a group III-only gene.

Shortly after this, Jackwood et al. Submitted the 1st full length sequence of the TCoV spike gene for two separate isolates of TCoV (Gen Bank Acc numbers AY342356 and AY342357). S1 was found to be 20% similar in amino acid sequence to that of IBV, and S2 was 27% similar. In 2004, the complete sequence of the spike gene and genes down stream to the polyadenylated tail of TCoV was published by Lin et al.[49] Their data indicated that the spike gene was 33% similar to IBV, but only 20% similar to BCoV, contradicting their previous report that only 1 out of 568 base pairs was different. This new report also showed a high degree of similarity between all structural genes after the spike gene, as well as the presence of the 3a, 3b, and E ORFs characteristic of group I and III coronaviruses, but not group II.

It had been proposed that because the spikes of TCoV and IBV were so dissimilar, yet everything after was not, that perhaps a crossover event occurred at some point between the two viruses.[44] Though not refuted, this theory is challenged by the high degree of similarity between the polymerase genes of the two viruses, suggesting that a crossover occurred that only replaced the spike gene. Of the spike genes sequenced, the closest sequence to TCoV is a very distant (~30% similarity) IBV, so there is no indication as to the origin of the spike.

When reading literature on TCoV it is important to note the years in which the research was done, and the properties associated with it. It is probable, though not certain, that research referring to a BCoV-like TCoV or hemagglutinating TCoV is referring to a contamination with BCoV, or BCoV that infected turkeys. References to the original bluecomb disease, where hemagglutination was not found, most likely refer to IBV-like TCoV that has been reported as of late. While the two viruses cause very similar diseases in young poults, antigenically and genotypically they are significantly different.

Conclusion.

Transmissible coronaviral enteritis of turkeys is an acute, economically devastating disease with an extremely high morbidity and varying mortality. The etiological agent of this

disease is now known to be turkey coronavirus (TCoV). There is no known vaccine for the virus, and all research for the past two decades has focused on classifying the virus rather than determining its pathogenesis. Further complicating matters is the lack of a reliable means of virus propagation such as cell culture. Recent findings of a region in the spike glycoprotein of TCoV perhaps corresponding to hypervariable region II of infectious bronchitis virus indicate the possibility of serotypes, which has not been reported to date. Further work examining this region, its potential to change, and its effect on serotype are needed. In concert with this research, a better means of propagation is also required, and here we present three projects to that end.

References

1. Adams, N. R. and M. S. Hofstad. Avian Diseases. 15(3):426-433. 1971.
2. Adams, N. R., M. S. Hofstad, and P. M. Gough. Avian Diseases.16(4):817-827. 1972.
3. Akin, A., T. L. Lin, C. C. Wu, T. A. Bryan, T. Hooper, and D. Schrader. Acta Virologica. 45:31-38. 2001.
4. Almeida, J. D., D. M. Berry, and C. H. Cunningham. Nature. 220:650. 1968.
5. Archetti, I. and F. L. Horsefall. Journal of Experimental Methods. 93:441-462. 1950.
6. Benbacer, L., K. E. Besnardeau, H. Laude, and B. Delmas. Journal of Virology. 71:734-737. 1997.
7. Breslin, J. J., L. G. Smith, F. J. Fuller, J. S. Guy. Virus Research. 65:187-193. 1999.
8. Breslin, J. J., L. G. Smith, H. J. Barnes, and J. S. Guy. Avian Diseases. 44:624-631. 2000.
9. Britton, P., S. Evans, B. Dove, M. Davies, R. Casais, and D. Cavangh. Journal of Virological Methods. 123:203-211. 2005.
10. Brown, T. P., A. Garcia, and L. Kelley. Avian Diseases. 41:604-609. 1997.
11. Brown, T. P., A. Garcia, and L. Kelley. Avian Diseases. 41:906-909. 1997.

12. Calibeo-Hayes, D., S. S. Denning, S. M. Stringham, J. S. Guy, L. G. Smith, and D. W. Watson. Avian Diseases. 47:149-153. 2003.
13. Callison, S. A, D. A. Hilt, and M. W. Jackwood. Avian Diseases. 45(2):492-499. 2001.
14. Callison, S. A. Molecular Characterization and sequence analysis of the spike glycoprotein from foreign and domestic infectious bronchitis viruses. Master’s Thesis, University of Georgia. 1998.
15. Carver, D. K., J. P. Vaillancourt, M. Stringham, J. S. Guy, H. J. Barnes. Avian Diseases. 45:985-991. 2001.
16. Caul, E. O., C. R. Ashley, and S. L. Egglestone. FEMS Microbiology Letters. 4:1-4. 1978.
17. Cavanagh, D. Avian Pathology. 30:109-115. 2001.
18. Cavanagh D. British Veterinary Journal. 48:199-222. 1992.
19. Cavanagh, D., K. Mawditt, M. Sharma, S. E. Drury, H. L. Ainsworth, P. Britton, and R. E. Gough. Avian Pathology. 30:355-368.
20. Coria M. F. and A. E. Ritchie. Avian Diseases. 17(4):697-704. 1973.
21. Cowen, B. S. and S. B. Hitchner. Avian Diseases. 19(1):6-11. 1974.

22. Dalton, K., R. Casais, K. Shaw, K. Stirrups, S. Evans, P. Britton, T. D. K. Brown, and D. Cavanagh. Journal of Virology. 75(1):125-133. 2001.

23. Dea, S. and P. Tijssen. American Journal of Veterinary Research. 50:2 226-231. 1989.

24. Dea, S. and P. Tijssen. Archives of Virology. 99:173-186. 1988.

25. Dea, S. and P. Tijssen. Canadian Journal of Veterinary Research. 52:53-57. 1988.

26. Dea, S., A. J. Verbeek, and P. Tijssen. Journal of Virology. 64(6):3112-3118. 1990.

27. Dea, S., A. Verbeek, and P. Tijssen. Avian Diseases. 35:767-777. 1991.

28. Dea, S., G. Marsolais, J. Beaubien, and R. Ruppanner. Avian Diseases. 30(2):319-326. 1985.

29. Dea, S., S. Garzon, H. Strykowski, and P. Tijssen. Veterinary Microbiology. 20:21-22. 1989.

30. Deshmukh, D. R. and B. S. Pomeroy. American Journal of Veterinary Research. 35(12):1549-1552.

31. Deshmukh, D. R., C. T. Larson, and B. S. Pomeroy. American Journal of Veterinary Research. 34(5):673-675. 1973.

32. Fong, S. G., S. Shen, F. P. L. Tay, and D. X. Liu. Biochemical and Biophysical Research Communications. 336(2): 417-423. 2005.

33. Gonder, E., B. L. Patel, and B. S. Pomeroy. American ournal of Veterinary Research. 37(12):1435-1439. 1976.

34. Guy, J. S. Avian Pathology. 29:207-212. 2000.

35. Guy, J. S. in *Diseases of Poultry.* (Iowa State University Press, Iowa) B. W. Calnek et al., eds. 2003.

36. Guy, J. S., H. J. Barnes, L. G. Smith, and J. Breslin. Avian Diseases. 41:583-590. 1997.

37. Guy, J. S., L. G. Smith, J. J. Breslin, and S. Pakpinyo. Avian Diseases. 44:334-341. 2002.

38. Guy, J. S., L. G. Smith, J. J. Breslin, J. P. Vaillancourt, and H. J. Barnes. Avian Diseases. 44:105-113. 2000.

39. Hattermann, K., M. A. Muller, A. Nitsche, S. Wendt, O. D. Mantke, and M. Niedrig. Archives of Virology. 150:1023-1031. 2005.

40. Heggen, C. L., M. A. Qureshi, F. W. Edens, H. J. Barnes, and G. B. Havenstein. Avian Diseases. 42:711-720. 1998.

41. Hodgson, R. Casais, B. Dove, P. Britton, and D. Cavanagh. Journal of Virology. 78(24):13804-13811. 2004.

42. Ismail, M. M., K. O. Cho, M. Hasoksuz., L. H. Saif, and Y. M. Saif. Avian Diseases. 45:157-163, 978-984. 2001.

43. Ismail, M. M., Y. Tang, and Y. M. Saif. Avian Diseases. 47:515-522. 2003.

44. Jackwood, M. W. D. A. Hilt, T. Boynton, and S. A. Callison. Proceedings of the the 4th International Symposium on Avian

Corona- and Pneumovirus. Rauischholzhausen, Germany. June 20-23. 2004.

45. Kawaguchi, T., K. Nomura, Y. Hirayama, and T. Kitagawa. Cancer Research. 47(16):4460-4464. 1987.

46. King, D. J. American Journal of Veterinary Research. 36(4):555-556. 1975.

47. Lai, M. M. C. and K. V. Holmes. in *Fields Virology*. (Lippincott Williams & Wilkins) Fields, B. N. et al. eds.

48. Lin, T. L., C. C. Loa, and C. C. Wu. Acta Virologica. 47:107-116. 2002.

49. Lin, T. L., C. C. Loa, and C. C. Wu. Virus Research. 106:61-70. 2004.

50. Lin, T. L., C. C. Loa, C. C. Wu, T. A. Bryan, T. Hooper, and D. Schrader. Avian Diseases. 46:466-472. 2002.

51. Lin, T. L., C. C. Loa, S. C. Tsai, C. C. Wu, T. A. Bryan, H. L. Thacker, T. Hooper, and D. Schrader. Veterinary Microbiology. 84:179-186. 2002.

52. Loa, C. C., T. L. Lin, C C. Wu, T. A. Bryan, H. L. Thacker, T. Hooper, and D. Schrader. Journal of Virological Methods 104:187-194. 2002.

53. Loa, C. C., T. L. Lin, C. C. Wu, T. A. Bryan, H. L. Thacker, T. Hooper, and D. Schrader. Avian Diseases. 44:498-506. 2000.

54. Loa, C. C., T. L. Lin, C. C. Wu, T. Bryan, T. Hooper, and D. Schrader. Veterinary Immunology and Immunopathology. 88:57-64. 2002.

55. Loa, C. C., T. L. Lin, C. C. Wu, T. Bryan, T. Hooper, and D. Schrader. Comparative Immunology, Microbiology, and Infectious Diseases. 25:127-138. 2002.

56. Loa, C. C., T. L. Lin, C. C. Wu, T. Bryan, T. Hooper, and D. Schrader. Poultry Science. 80:1416-1424. 2001.

57. Machamer, C. E., M. G. Grim, and A. Esquela. Molecular Biology of the Cell. 4:695-704. 1993.

58. Miguel, B., G. T. Pharr, and C. Wang. Archives of Virology. 147:2047-2056. 2002.

59. Moore, K. M., J. D. Bennet, B. S. Seal, M. W. Jackwood. Virus Genes. 17(1):63-83. 1998.

60. Moore, K. M., M. W. Jackwood, and D. A. Hilt. Archives of Virology. 142:2249-2256. 1997.

61. Moscovici, C., M. G. Moscovici, H. Jimenez, M. M. Lai, M. J. Hayman, and P. K. Vogt. Cell. 11(1):95-103. 1977.

62. Nagaraja, K. V. and B. S. Pomeroy. American Journal of Veterinary Research. 41(8):1283-1284. 1980.

63. Nagaraja, K. V. and B. S. Pomeroy. American Journal of Veterinary Research. 39(9):1463-1465. 1978.

64. Nagaraja, K. V. and B. S. Pomeroy. American Journal of Veterinary Research. 41(6):915-917. 1980.

65. Naqi, S. A., B. Panigrahy, and C. F. Hall. Avian Diseases. 16(4):937-939. 1972.

66. Naqi, S.A., B. Panigrahy, C. F. Hall. American Journal of Veterinary Research. 36(4):548-551. 1975.

67. Pakpinyo, S., D. H. Ley, H. J. Barnes, J. P. Vaillancourt, and J. S. Guy. Avian Diseases. 46:360-369. 2002.

68. Pakpinyo, S., D. H. Ley, H. J. Barnes, L. P. Vaillancourt, and J. S. Guy. Avian Diseases. 47:396-405. 2003.

69. Panigrahy, B., S. A. Naqi, and C. F. Hall. Avian Diseases. 17(2):430-438. 1973.

70. Patel, B. L., B. S. Pomeroy, E. Gonder, and C. E. Cronkite. American Journal of Veterinary Research. 37(9):1111-1112. 1976.

