Investigation into the variation of Infectious Bronchitis virus serotypes in KwaZulu-Natal poultry flocks

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List of Abbreviations

BLAST	Basic Local Alignment Search Tool
bp	base pair
cDNA	complimentary deoxyribonucleic acid
$CHCl_3$	chloroform
ddH_2O	double distilled water
DEPC water	Diethylpyrocarbonate treated water
dNTP	dioxynucleotide triphosphate
E	Envelope protein
E.coli	*Escherichia coli*
ELISA	Enzyme Linked Immunosorbant Assay
IBV	infectious bronchitis virus
IFA	immuno-fluoresence assay
kbp	kilobase pairs
KZN	KwaZulu-Natal Province
M	Membrane protein
Mass	Massachusetts strain/ serotype
M-MuLV	Moloney murine leukemia virus
N	Nucleocapsid protein
ng	nanogram
nM	Nanomol

OIE	World Organization for Animal Health (Office International des Epizoooties)
pmol	picomol
RFLP	Restriction Fragment Length Polymorphism
RNA	ribonucleic acid
RSA	Republic of South Africa
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RVL	Rainbow Veterinary Laboratory
S1	Spike glycoprotein 1
S2	Spike glycoprotein 2
SPF	Specific Pathogen Free
µl	Microliter
µM	Micromol
VNT	Virus Neutralization Test

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Summary

Investigation into the variation of Infectious Bronchitis virus serotypes in KwaZulu- Natal poultry flocks

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Infectious bronchitis virus (IBV) is a member of family *Coronaviridae* and is classified into group 3 of the Coronaviruses. The virus is a single-stranded positive-sense RNA virus with a genome of 27kbp. IBV is a highly infectious disease of chickens that results in high morbidity with moderate to severe mortality depending on the strain involved, age of the birds, and immune status of the chickens. Multiple worldwide investigations indicate that differentiation within the S1 glycoprotein gene can lead to serotype variation within the IBV species. In this study 46 isolates collected over two years from broiler and broiler breeder flocks and eight historical isolates were analyzed. Forty one isolates originated from the KwaZulu-Natal region whilst the remaining thirteen were isolated from 4 other poultry-dense provinces. The S1 gene was sequenced and compared to determine variation between South African isolates, as well as global sequences submitted to Genbank.

The results indicate the division of isolates analyzed into 2 different clades of IBV within the province. The most prevalent genotype was similar to IBV Mass strain detected in 79% of the full S1 sequences. Variation up to 22.3% was detected within local strains, supporting the hypothesis that multiple IBV serotypes may co-circulate in the same region simultaneously. Additionally, more conservation was observed among Mass serotypes versus QX-like serotypes, implying that vaccine use can influence the variability within the IBV population. Higher variability was found in the first half of the S1 gene in comparison to the last half of the S1 gene. This is in agreement with previous findings that the hypervariable regions of the S1 gene are

located within the first 450 base pairs. This study offers the first published consolidation of IBV isolates from South Africa and identifies variation within the IBV population of the SA broiler flock. Previous publications list four or five IBV isolates whilst this study describes variation found in 54 isolates spanning 32 years. In addition this study provides the insight into the prevalence of IBV variation in poultry flocks due to the large number of isolates. The comparative use of geno- and serotyping for South African IBV isolates is also described for the first time in this study.

Keywords: infectious bronchitis virus, poultry, S1 protein, Mass serotype, QX-like serotype, RT-PCR

Chapter 1

General Introduction

Infectious bronchitis virus (IBV) is a highly contagious, ubiquitous virus of poultry that causes disease characterized by upper respiratory or urogenital lesions. IBV is a listed disease according to the International Organization for Animal Health (OIE) and can result in large losses due to mortality or loss of production. The disease has a worldwide distribution. The virus is shed through both the upper respiratory system as well as through faeces and may be detected within the bird's gastrointestinal system for several weeks or months (Cavanagh & Naqi, 1997).