71. Patel, B. L., E. Gonder, and B. S. Pomeroy. American Journal of Veterinary Research. 38(9):1407-1411. 1977.

72. Pendleton, A. R. and C. E. Machamer. Journal of Virology. 79(10):6142-6151. 2005.

73. Pomeroy, B. S., C. T. Larsen, D. R. Deshmukh, and B. L. Patel. American Journal of Veterinary Research. 36(4):553-554. 1975.

74. Pomeroy, M. S. and J. M. Sieburth. Proc. Book, AVMA. 321-327. 1953.

75. Ritchie, A. E., D. R. Deshmukh, C. T. Larsen, and B. S. Pomeroy. Avian Diseases. 17:546-558. 1973.

76. Reynolds, D. L. in A Laboratory Manual For the Isolation and Identification of Avian Pathogens. 4th edition. (Rose Printing, Tallahassee Florida) Swayne, D. et al. eds. 1998.

77. Sellers, H. S., T. Brown, M. Jackwood, L. Kelley, and A. Garcia. 134th AVMA Convention. Session B, July 1997.

78. Sieburth, J. M. and B. S. Pomeroy. Proc. Book, AVMA. 301-306. 1955.

79. Sieburth, J. M. and B. S. Pomeroy. Journal of American Veterinary Medical Association. 128:509-513. 1956.

80. Spackman, E., D. Kapczynski, and H. Sellers. Avian Diseases. 49:86-91. 2005.

81. Stephenson, C. B., D. B. Casebolt, and N. N. Gangopadhyay. Virus Research. 60:181-189. 1999.

82. Stern, D. F. and B. M. Sefton. Journal of Virology. 44:804-812. 1982.

83. Tijssen, P., A. H. Verbeek, and S. Dea. in *Coronaviruses and Their Diseases.* (Plenum Press, New York) D. Cavanagh and T. D. K. Brown, eds. 457-460.

84. Tompkins, W. A. F., A. W. Watrach, J. D. Schmale, R. M. Schultz, and J. A. Harris. Journal of the National Cancer Institute. 52:101-106. 1974.

85. Truscott, R. B. and E. W. Morin. Avian Diseases. 8:27-35. 1964.

86. Truscott, R. B., M. C. Connell, A. E. Ferguson, and C. G. Wills. Avian Diseases. 4:391-401. 1960.

87. Velayudhan, B. T., H. Shin, V. C. Lopes, T. Hooper, D. A. Halvorson, and K. V. Nagaraja. Journal of Veterinary Diagnostics and Ivestigations. 15:592-596. 2003.

88. Verbeek, A. H. and P. Tijssen. Journal of General Virology. 72:1659-1666. 1991.

89. Verbeek, A. H., S. Dea, and P. Tijssen. Archives of Virology. 121:199-211. 1991.

90. Wang, C. H and Y. C. Huang. Archives of Virology. 145:291-300. 2000.

91. Watson, D. W., J. S. Guy, and S. M. Stringham. Medical Entomology. 37:480-483. 2000.

92. Wimley, W. C., B. Sainz Jr., , J. M. Rausch, W. R. Gallaher, and R. F. Garry. Biochemistry. 44:947-958. 2005.

93. Yu, M., M. M. Ismail, M. A. Qureshi, R. N. Dearth, H. J. Barnes, and Y. M. Saif. Avian Diseases. 44:297-304. 2000.

94. Zhang, X., K. G. Kousoulas, and J. Storz. Virology. 185:847-852. 1991.

Chapter 2

SEQUENCE ANALYSIS OF THE HYPERVARIABLE REGION OF THE S1 GENE FOLLOWING *IN VITRO* PASSAGE OF TURKEY CORONAVRIRUS[1]

[1]Boynton, T. O., M. W. Jackwood, S. A. Callison, and D. A. Hilt. To be included in a submission to Journal of Virology.

Summary

The mutational rate of a hypervariable region (HVR) in the S1 gene of turkey coronavirus (TCoV) was investigated. In coronaviruses, sequences in the HVRs of the S1 gene correlate with epitopes on the expressed protein that induce protective immune responses. Sequence differences in the HVR were observed for different TCoV isolates indicating the presence of different genotypes. The effect of these differences on serotype is unknown. The frequency of genetic changes in this region was determined by sequencing the HVR of two of the TCoV isolates serially passaged in embryonating turkey eggs. Mutational rates of 3.0×10^{-2} nucleotides per passage and 4.5×10^{-2} amino acids per passage were determined for the Tx strain. Rates of 8.5×10^{-5} nucleotides per passage and 2.6×10^{-4} amino acids per passage were calculated for the R strain. The mutational rate and localization of mutations in the Tx strain strengthens the existence of this HVR, and this region may also play a role in serotype.

Introduction

Turkey coronaviral enteritis (TCE) of turkeys is an acute, contagious disease characterized by diarrhea, inappetance, ruffled feathers, decreased body weight, and a general loss in production (8). Morbidity is usually high and mortality varies greatly, depending on host and environmental factors (13). Secondary co-infections such as *E. coli* are responsible for the higher mortality rates often seen clinically (7, 14). The causative agent of this disease is turkey coronavirus (TCOV). This virus has also been implicated as a main factor in poult enteritis and mortality syndrome (PEMS) and spiking mortality of poults (SMT), diseases with similar signs as TCE, but with much higher mortality rates (2, 20).

Coronaviruses are positive sense RNA viruses, with a genome of ~30 kb encoding several structural and non-structural proteins (16). One of these proteins, the spike, is responsible for inducing neutralizing antibodies (12). The spike is composed of two subunits, S1 and S2, cleaved from a precursor polypeptide. The spike protein mediates attachment to the host sell and is important for pathogenicity, though changes in the spike are not solely responsible.

Coronaviruses are subdivided into three major antigenic groups based on differences in serology and sequencing (15, 19). Early studies put TCoV into group II and as a close relative of

bovine coronavirus (5, 18). Recent antigenic and nucleotide studies have instead placed the virus into group III alongside infectious bronchitis (IBV)(1, 6, 17).

In IBV, now known to be the closest relative of TCoV, the s1 subunit contains three hypervariable regions (HVRs), which are associated with serotypes specific neutralizing epitopes (3). Complete sequence of the entire S gene of two TCoV isolates indicates the possible presence of a HVR comparable to HVRII in IBV between residues 123-158 (Genbank Acc. Nos. AY342356 and AY342357). Alignment of available sequence of this region in other TCoV isolates gives further proof of this HVR (data not shown). Although it appears that different TCoV genotypes occur, it is not clear if different serotypes exist.

In IBV, estimated mutational rates range from 10^{-3} to 10^{-5} point mutations per nucleotide (10). With a genome of 30 kb, an average of three mutations per replication can occur. If these mutations occur in an antigenically important region and become fixed overtime, new serotypes can emerge, leading to vaccines which no longer provide adequate protection to field strains.

The mutational rate of TCoV is currently not known, nor is the rate of change in the proposed hypervariable region. The region could be an actual HVR, or the sequence difference may have just occurred selectively over long periods of time. If it is hypervariable, then changes should be observed fairly quick.

The purpose of this experiment was to gain a better understanding of this region by examining the sequence changes within the S1 gene over time.

Materials and Methods

Virus Strains. Two strains of TCoV were recovered from turkey poults in Texas and designated Tx and R. These isolates were obtained from Purdue University, West Lafayette, IN courtesy of Tom Hooper.

Propagation of TCoV. Virus was propagated in specific-pathogen-free (SPF) turkey eggs. Briefly, 23 day old embryos were inoculated with viral strains via the chlorioallantoic sac route. Inoculated eggs were incubated for 72 hours, after which intestines were harvested. Intestines were then homogenized in PBS containing antibiotics, and then centrifuged at low speed. The supernatant was collected and filtered to 0.2 μm and stored at -80^{o}C for use as inoculum in further propagations.

RNA Isolation and RT-PCR. RNA was isolated from each pass using the High Pure Isolation kit (Roche) according to the manufacturer's recommendations. Briefly, 200 μl of virus-containing homogenate from each passage was added to appropriate buffers and columns and eluted to 75 μl. To amplify the HVR for sequencing, RT-PCR was performed using the Titan one-tube RT-PCR

system (Roche) according to the manufacturer's recommendations and as described by Jackwood et al. (9), with the exception of $MgCl_2$ concentration being increased to 1.0 mM. Primers designed to amplify the HVR were designated TCV 163F S1 (5'-GAT TTT TAT AGT CCA GAT GT-3') and TCV 906R S1 (5'-ATC AAA ATC CCA AGA ATA AG-3'). The amplification conditions were set as follows: 42^{o}C for 1 hour, followed by 10 cycles of 94^{o}C for 30 s, 45^{o}C for 30 s, and 68^{o}C for 90 s. This was repeated 25 more times with an addition of 5 s every cycle to the 68^{o}C step. A final cycle was performed at 68^{o}C for 7 min.

Sequencing and Analysis. To sequence the HVR, RT-PCR products were purified using a QIAquick© Gel Extraction kit (Qiagen) according to the manufacturer's recommendations. PCR product was then used in the ABI Prism BigDye™ Terminator kit. Sequencing was then performed using an ABI prism™ 310 genetic analyzer. Analysis and alignments were carried out using the Clustal V method in MacDNAsis© software (Hitachi, Ltd.).

Results

The Tx isolate was passaged 50 times and the R isolate 30 times in embryonating turkey eggs. Virus presence was confirmed after each passage by RT-PCR, which yielded a ~750 bp fragment as expected (data not shown). Once purified, sequence data was obtained from amplified products. A consensus sequence

containing a continuous open reading frame (ORF) was obtained from each passage using three independent sequencing runs. Comparison with available S1 sequences on GenBank (www.ncbi.nlm.nih.gov) verified the ORFs.

Sequence was examined for passages 1, 10, 20, 30, 40, and 50 for the Tx strain (figure 1a and 1b). No sequence changes were observed for passages 10, 20, and 30 when compared to passage 1. After 40 passages, mutations were observed at nucleotides 183, 189, 193, 198, 225, and 252 (from the ATG start site). After 50 passages, additional mutations were observed at nucleotides 246, 252, 271, 380, 411, and 413. Passage 50 was found to be 1.5% different than passage 1, which results in a mutational rate of $3x10^{-2}$% nucleotide changes per passage.

Amino acid mutations were also deduced from the nucleotide sequence of the Tx passages (figure 1c and 1d). After 40 passages, changes occurred at residues 65 and 84. After 50 passages, additional mutations were also observed at residues 84, 91, 127, and 138. The nucleotide at position 252 changed from C to A between passages 30 and 40, and then changed to a T after 50 passages. This change caused amino acid 84 to revert back to its original residue, despite a different nucleotide. The mutational rate for amino acids was $4.6x10^{-2}$ residues per passage. Exact nucleotide and amino acid changes are presented in Table 1A.

Sequence was examined for passages 1, 11, 20, and 30 for the R strain (figure 2a and 2b). No sequence changes were observed for passages 11 and 20 when compared to passage 1. After 30 passages, nucleotide mutations were observed at positions 578 and 702 from the ATG start site.