First described in 1936 in the United States of America, IBV has since been described throughout North America, Europe, the Middle East, Asia, the Pacific islands as well Northern and Southern Africa. The emergence of variation within the IBV population is widely described with the first evidence thereof published in 1951 when Jungherr *et al* demonstrated that the Connecticut isolation neither cross-neutralized nor cross- protected against the traditional Massachusetts isolate of the 1940s.

Variation in IBV strains has been investigated and documented in various areas of the world. Similarly to South Africa pre-2013, Brazil only allows the use of Mass serotype vaccines and the epidemiological survey of isolates between 2003 - 2009 demonstrated that one fifth of Brazilian isolates had poor genetic similarity to Mass isolates (Chacon *et al* 2011). Latin America as a whole has several enzootic serotypes identified from Mexico and Argentina as well as Ark and 4/91 serotypes (De Wit *et al* 2011). Studies into IBV in China during 2010 reported being able to classify 56 isolates into 19 differing reference strains with LX types being most dominant (Ma *et al* 2012). This showed some slight variation from the 2009 isolate survey that promoted QX -like strains as the predominant Chinese IBV (Sun *et al* 2011). Xu *et al* (2007) previously found that the A2 serotype was common amongst study isolates and postulated that disease outbreaks may be the result of infections with multiple strains of IBV. Studies conducted within poultry flocks in the Middle East demonstrated IBV variation with the well-publicized Israeli variants and

subsequent IBVar 1 and IBVar2 attenuated vaccine use. The presence of IBV other than Mass serotype was shown by Gharaibeh in 2007. Mahmoud and coworkers (2011) demonstrated Iraqi strains of IBV in their poultry flocks along with 4/91 and Egypt/Benisuef/01, Australian IBV strains are genetically distinct in comparison to the rest of the world, including highly nephropathogenic isolates. The emergence of respirotropic serotypes began in the 1980's. Interestingly, New Zealand only started reporting problems due to IBV and has only begun to be a problem from the late 1980's, over 50 years after the initial description of the disease in the United States of America (De Wit, 2011).

A vast amount of IBV work has been performed with regards to the United States and European variants. The 793/B strain has persisted within the United Kingdom and variation within the serotype can be as high as 25%. This high variation is cited as a possible reason for the persistence despite the use of heterologous vaccines (Adhzar & Gough 1997). Meulemans *et al* (2001) investigated Belgian isolates retrospectively over a 10 year period and found that Mass types accounted for 50% of isolates with D274, B1648 and 793/B accounting for the other half. A very detailed study of Spanish isolates over 14 years showed a dramatic shift from the 793/B strain to te Italy02 strain, with detailed analyses regarding possible recombination events (Dolz *et al* 2008). Investigations in Russia clearly demonstrated the existence of predominantly Mass type but other European and Chinese strains were also found. In addition, six novel Russian IBV genotypes were described (Bochkov *et al* 2006). The earliest discovery of IBV variation was reported in The United States and serotypes such as Arkansas, Connecticut, and Delaware have been described from the 1950's (Jackwood, 2012). In 2001, Lee and Jackwood demonstrated the emergence of the serotype Georgia 98 which was genetically similar to DE072, but serotypically different. This proved to be a rather virulent strain to the local poultry market.

The first published reports of IBV from South Africa were by Morley and Thompson in 1984. This was confirmed as an unknown variant by Cook *et al* in 1999. These viruses were associated with swollen head syndrome experienced in infected flocks throughout Southern Africa. Novel IBV has been described in Nigeria (De Wit, 2011) as well as Zimbabwe, where a QX-like IBV strain has been reported (Jackwood, 2012).

To date there has however been neither published accounts of disease associated with South African IBV strains nor the ability of current vaccines to offer protection against South African field viruses.

Practiced control programs are heavily based on the use of live attenuated and inactivated vaccines to offer virus-neutralizing humoral and cell-mediated immunity. The standard control also has weighting of biosecurity and the treatment of infected flocks with secondary supportive treatments. Direct losses due to mortality, loss of growth and downgrades are caused by IBV infection. Furthermore, additional control measure and treatment costs are also incurred.