These two changes resulted in amino acid substitutions at both locations (residues 193 and 234, figure 2c and 2d). Mutational rates were found to be $8.6x10^{-5}$% nucleotide changes per passage and $2.6x10^{-4}$% amino acid changes per passage. Exact nucleotide and amino acid substitutions can be seen in Table 1B.

Discussion

Although information exists on the mutational rate of infectious bronchitis virus, none is available for TCoV. In this study we examined a proposed HVR and surrounding sequence in the TCoV spike gene following serial passages in embryonating turkey eggs and observed mutations after 40 and 50 passages for the Tx strain and after 30 passages for the R strain.

The mutational rate of the Tx strain shows that every 4 passages a nucleotide can change. During outbreaks of turkey coronaviral entiritis, turkey coronavirus can spread throughout the exposed flock and possibly the entire farm, providing numerous opportunities for changes to occur in a single

outbreak. Under immune pressure, mutations may occur even faster, and be selected towards escaping the host defenses (10).

The changes seen in the Tx strain were not throughout the 780 bp sequence, but localized to the proposed hypervariable region (nucleotides 370-474, figure 1a) similar to HVRII in IBV, as well as another region upstream. This second region may constitute another HVR similar to HVR I in IBV, though this new region does not appear to contain major amino acid differences between strains. Changes were not seen downstream even after 50 passages, suggesting a stability in these regions.

The mutational rate of the R strain was much lower than the Tx strain. Changes in nucleotides occurred approximately once in every 15 passages, though both changes occurred only after 30 passages, and it is worthwhile to note that changes seen in the Tx strain only occurred after 40 passages. It is likely that more changes will occur as the virus is serially passed further.

The changes seen in the Tx strain indicate further that the region is hypervariable. If these regions behave like their counterparts in IBV, then it is possible different serotypes of TCoV exist (3,12). Serotypes have not been reported for TCoV, but their presence would be extremely important when developing vaccines. A vaccine raised against the R strain may not be efficacious against the Tx strain, and further research is needed in this area. Despite the fact that HVRs in IBV

correspond to serotypes, evidence also exists that, though the spike protein of IBV varies greatly, IBV can change slowly. Mutations in the spike protein are not always fixed overtime, and do not necessarily equate to evolution of the virus (4).

References

1. **Breslin, J. J., L. G. Smith, F. J. Fuller, and J. S. Guy.** 1999. Sequence analysis of the matrix/nucleocapsid gene region of turkey coronavirus. Intervirology. 42:22-29.
2. **Brown, T. P., A. Garcia, and L. Kelly**. 1997. Spiking mortality of poults: I. Experimental reproduction in isolation facilities. Avian Dis. 41:604-609.
3. **Cavanagh, D. P., J. Davis, and A. Mockett.** 1988. Amino acids within hypervarible region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralizing epitopes. Virus Res. 11:141-150.
4. **Cavanagh, D. , K. Mawditt, A. Adzhar, R. E. Gough, J. P. Picault, C. J. Naylor, D. Haydon, K. Shaw, and P. Britton.** 1998. Does Ibv change slowly despite the capacity of the spike protein to vary greatly? Adv Exp Med Biol. 440:729-734.
5. **Dea, S., A. J. Verbeek, and P. Tijssen.** 1990. Antigenic and genomic relationships among turkey and bovine enteric coronaviruses. J. Virol. 64:3112-3118.
6. **Guy, J. S., H. J. Barnes, L. G. Smith, and J. Breslin.** 1997. Antigenic characterization of a turkey coronavirus identified in poult enteritis- and mortality syndrome-affected turkeys. Avian Dis. 41:583-590.
7. **Guy, J. S., L. G. Smith, J. J. Breslin, J. P. Vaillancourt, and H. J. Barnes.** 2000. High mortality and growth depression

experimentally produced in young turkeys by dual infection with enteropathogenic Escherichia coli and turkey coronavirus. Avian Dis. 44:105-13.

8. **Holmes, K.V., and M. M. C. Lai.** 1996. Coronaviridae: the viruses and their replication. In: Fields, B. N., Knipe, D. M., Howly, P. M. (Eds), Fundamental Virology, 3rd ed., Vol. 1. 2 Vols. Lippincott-Raven, Philadelphia, PA, pp. 1075-1093.
9. **Jackwood, M. W., M. Yousef, and D. A. Hilt.** 1997. Further development and use of a molecular serotype identification test for infectious bronchitis virus. Avian Dis. 41:105-110.
10. **Lee, C. and M. W. Jackwood.** 2001. Origin and evolution of Georgia 98 (GA98), a new serotype of avian infectious bronchitis virus. Virus Res. 80:33-39.
11. **Moore, K. M., J. D. Bennett, B. S. Seal, and M. W. Jackwood.** 1998. Sequence comparison of avian infectious bronchitis virus s1 glycoproteins of the Florida serotype and five variant isolates from Georgia and California. Viruse Genes. 17:63-83.
12. **Moore, K. M., M. W. Jackwood, and D. A. Hilt.** 1997. Indentification of amino acids involved in a serotype and neutralization specific epitope within the s1 subunit of avian infectious bronchitis virus. Arch Virol. 142:2249-2256.
13. **Nagaraja, K.V., and B. S. Pomeroy.** 1997. Coronaviral enteritis of turkeys (bluecomb disease). In: Calnek, B. W.,

Barnes, H. J., Beard, C. W., McDougald, L. R., Saif, Y. M. (Eds.), Diseases of Poultry, 10th ed. Iowa State University Press, Ames, IA, pp. 686-692.

14. **Pakpinyo, S., D. H. Ley, H. J. Barnes, J. P. Vaillancourt, and J. S. Guy.** 2003. Enhancement of enteropathogenic Escherichia coli pathogenicity in young turkeys by concurrent turkey coronavirus infection. Avian Dis. 47:396-405.
15. **Pederson, N. C.** 1978. Antigenic relationship of feline infectious peritonitis virus to coronaviruses of other species. Arch Virol. 58:45-53.
16. **Siddell, S. G.** 1995. The coronaviridae, an introduction. In: Siddell, S. G. (ed), Coronaviridae. Plenum, New York. pp. 1-9.
17. **Stephenson, C. B., D. B. Casebolt, and N. N. Gangopadhyay.** 1999. Phylogenetic analysis of a highly conserved region of the polymerase gene from 11 coronaviruses and development of a consensus polymerase chain reaction assay. Virus Res. 60:181-189.
18. **Verbeek, A. and P. Tijssen.** 1991. Sequence analysis of the turkey enteric coronavirus nucleocapsid and membrane protein genes: a close genomic relationship with bovine coronavirus. J. Gen. Virol. 72:1659-1666.
19. **Williams, A. K., L. Wang, L. W. Sneed, and E. W. Collisson.** 1993. Analysis of a hypervariable region in the 3′ non-coding

end of the infectious bronchitis virus genome. Virus Res. 28:29-27.

20. **Yu, M., M. M. Ismail, M. A. Qureshi, R. N. Dearth, H. J. Barnes, and Y. M. Saif.** 2000. Viral agents associated with poult enteritis and mortality syndrome: the role of a small round virus and a turkey coronavirus. Avian Dis. 44:297-304.

Figure 1. Sequence analysis of TCoV isolate Tx through 50 passages in SPF turkey embryos. A) Clustal V alignment of nucleotide sequence of passages 1, 10, 20, 30, 40, and 50. Mutations are shown in red. B) Phlyogenetic tree depicting percent similarities between nucleotide sequence for each passage. C) Clustal V alignment of translated amino acid sequence of passages. Mutations are shown in red. D) Phylogenetic tree depicting percent similarities between amino acid sequence for each passage.

A.

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                    10         20         30         40         50
Tx p1          1 GATTTTTATA GTCCAGATGT CATGCGACCG TCAGACGGTG CGTATATACA      50
Tx p10         1 GATTTTTATA GTCCAGATGT CATGCGACCG TCAGACGGTG CGTATATACA      50
Tx p20         1 GATTTTTATA GTCCAGATGT CATGCGACCG TCAGACGGTG CGTATATACA      50
Tx p30         1 GATTTTTATA GTCCAGATGT CATGCGACCG TCAGACGGTG CGTATATACA      50
Tx p40         1 GATTTTTATA GTCCAGATGT TATGCGCCCG CCAGATGGTG CGTATATACA      50
Tx p50         1 GATTTTTATA GTCCAGATGT TATGCGCCCG CCAGATGGTG CGTATATACA      50

                   60         70         80         90        100
Tx p1         51 ATCCGGTTAT TACGAACCAC TTTTTACAGG TTGCTTTAAC CAAACTAATC     100
Tx p10        51 ATCCGGTTAT TACGAACCAC TTTTTACAGG TTGCTTTAAC CAAACTAATC     100
Tx p20        51 ATCCGGTTAT TACGAACCAC TTTTTACAGG TTGCTTTAAC CAAACTAATC     100
Tx p30        51 ATCCGGTTAT TACGAACCAC TTTTTACAGG TTGCTTTAAC CAAACTAATC     100
Tx p40        51 ATCCGGTTAT TATGAACCAC TTTTTACAGG TTGCTTTAAA CAAACTAATC     100
Tx p50        51 ATCCGGTTAT TATGAACCAC TTTTTACAGG TTGTTTTAAT CAAACTAATC     100

                  110        120        130        140        150
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Tx p10       101 AAACTGATAC CACCTGTAAA AATGGGCTTT ATGCAGGGTC GCCAGGTAAT     150
Tx p20       101 AAACTGATAC CACCTGTAAA AATGGGCTTT ATGCAGGGTC GCCAGGTAAT     150
Tx p30       101 AAACTGATAC CACCTGTAAA AATGGGCTTT ATGCAGGGTC GCCAGGTAAT     150
Tx p40       101 AAACTGATAC CACCTGTAAA AATGGGCTTT ATGCAGGGTC GCCAGGTAAT     150
Tx p50       101 AAACTGATTC CACCTGTAAA AATGGGCTTT ATGCAGGGTC GCCAGGTAAT     150

                  160        170        180        190        200
Tx p1        151 TTTACTATAC AAGGTGGTTT TCTTCAAAAT TATGATGCTA TTGGCATAAT     200
Tx p10       151 TTTACTATAC AAGGTGGTTT TCTTCAAAAT TATGATGCTA TTGGCATAAT     200
Tx p20       151 TTTACTATAC AAGGTGGTTT TCTTCAAAAT TATGATGCTA TTGGCATAAT     200
Tx p30       151 TTTACTATAC AAGGTGGTTT TCTTCAAAAT TATGATGCTA TTGGCATAAT     200
Tx p40       151 TTTACTATAC AAGGTGGTTT TCTTCAAAAT TATGATGCTA TTGGCATAAT     200
Tx p50       151 TTTACTATAC AAGGTGGTTT TCTTCAAAAT TATGATGCTA TTGGCATAAT     200

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Tx p10       201 GTTTTGGTGG GGCATTGGTA CTAATGCAGA TAAAAAATCC AATGATCCCA     250
Tx p20       201 GTTTTGGTGG GGCATTGGTA CTAATGCAGA TAAAAAATCC AATGATCCCA     250
Tx p30       201 GTTTTGGTGG GGCATTGGTA CTAATGCAGA TAAAAAATCC AATGATCCCA     250
Tx p40       201 GTTTTGGTGG GGCATTGGTA CTAATGCAGA TAAAAAATCC AATGATCCCA     250
Tx p50       201 GTTTTGGTGG GGCATTGATA CTAATGCAGA TAAAAAATCC AATGATCCTA     250

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Tx p20       251 TTTTTAATTT AACATGGGGC AACTTTTTCC TTAATTCTAA GAATTTTACA     300
Tx p30       251 TTTTTAATTT AACATGGGGC AACTTTTTCC TTAATTCTAA GAATTTTACA     300
Tx p40       251 TTTTTAATTT AACATGGGGC AACTTTTTCC TTAATTCTAA GAATTTTACA     300
Tx p50       251 CTTTTAATTT AACATGGGGC AACTTTTTCC TTAATTCTAA GAATTTTACA     300

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Tx p40       301 GGTTTCCCTA AAGTTAAAAG TGTTATATTC ATTGCCACTG GAGATATTTT     350
Tx p50       301 GGTTTCCCTA AAGTTAAAAG TGTTATATTC ATTGCCACTG GAGATATTTT     350

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Tx p20       351 TGTAAATGGT GTTTTAATGG GTGTTTATAA TCTAAATTTT ACGCAAACCT     400
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Tx p40       351 TGTAAATGGT GTTTTAATGG GTGTTTATAA TCTAAATTTT ACGCAAACCT     400
Tx p50       351 TGTAAATGGT GTTTTAATGG GTGTTTATAA TCTAAATTTT ACGCAAACCT     400
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Tx p10       401 TAACAATTTG GTTAGCACAG TGTGTTGGCA CAATGAAAGT TGTTATTTTA     450
Tx p20       401 TAACAATTTG GTTAGCACAG TGTGTTGGCA CAATGAAAGT TGTTATTTTA     450
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Tx p50       401 TAACAATTTG GTTAGCACAG TGTGTTGGCA CAATGAAAGT TGTTATTTTA     450
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Tx p50          501 TGAACCCTGC ACAGGAGACA CTACTATTAA TAAGTTACGT TGCGCTTATC 550

                          560         570         580         590         600
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Tx p50          651 TAGTCAAGAG GTACATGAAT TTTATGTAGC TCTTAAAGGA GATCCTGTTA 700

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Tx p40          701 ATTACAATCA AAGTTGTGTA GACTCTAAGT ACTCATTCTT TAAATTAAAG 750
Tx p50          701 ATTACAATCA AAGTTGTGTA GACTCTAAGT ACTCATTCTT TAAATTAAAG 750

                          760         770         780         790         800
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Tx p20          751 TGTAATAATA CTTATTCTTG GGATTTTGAT .......... .......... 800
Tx p30          751 TGTAATAATA CTTATTCTTG GGATTTTGAT .......... .......... 800
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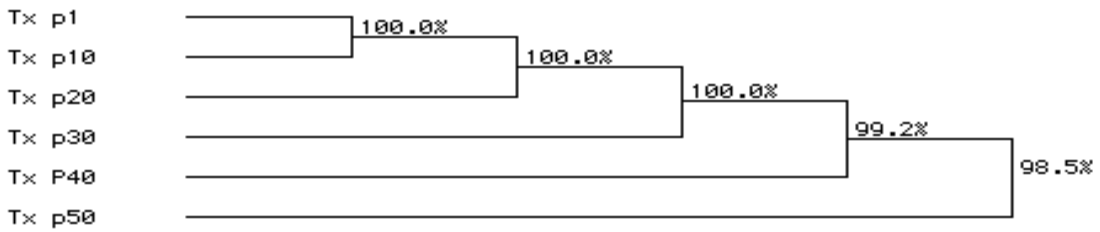
B.



C.

```
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Tx p10[Frame       1 DFYSPDVMRP SDGAYIQSGY YEPLFTGCFN QTNQTDTTCK NGLYAGSPGN       50
Tx p20[Frame       1 DFYSPDVMRP SDGAYIQSGY YEPLFTGCFN QTNQTDTTCK NGLYAGSPGN       50
Tx p30[Frame       1 DFYSPDVMRP SDGAYIQSGY YEPLFTGCFN QTNQTDTTCK NGLYAGSPGN       50
Tx p40[Frame       1 DFYSPDVMRP PDGAYIQSGY YEPLFTGCFK QTNQTDTTCK NGLYAGSPGN       50
Tx p50[Frame       1 DFYSPDVMRP PDGAYIQSGY YEPLFTGCFN QTNQTDSTCK NGLYAGSPGN       50

                          60         70         80         90        100
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Tx p10[Frame      51 FTIQGGFLQN YDAIGIMFWW GIGTNADKKS NDPIFNLTWG NFFLNSKNFT      100
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Tx p30[Frame      51 FTIQGGFLQN YDAIGIMFWW GIGTNADKKS NDPIFNLTWG NFFLNSKNFT      100
Tx p40[Frame      51 FTIQGGFLQN YDAIGIMFWW GIGTNADKKS NDPIFNLTWG NFFLNSKNFT      100
Tx p50[Frame      51 FTIQGGFLQN YDAIGIMFWW GIDTNADKKS NDPTFNLTWG NFFLNSKNFT      100

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Tx p10[Frame     101 GFPKVKSVIF IATGDIFVNG VLMGVYNLNF TQTLTIWLAQ CVGTMKVVIL      150
Tx p20[Frame     101 GFPKVKSVIF IATGDIFVNG VLMGVYNLNF TQTLTIWLAQ CVGTMKVVIL      150
Tx p30[Frame     101 GFPKVKSVIF IATGDIFVNG VLMGVYNLNF TQTLTIWLAQ CVGTMKVVIL      150
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Tx p20[Frame     151 RNSNALVHFS AGNVVAFEPC TGDTTINKLR CAYQQFNFST GFYDIDTFVP      200
Tx p30[Frame     151 RNSNALVHFS AGNVVAFEPC TGDTTINKLR CAYQQFNFST GFYDIDTFVP      200
Tx p40[Frame     151 RNSNALVHFS AGNVVAFEPC TGDTTINKLR CAYQQFNFST GFYDIDTFVP      200
Tx p50[Frame     151 RNSNALVHFS AGNVVAFEPC TGDTTINKLR CAYQQFNFST GFYDIDTFVP      200

                         210        220        230        240        250
Tx p1[Frame      201 VTPNITHLPY PDLKDNTSQE VHEFYVALKG DPVNYNQSCV DSKYSFFKLK      250
Tx p10[Frame     201 VTPNITHLPY PDLKDNTSQE VHEFYVALKG DPVNYNQSCV DSKYSFFKLK      250
Tx p20[Frame     201 VTPNITHLPY PDLKDNTSQE VHEFYVALKG DPVNYNQSCV DSKYSFFKLK      250
Tx p30[Frame     201 VTPNITHLPY PDLKDNTSQE VHEFYVALKG DPVNYNQSCV DSKYSFFKLK      250
Tx p40[Frame     201 VTPNITHLPY PDLKDNTSQE VHEFYVALKG DPVNYNQSCV DSKYSFFKLK      250
Tx p50[Frame     201 VTPNITHLPY PDLKDNTSQE VHEFYVALKG DPVNYNQSCV DSKYSFFKLK      250

                         260        270        280        290        300
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Tx p10[Frame     251 CNNTYSWDFD .......... .......... .......... ..........      300
Tx p20[Frame     251 CNNTYSWDFD .......... .......... .......... ..........      300
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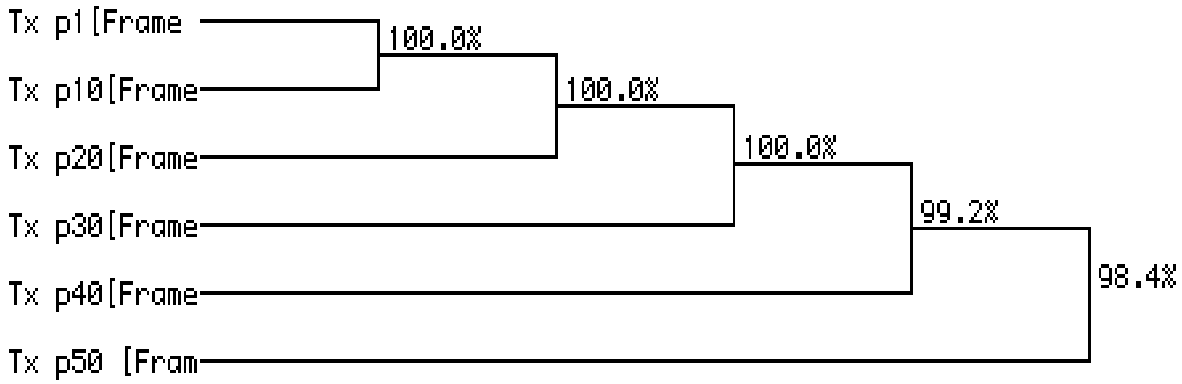
D.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98.4%

Figure 2. Nucleotide sequence analysis of TCoV isolate R through 50 passages in SPF turkey embryos. A) Clustal V alignment of passages 1, 10, 20, and 30. Mutations are shown in red. B) Phlyogenetic tree depicting percent similarities between nucleotide sequence for each passage.

A.

```
                    10         20         30         40         50
R p1           1 GATTTTTATA GTCCAGATGT TATGCGACCG CCGGATGGTG CGTATATACA      50
R p11          1 GATTTTTATA GTCCAGATGT TATGCGACCG CCGGATGGTG CGTATATACA      50
R p20          1 GATTTTTATA GTCCAGATGT TATGCGACCG CCGGATGGTG CGTATATACA      50
R p30          1 GATTTTTATA GTCCAGATGT TATGCGACCG CCGGATGGTG CGTATATACA      50

                    60         70         80         90        100
R p1          51 ATCCGGTTAT TATGAACCAC TTTTCACAGG TTGTTTTAAC CAAACTAATC     100
R p11         51 ATCCGGTTAT TATGAACCAC TTTTCACAGG TTGTTTTAAC CAAACTAATC     100
R p20         51 ATCCGGTTAT TATGAACCAC TTTTCACAGG TTGTTTTAAC CAAACTAATC     100
R p30         51 ATCCGGTTAT TATGAACCAC TTTTCACAGG TTGTTTTAAC CAAACTAATC     100

                   110        120        130        140        150
R p1         101 AAACCGAGTT CACCTGTAGA AATGGGCTTT ATGGAGGGTC GCCAGGTAAT     150
R p11        101 AAACCGAGTT CACCTGTAGA AATGGGCTTT ATGGAGGGTC GCCAGGTAAT     150
R p20        101 AAACCGAGTT CACCTGTAGA AATGGGCTTT ATGGAGGGTC GCCAGGTAAT     150
R p30        101 AAACCGAGTT CACCTGTAGA AATGGGCTTT ATGGAGGGTC GCCAGGTAAT     150

                   160        170        180        190        200
R p1         151 TTTACTATAC AAGGTGCTTT TCTTCAAAAT TATGATGCTA TTGGCATAAT     200
R p11        151 TTTACTATAC AAGGTGCTTT TCTTCAAAAT TATGATGCTA TTGGCATAAT     200
R p20        151 TTTACTATAC AAGGTGCTTT TCTTCAAAAT TATGATGCTA TTGGCATAAT     200
R p30        151 TTTACTATAC AAGGTGCTTT TCTTCAAAAT TATGATGCTA TTGGCATAAT     200

                   210        220        230        240        250
R p1         201 GTTTTGGTGG GGCATTAATA CTAAAGGCAA TGATCCTATT TTTAATTTAA     250
R p11        201 GTTTTGGTGG GGCATTAATA CTAAAGGCAA TGATCCTATT TTTAATTTAA     250
R p20        201 GTTTTGGTGG GGCATTAATA CTAAAGGCAA TGATCCTATT TTTAATTTAA     250
R p30        201 GTTTTGGTGG GGCATTAATA CTAAAGGCAA TGATCCTATT TTTAATTTAA     250

                   260        270        280        290        300
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R p20        251 CATGGGGCAA CTTTTTCTTT AATTCTAAGA ATTTTACAGG TTTCCCTAAA     300
R p30        251 CATGGGGCAA CTTTTTCTTT AATTCTAAGA ATTTTACAGG TTTCCCTAAA     300