The South African poultry industry is the single largest agricultural sub-sector accounting for an annual turnover of R31,7 billion per annum (SAPA Industry profile, 2012) with flock sizes ranging from several hundred to large complexes of 600 000 birds. Perdue and Seal (2000) listed the estimate losses per flock from an IBV infection as 10-20% in market value. This can devastate a poultry farm where margins are relatively small. The economic impact of this disease is thus of great importance.

The aim of this study was to investigate the serotypic and genotypic variation of the S1 gene from current IBV infections within the KwaZulu-Natal province of South Africa. In addition, these were compared to several local historic isolates and several current IBV isolates from other poultry dense provinces within South Africa.

Chapter 2

Literature Review

2.1 Infectious bronchitis virus infections in commercial chickens

Infectious bronchitis virus is distributed worldwide, with the first recorded case in chickens in 1930, in North Dakota, USA. Initial clinical signs included respiratory signs and were later broadened to include effects on egg production, egg shell quality and kidney pathology. Currently IBV has been isolated from all continents including Australia, with reports of varying serotypes. Infectious bronchitis virus (IBV) infection is characterized by a short incubation period of 18-24 hours and has been shown to replicate on many epithelial surfaces (Cavanagh & Naqi 1997; Cavanagh, 2005). Rapid infection of the respiratory system, kidney and oviduct can occur with replication also being seen in the Harderian gland after intra-ocular exposure. Infections can be unremarkable on their own unless birds are young. However, certain virulent strains of IBV can result in severe respiratory disease and mortality (Cavanagh & Naqi 1997, Cavanagh 2004). Co-infection studies showed that the combined infection of sub-lethal challenges of IBV and *E.coli* isolates resulted in mortality (Cook *et al* 1983, Cook *et al* 1991). Mortality rates vary between 14-82% in different IBV serotypes (Cavanagh & Naqi, 1997, Dhinakar Raj & Jones, 1997). Co-infection with *Mycoplasma* species also results in increased clinical signs. Several management factors were enumerated in an Australian study that had impacts on the severity of IBV infections with a nephrogenic strain. Disease was more noted in males, especially when subjected to cold stress, as well in chickens fed diets high in animal protein or calcium (Cummings, 1969).

Disease pathogenesis is as a result of direct infection of the epithelial cells of the respiratory, kidney and female reproductive system. Clinical signs of respiratory infection include depression, snicking, tracheal rales, head shaking and a nasal or ocular discharge. Post mortem lesions include hyperemia with a serous or catarrhal exudate of the trachea and thickening of the air sac wall. A caseous plug in the lower trachea can also be found (Cavanagh & Naqi 1997, Dhinakar Raj & Jones 1997, Cavanagh 2004, Benyeda *et al* 2009, Benyeda *et al* 2010, Dolz *et al* 2011, Cook *et al* 2012, Gelb *et al* 2013). *In vitro* inhibition of tracheal cilia activity has been well documented and is widely utilized as an *in vitro* pathogenicity indicator.

Histopathological changes to the respiratory system during infection include loss and thickening of the cilia as well as thickening of the lamina propria due to the hyperplasia and the infiltration of lymphoid cells (Cavanagh & Naqi 1997, Dhinakar Raj & Jones 1997, Benyeda *et al* 2010).

Replication of IBV within the female reproductive tract is significant at two points in the hens life, firstly at or after the point of lay, and secondly soon after hatch. The former is associated with a drop in egg production, varying between 2- 70% as well as egg shell pathology that includes misshapen eggs, weak or cracked eggshells and watery albumin. Infection during the grow-out phase results in no significant impact on the hen's ability to produce eggs later in life. Effects similar to that seen in respiratory cilia have been described in the cilia of the oviduct (Cavanagh & Naqi 1997, Dhinakar Raj & Jones 1997, Cavanagh 2004, Benyeda *et al* 2009, Benyeda *et al* 2010, Cook *et al* 2012). The pathogenesis of egg pathology is due to the reduction of albumin proteins ovomucin, lysozyme and others that comprise the thick albumin portion of the egg and therefore result in "watery whites" (Dhinakar Raj & Jones 1997).

Infections of the oviduct early in a hen's life can result in irreparable damage to the oviduct and the subsequent effect of "silent layers" or "false layers". This has been shown to be serotype-specific with some IBV isolates not producing these signs. In flocks with this type of infection, reported reductions in egg production were as high as 50%. Reproducible studies with IBV QX-like strains showed dilatation of the oviducts as early as 14 days post infection (Cavanagh & Naqi 1997, Dhinakar Raj & Jones 1997, Benyeda *et al* 2009, Benyeda *et al* 2010, Gelb *et al* 2013).

In an interesting study the role of IBV infection in male testicles was proven and the study also demonstrated that venereal transmission of the virus in a breeding flock was possible. IBV virus was successfully re-isolated from the tracheas of females inoculated with IBV positive semen. In these birds oviduct pathology was also present (Gallardo *et al* 2011). The challenge study of Benyeda *et al* (2010) however could not detect the presence of IBV antigens using immunohistochemistry in testis from a challenge model growing birds to 60 weeks.

Nephrotropic strains of IBV were first investigated in the 1950s and were characterized by severe nephrosis and urate deposits within the collecting tubules.

Mortality rates are very high in infections of young broiler flocks. Flocks infected with this type of IBV tend to recover from a mild respiratory infection after 3-5 days and present with ruffled feathers, watery diarrhea and wet litter thereafter. Pathology of the kidneys includes swelling with pallor and urate crystals in the tubules. In some cases visceral gout may also be seen. Histopathological lesions are that of interstitial nephritis with interstitial oedema initially followed dilation of the collecting by thickening of the lamina propria of the mucous membrane of the collecting ducts. Cellular infiltrates comprise mostly lymphocytes and histiocytes in the form of macrophage cells (Cavanagh & Naqi 1997, Dhinakar Raj & Jones 1997, Benyeda *et al* 200, Benyeda *et al* 2010, Dolz *et al* 2011, Gelb *et al* 2013). An Israeli case study by Meir reported poor growth rate and low broiler slaughter weights with a nephrogenic IBV challenge in 2001 (Meir *et al* 2004)

As a family, many coronaviruses are responsible for enteric infections but the role of IBV in enteric infection has not always been clear. IBV strain 793/B was shown to have a greater affinity for the gastrointestinal tract versus the respiratory system (Dhinakar Raj & Jones 1997). Certain IBV isolates have been linked in the etiology of proventriculitis and dilation of the glandular stomach with the QX serotype infection. (Yu dong 1998, Liu *et al* 2009(b)). In a recent study, however, challenge with QX-like IBV was not able to produce any pathology of the glandular stomach (Benyeda *et al* 2010).

Bilateral pectoral myopathy has been described in 793/B IBV infections however the pathogenesis is unknown. The formation of immune complexes in capillaries are, however, similar to those found in the kidney, and are postulated as a possible cause (Dhinakar Raj & Jones 1997). Other organs that IBV has been isolated from but no pathology demonstrated include the Harderian gland as well as the bursa of Fabricius (Cavanagh & Naqi 1997, Dhinakar Raj & Jones 1997).

2.2 Molecular characteristics of Coronaviruses and Infectious Bronchitis Virus

Coronaviruses are enveloped single-stranded positive sense RNA viruses widely distributed amongst mammals and birds. The molecular biology of coronaviruses was first described by Sturman and Holmes in 1983 and then updated in 1997 and again in 2006 by Paul Masters. Viron size is on average 80-120 nm with club-like surface projections of the Spike protein that are 17-20nm in size. The spikes resemble projections from a crown and thus the family identifying name of *Corona* meaning crown. In certain group 2 two coronaviruses smaller shorter 5-10nm projections are seen these are known as the haemagglutinin-esterase (HE) proteins.