                   310        320        330        340        350
R p1         301 GTTAAAAGTG TTATATTCAT TGCCACTGGA GATATTTTTG TAAATGGCGT     350
R p11        301 GTTAAAAGTG TTATATTCAT TGCCACTGGA GATATTTTTG TAAATGGCGT     350
R p20        301 GTTAAAAGTG TTATATTCAT TGCCACTGGA GATATTTTTG TAAATGGCGT     350
R p30        301 GTTAAAAGTG TTATATTCAT TGCCACTGGA GATATTTTTG TAAATGGCGT     350

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R p11        351 TTTAATGGGT ATTTATAATC TAAATTTTAC GCAAAACTTA ACAATTTGGT     400
R p20        351 TTTAATGGGT ATTTATAATC TAAATTTTAC GCAAAACTTA ACAATTTGGT     400
R p30        351 TTTAATGGGT ATTTATAATC TAAATTTTAC GCAAAACTTA ACAATTTGGT     400

                   410        420        430        440        450
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R p11        401 TAGCACAGTG TGTTGGCACA ATGAAAGTTG TTATTTTACG TAATAGTAAT     450
R p20        401 TAGCACAGTG TGTTGGCACA ATGAAAGTTG TTATTTTACG TAATAGTAAT     450
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                   460        470        480        490        500
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R p20        451 GCTCTAGTTC GGTTTTCAGC TGGCAACGTA GTTGCTTTTG AACCCTGTAC     500
R p30        451 GCTCTAGTTC GGTTTTCAGC TGGCAACGTA GTTGCTTTTG AACCCTGTAC     500

                   510        520        530        540        550
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R p11         501 AGGAGACACT ACTATTAATA AGTTACGTTG CGCTTATCAG CAATTTAATT      550
R p20         501 AGGAGACACT ACTATTAATA AGTTACGTTG CGCTTATCAG CAATTTAATT      550
R p30         501 AGGAGACACT ACTATTAATA AGTTACGTTG CGCTTATCAT CAATTTAATT      550

                      560        570        580        590        600
R p1          551 TTTCTACAGG ATTTTATGAC ATAGATACTT TTGTACCTGT GACATCTAAT      600
R p11         551 TTTCTACAGG ATTTTATGAC ATAGATACTT TTGTACCTGT GACATCTAAT      600
R p20         551 TTTCTACAGG ATTTTATGAC ATAGATACTT TTGTACCTGT GACATCTAAT      600
R p30         551 TTTCTACAGG ATTTTATGAC ATAGATACTT TTGTACCTGT GACATCTAAT      600

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R p11         601 ATTACATATT TACCTTACCC AGATTTAAAA GATAATACTG GTCAACAGGT      650
R p20         601 ATTACATATT TACCTTACCC AGATTTAAAA GATAATACTG GTCAACAGGT      650
R p30         601 ATTACATATT TACCTTACCC AGATTTAAAA GATAATACTG GTCAACAGGT      650

                      660        670        680        690        700
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R p11         651 ATATTCTTTT TATGTAGCTC TTAAAGGAGA TTCTGTTAAT TACAATCAAA      700
R p20         651 ATATTCTTTT TATGTAGCTC TTAAAGGAGA TTCTGTTAAT TACAATCAAA      700
R p30         651 ATATTCTTTT TATGTAGCTC TTAAAGGAGA TTCTGTTAAT TACAATCAAA      700

                      710        720        730        740        750
R p1          701 GTTGTGTAGA CTCTAAGTAC CCATTCTTTA AATTAAAGTG TAATAATACT      750
R p11         701 GTTGTGTAGA CTCTAAGTAC CCATTCTTTA AATTAAAGTG TAATAATACT      750
R p20         701 GTTGTGTAGA CTCTAAGTAC CCATTCTTTA AATTAAAGTG TAATAATACT      750
R p30         701 GTTGTGTAGA CTCTAAGTAC CCATTCTTTA AATTAAAGTG TAATAATACT      750

                      760        770        780        790        800
R p1          751 TATTCTTGGG ATTTTGAT.. .......... .......... ..........      800
R p11         751 TATTCTTGGG ATTTTGAT.. .......... .......... ..........      800
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R p30         751 TATTCTTGGG ATTTTGAT.. .......... .......... ..........      800
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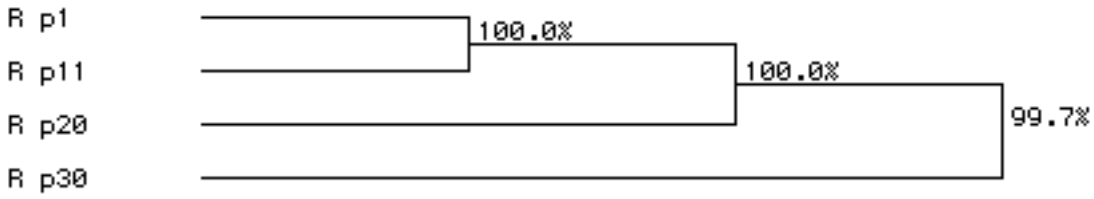
B.



C.

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                          60         70         80         90        100
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R p11[Frame       51 FTIQGAFLQN YDAIGIMFWW GINTKGNDPI FNLTWGNFFF NSKNFTGFPK     100
R p20[Frame       51 FTIQGAFLQN YDAIGIMFWW GINTKGNDPI FNLTWGNFFF NSKNFTGFPK     100
R p30[Frame       51 FTIQGAFLQN YDAIGIMFWW GINTKGNDPI FNLTWGNFFF NSKNFTGFPK     100

                         110        120        130        140        150
R p1[Frame 1     101 VKSVIFIATG DIFVNGVLMG IYNLNFTQNL TIWLAQCVGT MKVVILRNSN     150
R p11[Frame      101 VKSVIFIATG DIFVNGVLMG IYNLNFTQNL TIWLAQCVGT MKVVILRNSN     150
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R p30[Frame      101 VKSVIFIATG DIFVNGVLMG IYNLNFTQNL TIWLAQCVDT MKVVILRNSN     150

                         160        170        180        190        200
R p1[Frame 1     151 ALVRFSAGNV VAFEPCTGDT TINKLRCAYQ QFNFSTGFYD IDTFVPVTSN     200
R p11[Frame      151 ALVRFSAGNV VAFEPCTGDT TINKLRCAYQ QFNFSTGFYD IDTFVPVTSN     200
R p20[Frame      151 ALVRFSAGNV VAFEPCTGDT TINKLRCAYQ QFNFSTGFYD IDTFVPVTSN     200
R p30[Frame      151 ALVRFSAGNV VAFEPCTGDT TINKLRCAYH QFNFSTGFYD IDTFVPVTSN     200

                         210        220        230        240        250
R p1[Frame 1     201 ITYLPYPDLK DNTGQQVYSF YVALKGDSVN YNQSCVDSKY PFFKLKCNNT     250
R p11[Frame      201 ITYLPYPDLK DNTGQQVYSF YVALKGDSVN YNQSCVDSKY PFFKLKCNNT     250
R p20[Frame      201 ITYLPYPDLK DNTGQQVYSF YVALKGDSVN YNQSCVDSKY PFFKLKCNNT     250
R p30[Frame      201 ITYLPYPDLK DNTGQQVYSF YVALKGDSVN YNQSCVDSKY PFFKLKCNNT     250

                         260        270        280        290        300
R p1[Frame 1     251 YSWDFD.... .......... .......... .......... ..........     300
R p11[Frame      251 YSWDFD.... .......... .......... .......... ..........     300
R p20[Frame      251 YSWDFD.... .......... .......... .......... ..........     300
R p30[Frame      251 YSWDFD.... .......... .......... .......... ..........     300
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D.

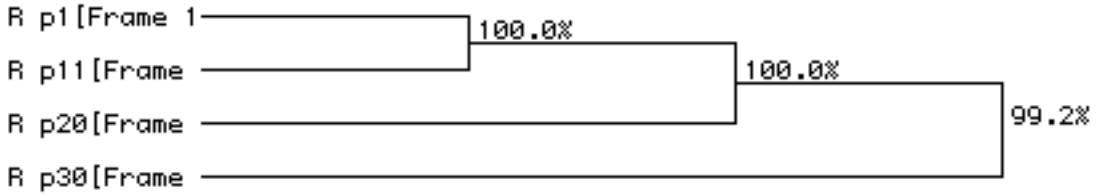


Table 1. Exact mutations and mutational rate seen after serial passaging of A) Tx strain and B) R strain. Nucleotide and Amino Acid positions are relative to the ATG start site of the S1 gene.

A.

Tx Strain	Nucleotide Mutations	Amino Acid Mutations
40 passages	183(C→T), 189(A→C), 193(T→C), 198(C→T), 225(C→T), 252(C→A)	65(Ser→Pro), 84(Asn→Lys)
50 passages	246(C→T), 252(A→T), 271(A→T), 380(G→A), 411(C→T), 413(T→C)	84(Lys→Asn), 91(Thr→Ser), 127(Gly→Asp), 138(Ile→Thr)
Mutational Rate	$3x10^{-2}$% nucleotide changes per passage	$4.6x10^{-2}$% amino acid change pers passage

B.

R Strain	Nucleotide Mutations	Amino Acid Mutations
30 Passages	578(G→), 702(G→T)	193(Gly→Asp), 294(Gln→His)
Mutational Rate	$8.6x10^{-5}$% nucleotide changes per passage	$2.6x10^{-4}$% amino acid change per passage

Chapter 3

SEROLOGICAL RELATIONSHIPS AMONG DIFFERENT GENOTYPES OF TURKEY CORONAVIRUS: DEVELOPMENT OF A NOVEL VIRUS NEUTRALIZATION ASSAY[1]

[1]Boynton, T. O., M. W. Jackwood, S. A. Callison, and D. A. Hilt. To be included in a submission to Journal of Virology.

Summary

The serological relationship between two strains of turkey coronavirus (TCoV) was studied. Recent sequence evidence suggests the presence of a hypervariable region within the s1 subunit of the spike gene. In infectious bronchitis virus, the closest relative of TCoV, hypervariable regions can be directly correlated to serotype. Because no previous accounts of different serotypes within TCoV have been reported, the effect of this region is not known. To study this, a novel means of virus neutralization was developed using real-time RT-PCR as a quantifiable means of detection. An indirect ELISA utilizing whole virus as antigen was also developed. Data gathered from these tests suggest that serological differences do exist between these strains, though the hypervariable region may not be the only sequences involved.

Introduction

Transmissible coronaviral enteritis (TCE) of turkeys is a disease characterized by diarrhea, inappetance, ruffled feathers, decreased body weight, and a general loss in production. Mortality is usually low during outbreaks, but morbidity can be as high as 100% (14, 6). The causative agent of this disease is Turkey Coronavirus (15). This virus has also been implicated as a cofactor in two other diseases, poult enteritis and mortality syndrome (PEMS) and spiking mortality of turkeys (SMT)(2, 22). Both exhibit similar clinical signs as TCE, but with much higher mortality rates. This is due to the presence of other viruses and enteropathogenic *E. Coli* (EPEC). Turkey coronavirus and EPEC exhibit a synergistic relationship with each other, increasing the severity of both infections that would otherwise be mild separately (4).

Coronaviruses are positive sense RNA viruses in the *Coronaviridae*, who along with *Arteriviridae* make up the order *Nidovirales*. Their genome consists of a single stranded molecule of RNA ~30kb which is transcribed into several nested mRNAs (17). Roughly three quarters of this genome is devoted to two overlapping open reading frames (ORF) encoding the polymerase and several proteases. The 3’ end of the genome is transcribed into several mRNA segments that encode the structural proteins. One of these proteins, the Spike protein,

is associated with virus neutralization, attachment, and membrane fusion (13). It is post-translationally cleaved into two subunits, S1 and S2.

The spike gene of infectious bronchitis virus (IBV), which is the closest relative of TCoV, contains three hypervariable regions (HVR) within the S1 subunit (12). These hypervariable regions are known to correlate to neutralizing antibody-inducing epitopes among different serotypes (21, 7). Analysis of available sequence data from several strains of TCoV suggests the presence of a HVR in relatively the same position as HVRII of IBV (data not shown). The effect of this region on serology in TCoV is not known.

One means of examining serological relationships is the enzyme-linked Immunosorbent assay (ELISA). Using a colormetric scale, this test can determine if antibodies present in samples react to specific antigens. Several ELISAs have been developed for TCoV using various types of antigens (4, 9), but none have been used to study polyclonal sera produced against different strains. While ELISA tests provide valuable data concerning antibody-antigen interaction, they yield no data concerning virus neutralization.

Classically, serotypes are calculated via the method developed by Archetti and Horsfall (1). By this method, a numerical index is created that is interpreted as the antigenic

relatedness between the two viruses. It is used to determine if two viruses fall within the same serotypes. The index is found by finding the neutralizing titer of antisera raised against both viruses, according to the formula, $r^2=(hetero_2/homo_1)X(hetero_1/homo_2)$, where r=index, $hetero_2$=the heterologous titer of virus 2, $homo_1$=the homologous titer of virus 1, $hetero_1$=the heterologous titer of virus 1, and $homo_2$=the homologous titer of virus 2. The virus neutralization test utilizes the appearance of clinical signs in specific-pathogen free (SPF) embryonating turkey eggs. While TCoV replicates in SPF eggs readily, it does not consistently produce clinical signs making it difficult to conduct virus neutralization tests.

Recently, researchers in our lab have developed a real-time RT-PCR (RRT-PCR) test to detect IBV in clinical samples (3). This test amplifies a region in the 5’ UTR of the IBV genome. The test has also been found to adequately detect and quantify TCoV. Amplicon quantification is expressed in C_T values, which are the cycle thresholds that exponential amplification of PCR products is first detected (18). The higher the C_T value, the less RNA present. Several quantitative RRT-PCR tests have been described that detect different viruses and are much more sensitive than standard virus isolation (5, 8, 20). Along these same lines, Santen *et al*. recently described a novel method of virus neutralization involving RRT-PCR to detect the presence of

chicken anemia virus replicating *in vitro* following neutralization by specific antibodies (16).

In this study, an ELISA was used to judge antibody-antigen interactions between two strains TCoV. In addition, a novel means of virus neutralization was used to test serotype specificity in these strains.

Materials and Methods

Viruses. Two isolates of TCoV were recovered from turkey poults in Texas and designated Tx and R. These isolates were obtained from Purdue University, West Lafayette, IN courtesy of Tom Hooper.

Virus Isolation. Intestines were collected from embryos and placed in separate Lysing Matrix D tubes (Q-biogene) in 200 μl of PBS and homogenized in a FastPrep® instrument (Q-biogene) for 40 s at 4.0 m/s. Tubes were centrifuged at 2500 rpm in a standard tabletop centrifuge for 5 min and supernatants were collected.

RNA was isolated from supernatants using the High Pure Isolation kit (Roche) according to the manufacturer's recommendations. Briefly, 200 μl of virus-containing homogenate from each sample was added to appropriate buffers and columns and eluted to 75 μl.

The presence of TCoV was detected by RRT-PCR as described by Callison *et al*.(3) Briefly, a Quantitect Probe RT-PCR kit (Qiagen) was used and PCR was carried out using a SmartCylcler instrument (Cephied). Primers and probe were specific for a 150 bp region in the 5’ untranslated region (UTR) of TCoV.

TCoV Purification. TCoV was purified using a sucrose cushion as described by Loa *et al*. (10) Briefly, TCoV-containing intestinal homogenates were centrifuged at 100,000 x *g* in PBS buffer (pH 7.4) on top of a 5 ml cushion of 60% sucrose. Fractions were taken from the opalescent band generated above the sucrose and tested for the presence of virus. Those fractions containing high amounts of TCoV were pooled for use in the ELISA.

Titration of TCoV. Titrations of virus infectivity were performed in specific-pathogen free (SPF) turkey embryos and expressed in median (50%) PCR-positive doses (PCR_{50}), whereby a unit of PCR_{50} would represent the amount of TCoV capable of resulting in PCR-positive samples in 50% of the hosts. Tenfold dilutions of virus were inoculated in groups of 5 23-day old embryos via the chlorioallantoic sac route an intestines were harvested after 3 days. Embryos positive by RRT-PCR were recorded and titrations were determined using the Reed and Muench method (19).

Antibody Production. Turkey polyclonal antibodies against TCoV isolates were produced in SPF turkey poults. Poults at three weeks of age were inoculated intratracheally with $1x10^6$ PCR_{50} of sucrose-cushion purified TCoV per bird. Poults were then boosted identically after two weeks, and antibodies were harvested 1 week after boost.

Rabbit polyclonal antibodies against TCoV were also produced. Rabbits were inoculation with $1x10^6$ PCR_{50} sucrose-cushion purified TCoV along with Freund's adjuvant and boosted 1 week later. After two weeks, serum was harvested and verified positive by ELISA. Pre-immunized sera was also collected and tested to ensure no antibodies against similar coronaviruses were present.

Both turkey and rabbit antisera were verified using a commercial IDEXX IBV ELISA kit using modified secondary antibodies (anti-turkey and anti-rabbit IgG conjugated with HRP) according to the manufacturers recommendations.

Indirect Antibody ELISA. ELISA was carried out using the KPL protein detector ELISA kit (Kirkgaard & Perry Laboratories) as described by McKenzie et al (11). Briefly, plates were coated with equal volumes of sucrose-cushion purified TCoV and coating buffer, and incubated for 1 hour at room temperature then blocked with buffer containing BSA. Separate plates were coated with Tx antigen and R antigen. Turkey antibodies were serially

diluted from 1:2 to 1:64, and secondary anti-turkey antibodies were diluted 1:500. Elisa steps were carried out as recommended by KPL.

Virus Neutralization. Rabbit polyclonal antisera against the Tx and R isolates were serially diluted from 1:16 to 1:1024. Virus ($1x10^3$ PCR_{50}/ml) was mixed with an equal volume of each serum dilution and incubated at 37^o C for 30 min. Each mixture was then inoculated into five 23-day old SPF turkey embryos at 0.2 ml per egg. Positive controls were inoculated without the addition of antibodies, but diluted 1:2 in PBS and inoculated 0.2 ml per egg. Intestines were harvested from inoculated and negative control embryos after three days and tested by RRT-PCR. Homologous neutralization and heterologous neutralization was carried out for both the Tx and R isolates.

Results

Titrations. The results of the titrations are presented in tables 1 and 2. The Tx isolate was found to have a titer of $10^{7.7}$ PCR_{50} doses/ml. The R isolate was found to have a titer of $10^{5.3}$ PCR_{50} doses/ml.

Antibodies. Turkey and Rabbit antibodies were found to be positive at the standard dilution of 1:500 by the IDEXX IBV ELISA.

ELISA. In plates coated with Tx antigen, titers were recorded as 16 for Tx antisera and 4 for R antisera. In plates coated

with R antigen, Tx antisera was found to be negative and R antibodies were found to have a titer of 16. Positive controls of IBV antisera were also found to be positive. Negative samples exhibited no activity.

Virus Neutralization. Virus neutralization data obtained by RRT-PCR is presented in tables 3-8 and figures 1 and 2. All negative controls were found to be negative, and all positive controls contained virus. For the Tx strain of TCoV, given an average positive control C_T value of ~19, the titer of the homologous neutralization was 64. The heterologous titer appears to be around 128. For the R strain, given an average positive control C_T value of ~13, the titer of the homolgous neutralization was $\geq$1024. The heterologous titer was 16. These titers were recorded as the titer above the first titer to drop below the average positive control.

Discussion

The results from the ELISA test indicate that there are differences between the antibodies produced against the Tx TCoV strain and R TCoV Strain. Using plates coated with Tx antigen, strain specific Tx antisera had a titer of 1:16, whereas the same antisera did not react at all against a plate coated with R antigen. Likewise, strain specific R antisera had a titer of 1:16 when reacted against its own antigen, yet had only a titer of 1:4 when reacted against Tx antigen. Having high homologous

titers and low heterologous titers suggests that there are perhaps different serotypes, though it is distinctly possible the different antibodies present in the sera are not neutralizing.

Data obtained from the virus neutralization assay again suggests serological differences between the two TCoV strains. Using a scatter plot (figs. 1 and 2) neutralization can be inferred in samples where the Ct value given is higher than the highest positive control. From figure 1, as well as the titers found by using average Ct value, it appears that both antiseras neutralized Tx antigen at about the same dilution; in this case it appears that R antisera neutralized somewhat better than Tx antisera. In the R TCoV neutralization studies however, R strain-specific antisera neutralized the virus as would be expected, and Tx strain-specific antisera for the most part did not, which supports the possibility that they are different serotypes.

There are two possible reasons for the outcomes seen from these virus neutralizations. If the R strain contained all the neutralizing epitopes found on Tx as well as others not present, then the data would make sense. Antisera raised against the R antigen would provide antibodies capable of neutralizing both viruses. Antisera raised against only the Tx strain, however, would fail to neutralize R as this data suggests.

The other possible explanation would be that the neutralization did not work as efficiently as was hoped. The standard deviations of the two positive control groups are important to note. In the R strain, the standard deviation was 0.66, yet the Tx positive Ct values ranged from 12-30. The errant values detected for the Tx strain are perhaps explained by the inoculation of SPF embryonating turkey eggs which cannot be controlled as well as systems like cell culture. The proper way to inoculate TCoV is by the yolk sac route, but inoculating 23 day-old eggs via this route is difficult. Because of this, it was decided that inoculation by the chlorioallantoic sac route instead of the yolk sac would be the easiest to ensure the same inoculation every time. While this was the best option, it is not known if the uptake of TCoV via this method is always the same in every embryo. Another explanation for these results may be the presence of subpopulations within the Tx isolate inoculated. This might provide different subsets of antibodies resulting in differences in data. But, since all embryos were inoculated with the same TCoV suspension that was used to produce antibodies, the effect should be minimal.

Despite the different explanations for our results, it appears that serological differences do exist between these two viruses. Homologous and heterologous neutralization reactions for the R strain of TCoV are clearly different. Different

serotypes suggest that vaccines developed with a specific TCoV strain may not be protective against future outbreaks of TCE.

References.

1. **Archetti, I. and F. L. Horsfall Jr.** 1950. Persistent antigenic variation of influenze A viruses after incomplete neutralization in ovo with heterologous immune serum. Jour Exp Meth. 92:441-462.
2. **Brown, T. P., A. Garcia, and L. Kelly.** 1997. Spiking mortality of poults: I. Experimental reproduction in isolation facilities. Avian Dis. 41:604-609.
3. **Callison, S. A., M. W. Jackwood, C. W. Lee, and D. A. Hilt.** 2004. Development of a real-Time RT-PCR assay for the detection of infectious bronchitis virus in biological samples. Proc. 141st Annual Meeting of the American Veterinary Medical Association, Philadelphia, PA. July 24-28.
4. **Guy, J. S., L. G. Smith, J. J. Breslin, and S. Pakpinyo.** 2002. Development of a competitive enzyme-linked immunosorbent assay for detection of turkey coronavirus antibodies. Avian Dis. 46:334-341.
5. **Hussy, D., N. Stauber, C. M. Leutenegger, S. Rieder, and M. Ackermann.** 2001. Quantitative fluorogenic PCR assay for measuring ovine herpesvirus 2 replication in sheep. Clin. Diagn. Lab. Immunol. 8:123-128.
6. **Ismail, M. M., K. O. Cho, M. Hasoksuz, L. J. Saif, and Y. M. Saif.** 2001. Antigenic and genomic relatedness of turkey-

origin coronaviruses, bovine conronaviruses, and infectious bronchitis virus of chickens. Avian Dis. 45:978-84.