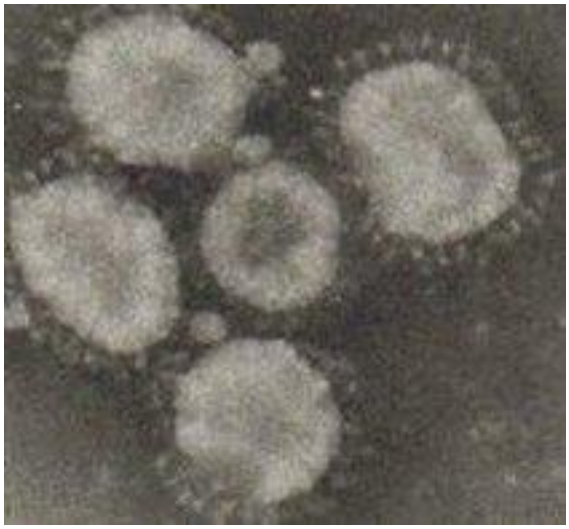

Figure 1. Electron Micrograph of IBV virus particles (Cook, 1983)

Coronaviruses are classified into two genera within the family of *coronaviridae*, order *Nidovirales*. The most notable features of the nidoviruses are that they undergo gene expression through transcription of multiple 3′ nested subgenomic RNAs. The order is named based on this salient characteristic using the Latin *Nido* meaning nested. Other characteristics of the order include expression of the replicase polyprotein through ribosomal frameshifting, unique enzymatic activities among the replicase protein products, a viron membrane envelope and a multi-spanning integral membrane protein for the order (Masters 2006).

Members of the coronaviruses have been classified into three groups originally based on their antigenic relationships and later with genome sequence comparisons becoming the basis for the classification system. The first two groups of the family

contain viruses that have mammalian hosts whilst group 3 contains viruses with avian hosts. Some notable group 1 coronaviruses include porcine transmissible gastroenteritis virus (TGEV), feline infectious peritonitis virus (FIPV), canine coronavirus (CCoV) and human coronavirus (HCoV). Mouse hepatitis virus (MHV) is a member of group 2 along with bovine coronavirus (BCoV) and SARS coronavirus (SARS-CoV). Group 3 contains viruses that affect birds notably Infectious Bronchitis virus (IBV), turkey coronavirus (TCoV) and various viruses that have hosts such as ducks, geese, pigeons and pheasants (Cavanagh 2005, Masters 2006). There is however evidence that coronaviruses isolated from other galliform birds other than chickens are closely related to IBV and in some cases probably are IBV (Cavanagh 2005). All coronaviruses have a genome encoding for at least four structural proteins. The spike protein (S) is responsible for surface attachment to host cells and fusion of the envelope with the host cell or endosomal membrane. The membrane glycoprotein (M) also formally called E1, gives the viron envelope its shape. M is a multi-spanning membrane protein with a small amino terminal domain located on the exterior of the viron. The nucleocapsid protein (N) is the protein component of the helical nuclocapsid that binds the genomic RNA in beads-on-a-string fashion. The envelope protein (E) is a small polypeptide ranging from 76-109 amino acids and is the only small constituent of the coronavirus. Due to its small size it was also the last recognized structural protein (Spaan *et al* 1988, Cavanagh 2005, Masters 2006). Interspersed among the structural genes are small genes encoding for non-structural proteins. Examples of two of these genes are number 3 and 5, both of which encode for two proteins each. Protein 3a, 3b, 5a and 5b are found in IBV and TCoV but are not required in viral replication as virus replicates to similar titres in organ cultures containing recombinant IBV with these genes deleted Casais *et al (*2005).

Infectious bronchitis virus is comprised of a 27.569 kbp genome contains the four coronavirus structural protein genes, non-structural protein genes 3 and 5 and its viral replicase gene. The order of the genes is 5′ Rep-S-3 -E-M-5-N 3′ and during replication IBV produces 5 subgenomic mRNA’s (Boursnell 1987, Spaan 1988).

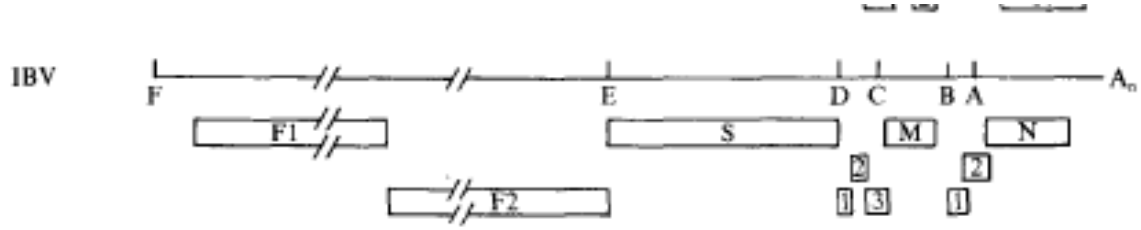

Figure 2. Organisation of the IBV genome (Spaan,1988)

Figure 3. An alternative schematic overview of IBV genomic organization (http://www.infectious-bronchitis.com/microbiology-virus.asp)

The naming of IBV isolates has been an area of area of disparity in the past but the current accepted methodology is similar to the identification system used for avian ortho- and paramyxovirus. Briefly the system describes the host species of isolation followed by the country of isolation. Country abbreviations are accessible on http://www.craytions/com/country.html and in the instance of a large country or large amounts of isolates the province or state may be used as a second geographic description. The isolate reference number is then inserted followed by the year of isolation. In papers where only one host species is described as in this study the description of the species has been be omitted (Cavanagh 2001(a)).

2.3 Spike protein - its role in pathogenicity and immunogenicity.

The spike glycoproteins possess a hydrophobic profile. Infectious bronchitis virus S proteins undergo a post translational cleavage into a S1 and S2 sub-protein. Inter-peptide disulphide bonds are not involved in the quaternary structure and thus S1 can be removed from virons by urea treatment. In addition, this resulted in the proposal that the S1 protein formed the bulbous “club” of the spike whilst anchored to the virus envelope through the S2 protein (Spaan 1988).

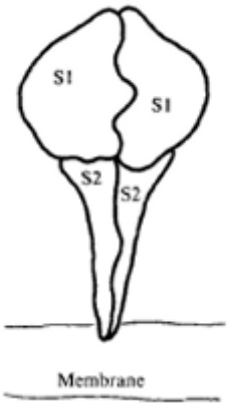

Figure 4. A speculative model for the S protein of IBV (Cavanagh 1983)

Immunizations of purified S1 protein offered between 71% to 80% protection during organ culture challenge whilst none was offered with immunization of N or M protein (Ignjatovic & Galli 1994). Some mild immune stimulation benefits were noted in another study with prior exposure to N protein before vaccination (Boots *et al* 1992). The immunogenicity of the S1 and S2 protein did not differ in regard to induction of cross-reactive antibodies but a cross reactive epitope was only identified on the S2 protein (Ignjatovic & Galli 1994). Both the S1, S2 and M proteins have been shown to induce cell-mediated immunity with the N gene proposed as carrying a T cell epitope (Ignjatovic & Galli 1994). However the most active virus neutralizing antibody sites are found on S1 (Koch *et al* 1990, Karaca & Gelb 1992, Parr & Collisson 1993, Cavanagh & Ellis 1997).

The S1 protein was shown to be responsible for attachment to the host cell membrane as well as for the induction of neutralizing and haemagglutination-inhibiting antibodies (Kant *et al* 1992, Koch *et al* 1992). Two hyper-variable regions (HVR) had been demonstrated between within the 50–159 residue region of the S1

gene. HVR1 lies within region 38-51 whilst HVR 2 between amino acids 99-115 (Cavanagh *et al* 1988, Cavanagh *et al* 1992, Wang *et al* 1997). Certain authors have also described a 3rd HVR between amino acids 274 and 387 (Lee & Jackwood 2001).