7. **Lee, C. and M. W. Jackwood.** 2001. Origin and evolution of Georgia 98 (GA98), a new serotype of avian infectious bronchitis virus. Virus Res. 80:33-39.

8. **Leutenegger, C. M., D. Klein, R> Hofmann-Lehmann, C. Mislin, U. Hummel, J. Boni, F. Boretti, W. H. Guenzburg, and H. Lutz.** 1999. Rapid feline immunodeficiency virus provirus quantitation by polymerase chain reaction using the TaqMan fluorogenic real-time detection system. J. Virol. Methods. 78:105-116.

9. **Loa, C. C., T. L. Lin, C. C. Wu, T. A. Bryan, H. L. Thacker, T. Hooper, and D. Schrader.** 2000. Detection of antibody to turkey coronavirus by antibody-capture enzyme-linked immunosorbent assay utilizing infectious bronchitis virus antigen. Avian Dis. 44:498-506.

10. **Loa, C. C., T. L. Lin, C. C. Wu, T. A. Bryan, H. L. Thacker, T. Hooper, and D. Schrader.** 2002. Purification of turkey coronavirus by Sephacryl size-exclusion chromatography. J Virol Methods. 104:187-194.

11. **McKenzie, K., T. Boynton, and D. Hilt, M. W. Jackwood, and S. Callison.** 2004. Development of a TCoV specific ELISA for detection of turkey coronavirus antibodies. National Merck-Merial Scholars Symposium. July 28-Aug 1, Aubrun, AL.

12. **Moore, K. M., J. D. Bennet, B. S. Seal, and M. W. Jackwood.** 1998. Sequence comparison of avian infectious bronchitis virus s1 glycoproteins of the Florida serotype and five variant isolates from Georgia and California. Virus Genes. 17:63-83.

13. **Moore, K. M., M. W. Jackwood, and D. A. Hilt. 1997.** Identification of amino acids involved in a serotype and neutralization specific epitope within the s1 subunit of avian infectious bronchitis virus. Arch Virol. 142:2249-2256.

14. **Pomeroy, M. S. aqnd J. M. Sieburth.** 1953. Bluecomb disease of turkeys. Proc. Book, AVMA. pp. 321-327.

15. **Ritchie, A. E., D. R. Deshmukh, C. T. Larsen, and B. S. Pomeroy.** 1973. Electron microscopy of coronavirus-like particles characteristic of turkey bluecomb disease. Avian Dis. 17:546-558.

16. **Santen, V. L., B. Kaltenboeck, K. S. Joiner, K. S. Macklin, and R. A. Norton.** 2004. Real-time quantitative PCR-based serum neutralization test for detection and titration of neutralizing antibodies to chicken anemia virus. J Virol. Methods. 115:123-135.

17. **Siddell, S. G.** 1995. The coronaviridae, an introduction. In: Siddell, S. G. (Ed.) Coronaviridae. Plenum, New York. pp. 1-9.

18. **Song, Y., C. Liu, and S. M. Finegold.** 2004. Real-time PCR quantitation of clostridia in feces of autistic children. Appl Environ Microbiol. 11:6459-6465.

19. **Thayer, S. and C. W. Beard.** 1998. Serological Procedues. In: Swayne, D. E., J. R. Glisson, M. W. Jackwood, J. E. Pearson, and W. M. Reed (eds.) A laboratory manual for the isolation and identification of avian pathogens, 4th edition. Rose Printing, Tallahasse FL. pp. 255-266.

19. **Vogtlin, A., C. Fraefel, S. Albini, C. M. Leutenegger, E. Schraner, B. Spiess, H. Lutz, and M. Ackermann.** 2002. Quantification of feline herpesvirus 1 DNA in ocular fluid samples of clinically diseased cats by real-time TaqMan PCR. J. Clin. Microbio. 40:519-523.

20. **Wang, C. H. and Y.-C. Huang.** 2000. Relationship between serotypes and genotypes based on the hypervariable region of the s1 gene of infectious bronchitis virus. Arch Virol. 145:291-300.

21 **Yu, M., M. M. Ismail, M. A. Qureshi, R. N. Dearth, H. J. Barnes, and Y. M. Saif.** 2000. Viral agents associated with poult enteritis and mortality syndrome: the role of a small round virus and a turkey coronavirus. Avian Dis. 44:297-304.

Table 1. Calculation of the titer of Tx antigen by the 50% endpoint of RRT-PCR positives using the method of Reed and Muench. Embryos were inoculated with 0.1 ml virus.

Virus Dilution Inoculated	Embryos		Accumulated Numbers		Proportion Positive/total	Percent Positive
	Number Positive	Number Negative	Positive	Negative		
10^{-4}	5	0	17	0	17/17	100
10^{-5}	5	0	12	0	12/12	100
10^{-6}	5	0	7	0	7/7	100
10^{-7}	1	4	2	4	2/6	33.3
10^{-8}	1	4	1	8	1/9	11.1

Proportionate distance = (100-50)/(100-33.3) = 0.7.
Log factor = 1, so log of 50% endpoint equal -6.7
Titer = $10^{6.7}$ PCR_{50}/0.1 ml = $10^{7.7}$ PCR_{50}/ml

Table 2. Calculation of the titer of R antigen by the 50% endpoint of RRT-PCR positives using the method of Reed and Muench. Embryos were inoculated with 0.1 ml virus.

Virus Dilution Inoculated	Embryos		Accumulated Numbers		Proportion Positive/total	Percent Positive
	Number Positive	Number Negative	Positive	Negative		
10^{-3}	5	0	9	0	9/9	100
10^{-4}	3	2	4	2	4/6	66.7
10^{-5}	0	5	1	7	1/8	12.5
10^{-6}	0	5	1	12	1/13	7.6
10^{-7}	1	4	1	16	1/17	5.9
10^{-8}	0	5	0	21	0/21	0

Proportionate distance = (66.7-50)/(66.7-12.5) = 0.3.
Log factor = 1, so log of 50% endpoint equal -4.3
Titer = $10^{4.3}$ PCR_{50}/0.1 ml = $10^{5.3}$ PCR_{50}/ml

Table 3. Data obtained from the homologous and heterologous neutralization for the Tx strain of TCoV. Quantification of TCoV is expressed in Ct values, where the lower the value the more viral RNA present. A value of 40.00 indicates no RNA detected. SD = Standard deviation. Samples are indicated by their antibody dilution and sample number, and were compared to a positive[A] virus control and a negative[B] control.

Antisera Dilution	Average Homologous Ct Values ± SD	Average Heterologous Ct Values ± SD
1:16	28.61 ± 13.17	23.04 ± 8.44
1:32	23.51 ± 4.85	35.10 ± 0.52
1:64	26.77 ± 10.88	22.77 ± 11.21
1:128	18.76 ± 3.06	23.13 ± 6.77
1:256	17.02 ± 7.96	19.48 ± 6.54
1:512	15.71 ± 3.58	27.68 ± 1.76
1:1024	14.08 ± 2.39	20.03 ± 4.68

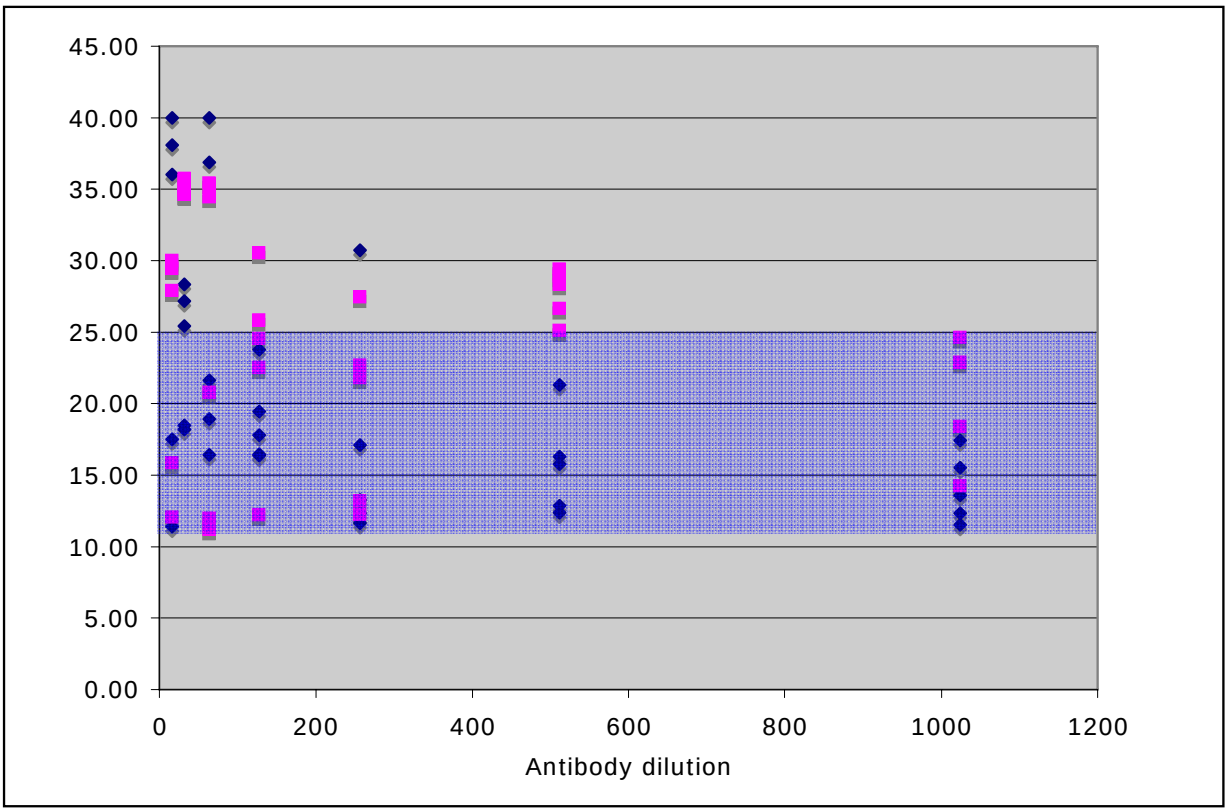
[A]Positive virus control average Ct value ± SD = 18.98 ± 6.87
[B]Negative virus control average Ct value ± SD = 40.00 ± 0

Table 4. Data obtained from the homologous and heterologous neutralization for the R strain of TCoV. Quantification of TCoV is expressed in Ct values, where the lower the value the more viral RNA present. A value of 40.00 indicates no RNA detected. SD = Standard deviation. Samples are indicated by their antibody dilution and sample number, and were compared to a positive[A] virus control and a negative[B] control.