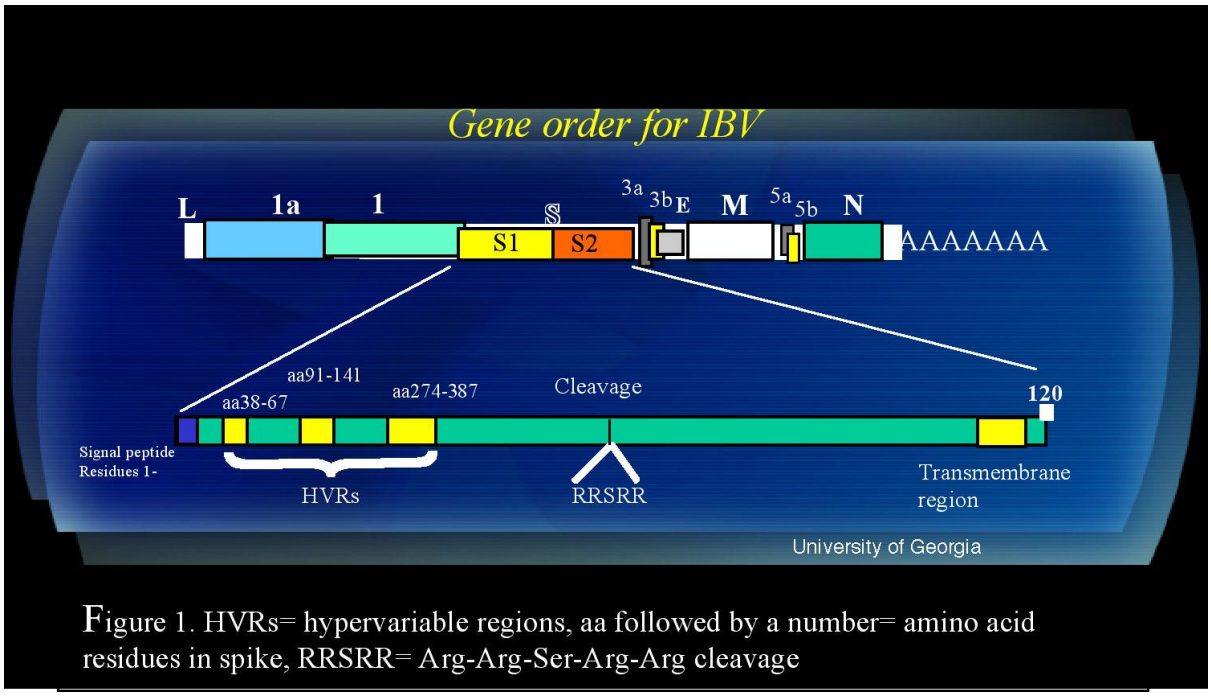

Figure 1. HVRs= hypervariable regions, aa followed by a number= amino acid residues in spike, RRSRR= Arg-Arg-Ser-Arg-Arg cleavage

Figure 5. Schematic illustration of hypervariable regions of S1 gene (http://mjackwoo.myweb.uga.edu/IBV%20web%20pages/IBVpersistence.html)

The high degree of variability has been attributed to the lack of proofreading by the viral RNA-dependent RNA polymerase and due to recombination events during replication (Toro *et al* 2012).

Variations in the HVR correlate with antigenic shifts in the S1 protein (Ignjatovic & Galli 1994). The S1 protein as the major inducer of immunity was evaluated through a cross-protection challenge study using IBV isolates of various S1 sequences. The results indicated that as the S1 peptide similarity diminished so too did the cross protective ability of the isolate (Cavanagh & Ellis 1997). The use of monoclonal and polyclonal antibodies were used to demonstrate that the most antigenic VN antibody-producing epitopes are found within the first and third quarters of the S1 gene (Cavanagh *et al* 1992). Early work was able to determine that the amino acid at position 45 of the Mass IBV-M41 serotype was responsible for the epitope that induces VN and haemagglutination-inhibiting antibodies (Cavanagh *et al* 1992). Shaw *et al* (1996) investigated Belgian nephrotropic IBV strains and reported that the