Antisera Dilution	Average Homologous Ct Values ± SD	Average Heterologous Ct Values ± SD
1:16	25.03 ± 11.79	14.26 ± 3.87
1:32	13.77 ± 1.89	12.39 ± 0.28
1:64	23.80 ± 9.69	13.78 ± 2.37
1:128	27.85 ± 8.96	12.51 ± 0.63
1:256	23.32 ± 13.23	13.42 ± 0.68
1:512	23.38 ± 8.42	12.85 ± 0.62
1:1024	18.79 ± 6.38	19.84 ± 7.58

[A]Positive virus control average Ct value ± SD = 12.82 ± 0.66
[B]Negative virus control average Ct value ± SD = 40.00 ± 0

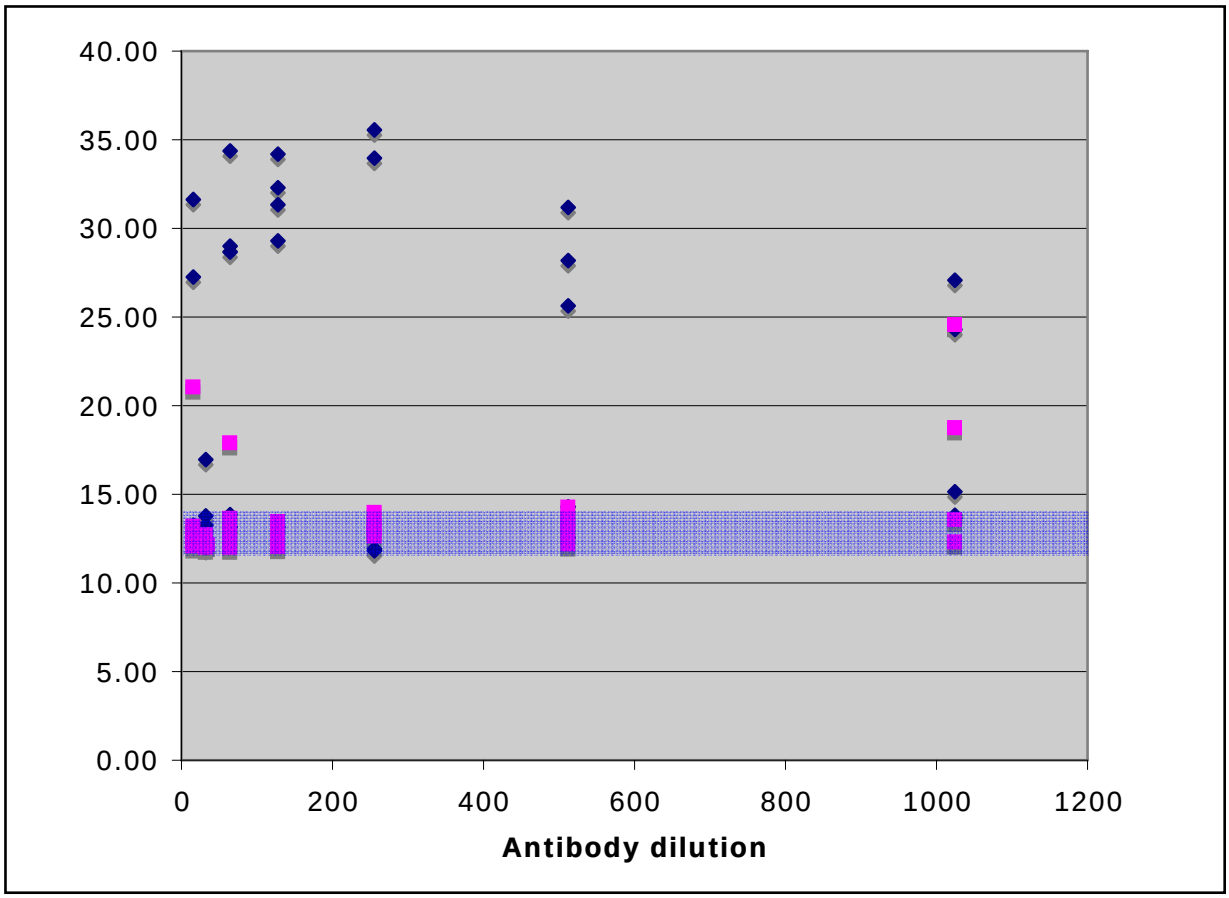
Figure 1. Scatter plots of real time RT-PCR analysis of two-way virus-neutralization tests for the Tx TCoV strain and type specific antisera against each virus. Diamonds indicate the homologous reaction and squares indicate the heterologous reactions. The blue box shows the average CT value for non-neutralized virus ± the standard deviation. Diamonds and squares above the blue box indicate neutralization has occurred (higher CT values= less virus present).



Tx Homologous Reaction

Tx Heterologous Reaction

Figure 2. Scatter plots of real time RT-PCR analysis of two-way virus-neutralization tests for the R TCoV strain and type specific antisera against each virus. Diamonds indicate the homologous reaction and squares indicate the heterologous reactions. The blue box shows the average CT value for non-neutralized virus ± the standard deviation. Diamonds and squares above the blue box indicate neutralization has occurred (higher CT values= less virus present).



◆ R Homologous Reaction

■ R Heterologous Reaction

Chapter 4

TCoV in Cell Culture

Currently, the biggest hindrance to working with TCoV is the lack of a good means of virus propagation. The only documented method is inoculation of specific-pathogen free (SPF) embryonating turkey eggs via the yolk sac or chlorioallantoic sac. While this method suffices in its means of replicating the virus, there are many drawbacks.

First and foremost, only two flocks of SPF turkeys are available in the U. S. One is at Ohio State University, and the other in Athens, Georgia, at the Southeast Poultry and Research Laboratory. This makes it difficult to obtain SPF eggs, which ensures that no other viruses present in the eggs will contaminate studies.

Other disadvantages to working with SPF turkey eggs and TCoV stem from the need to inoculate eggs at 23 days age. At this age, embryos are quite large, and ensuring inoculation through the yolk sac is difficult. Inoculation of eggs earlier in age alleviates this problem, but TCoV replicates inefficiently or not at all during this stage in the embryo’s

lifecycle. Why this occurs is not clear. Inoculation of the chlorioallantoic sac is achieved fairly easily in 23 day-old embryos, but unfortunately embryos do not take up the virus in a reproducible manner, and so variations in infectivity often occur. This hinders projects such as virus neutralizations, or anything that requires reproducibility of clinical signs.

In light of these disadvantages, it is clear that an easier *in vitro* method for propagation of TCoV is needed. To this end, research was done to attempt to adapt TCoV to cell culture, both primary and immortalized cell lines. While none of this research adequately propagated TCoV, a summary of the work undergone is presented:

Our first experimental procedure was to infect immortal cell lines with TCoV inoculum and serially passage the cells. Hoping for replication and cytopathic effect (CPE), each passage was observed and tested by PCR. The immortalized cell lines used were Vero cells, LMH cells, HRT-18 Cells, QT-35 cells, and immortalized feline kidney cells (FKC). Coronaviruses had been reported to grow in all of these except LMH, which is an immortal chicken cell line. These experiments yielded no results by PCR, and no CPE was observed after 20 passages.

Next, transfection of TCoV RNA into Vero cells was performed to ascertain the time point where the most virus was being released from cells, so that a standard for passaging

infected cells could be determined. Transfection was achieved using DMRIE-C reagent. RRT-PCR results indicated that viral output in the supernatant media of cell cultures did not increase from 0 hours post-transfection to 48 hours post-transfection. In fact, the amount of virus in cells and in supernatant media decreased from 0-48 hours, indicating no virus was released.

Failing at both these experiments, primary cell cultures were then investigated. TCoV is known to only infect turkey epithelial intestinal cells (TEICs) *in vivo*. Primary TEICs were created by trypsinizing chopped turkey intestines, and then placed in appropriate media-containing tissue culture flasks. Cells attached and became semi-confluent, but after 8-16 hours, fibroblasts over took the epithelial cells in the flask resulting in limited propogation of virus. Once again we were unable to demonstrate virus in these samples.

While working with primary cell culture, a novel method of viral propagation was also studied. Respiratory viruses are known to propagate well in tracheal organ cultures (TOCs), a method where rings are cut from a trachea and incubated in cell culture media. Viruses are able to enter the cells and replicate. Expanding on this method, we created intestinal ring cultures similarly. This method resulted in successfully yielding virus, but SPF embryos generated higher amount.

Production of intestinal rings also required the use of SPF embryos as well, so the method was abandoned. One advantage to the intestinal ring cultures was its ability to yield purer samples, as only the supernatant of the cell culture media was needed.

Next we tried variations on the theme of serial passaging. In the initial method, growth media was removed from cells followed by infection. After 1 hour of incubation, maintenance media was placed on the cells. After confluency of cells, flasks were frozen and thawed, and the entire contents of the flask used for 20 passages. In an alternative method, we tried trypsinizing the virus briefly before inoculation, a method known to aid in the infectivity of other coronaviruses such as bovine coronavirus. This had no effect. We tried simultaneous inoculation, where TCoV was introduced into the growth media when cells were first planted. Serially passaging these cells still led to no positive results. The final variation to be used was the addition of turkey allantoic fluid into the cell culture media. It was thought that enzyme contained within the fluid might aid in entry of virus to the cells. Yet again, no virus was detected after 3 passages and CPE was never observed.

While these results do not rule out the possibility of TCoV being adapted to an *in vivo* system, it clearly did not adapt to the cell limes examined here following a maximum of 20 passages,

and attempts at primary methods were unsuccesful. Perhaps further attempts in passaging would no doubt increase the chances of success, allowing the virus more opportunities to selectively mutate and adapt to the available environment. One strain of infectious bronchitis virus is known to be adapted to Vero cells, so it certainly seems possible to do the same for TCoV.

DISCUSSION

Transmissible coronaviral enteritis (TCE) of turkeys is a disease whose main effect on the industry is large economic loss, due to reduced weight gain and poor performance. In its initial outbreak in 1951, TCE cost the industry roughly one million dollars. Since then other outbreaks have occurred, but little research has been done into the differences between separate TCoV isolates, and whether or not distinct serological groups exist. Since recent evidence suggests the presences of a hypervariable region (HVR) within the S1 subunit of the spike gene, it is possible that these genetic variations result in different serotypes. The objectives of this project were to further characterize the HVR as well as to study the serological response in two TCoV isolates that were dissimilar in the HVR.

The first part of the project was to investigate the mutational rate of the HVR in the S1 gene of TCoV. In coronaviruses, sequences in the HVRs of the S1 gene correlate with epitopes on the expressed protein that induce protective immune responses. Sequence differences in the HVR were observed for different TCoV isolates indicating the presence of different genotypes. The effect of these differences on serotype is unknown. The frequency of genetic changes in this region was

determined by sequencing the HVR of two of the TCoV isolates serially passaged in embryonating turkey eggs. Mutational rates of $3.0x10^{-2}$ nucleotides per passage and $4.5x10^{-2}$ amino acids per passage were observed in the Tx strain. Rates of $8.5x10^{-5}$ nucleotides per passage and $2.6x10^{-4}$ amino acids per passage were seen in the R strain.

The higher mutational rate in the Tx strain provides convincing evidence for the existence of this HVR. The changes seen were localized to the putative HVR and upstream in what might be another region prone to hypervariability. If these regions behave like their counterparts in infectious bronchitis virus (IBV), then it is possible different serotypes of TCoV exist. Serotypes have not been reported for TCoV, and their presence is important when developing vaccines because generally different serotypes do not cross protect.

In the next phase of the project, the serological relationship between two strains of TCoV was studied. In IBV, the closest relative of TCoV, hypervariable regions can be directly correlated to serotype. To study the effect of this region, a novel means of virus neutralization (VN) was developed using real-time RT-PCR as a quantifiable means of virus detection. An indirect ELISA utilizing whole virus as antigen was also developed.

The results gathered from these tests indicated that serological differences do exist between these strains, though the HVR may not be the only factor involved in this. It is obvious from the ELISA that strain-specific antisera behaved differently when reacted against its homologous antigen and that of the other strain. In addition, the VN assay provided further data suggesting that the R strain could be neutralized by its own antisera, but not that of the a Tx strain.

Finally we attempted to adapt TCoV to cell culture. A suitable *in vitro* propagation assay for TCoV is needed, as currently the only method available is inoculation of SPF embryonating turkey eggs. Several coronaviruses have been reported to grow in immortalized cell lines, and it was hoped this could be accomplished with TCoV. All attempts to achieve this failed, including producing primary intestinal epithelial cells. Adaptation of the virus to cells different from its natural tropism will take time, and further attempts may prove successful.