S1 gene differed by 21-45% from vaccine strains whilst the S2 gene only had 10-12% differentiation. They also stated that when divided into 4 sections the first quarter of the S1 gene has considerably more variation than the latter three. McKinley and coworkers (2008) considered that as little as 0.25% sequence change resulted in rapid negative or positive selection. In contrast a similarity of between 89-99% was seen amongst four Chinese isolates and all were shown to share the same serotype namely A2 (Xu *et al* 2007). The 793/B strain was shown to display between 21-25% variation amongst field isolates, whilst still retaining the same serotype (Adhzar & Gough 1997).The ck/CH/LDL/971 strain of IBV was shown to have <80% homology between the vaccine and pathogenic strain as well as between itself and other IBV types (Liu *et al* 2009(b)).

Many studies have shown that protection against IBV infection after vaccination with a different serotype resulted in poorer immunity than vaccination with a similar serotype (Gelb *et al* 1989, Cavanagh & Ellis 1997). Evidence supports the greater emphasis on the S protein's role in inducing immunity than other structural proteins (Cavanagh & Ellis 1997). Despite relatively poor immunity being induced by inactivated IBV vaccines, it was shown that no immune protection is offered by inactivated IBV vaccines treated with urea to remove the S1 protein (Cavanagh *et al* 1983). The variability in the detection of antibody post vaccination in both SN tests does not correlate circulating antibodies to disease resistance (Gough & Alexander, 1977).

Spike protein sequences have shown between 20-50% variability on an amino acid or nucleotide basis (Cavanagh 2001(b)). Under laboratory conditions it has been demonstrated that production of recombinant IBV using Ark99 and Mass41 vaccines is possible. The cross-over area in these chimeric products was shown to occur mostly commonly upstream of the HVR, but this has not been observed in field samples (Wang *et al* 1997). Host-driven selection has been postulated in the past and can occur as demonstrated during egg passage of several 793/B isolates For example, serine in place of alanine at position 95 of the S1 gene was observed. The selection did not alter the pathogenicity but does illustrate the quasispecies theorem regarding IBV populations (Cavanagh *et al* 2005). Jackwood *et al* (2003) utilized real time PCR techniques and melting curves to visualize the existence of quasispecies within the IBV Beaudette strain.

During the development of an attenuated Chinese IBV strain, it was found that within the same pathogenic and attenuated IBV isolates at least two subpopulations existed. It was proposed that recombination, however unlikely, between the subpopulations and accumulated mutations of the S1 gene resulted in the apathogenic attenuated virus (Lui *et al* 2007). The same author then demonstrated a similar effect during egg passages of the LX strain and identified that the selection for subpopulations happened as early as the 3rd passage (Liu *et al* 2009(b)). Ma *et al* (2012) analyzed 56 IBV isolates of 2010 and proposed a recombination event between a LX strain and the tl/CH/LDT3/031 serotype that resulted in a new serotype.

This was further explored when vaccines re-passaged were inoculated into chickens and the resulting infections were re-isolated and sequenced. The study showed that variation could be found with within different vaccine batches from the same supplier, between the same vaccine serotype from different suppliers as well as within the same vaccine. Re-isolated virus was then proved to have subpopulations, again supporting the quasispecies theorem (McKinley *et al* 2008). The quasispecies model, although generally supported, has been criticized on the basis that it operates in an infinite population size in which genetic drift does not operate, neutral mutants are not considered and recombination events are not considered (Toro *et al* 2012).

Recent work involving co-infection with immunosuppressive infectious bursal disease virus and chicken anaemia virus revealed that varying subpopulations of IBV emerged and persisted in immune competent and immunodeficient chickens. This upheld the phenotypic drift model of genetic variation as a result of selection (Gallardo *et al* 2012). An analysis into the discovery of IBV strain GA98 demonstrated that the evolutionary and mutation rates of the S1 HVR were 1.5% and 2.5% respectively (Lee & Jackwood 2001). The authors also cautioned that the unguarded use of variant IBV vaccines could have led to the faster emergence of new serotypes within the IBV population.

The trial work involving cross-protection between various IBV strains has resulted in the terminology of “protectotype” being used to describe cross-protection from a specific IBV strain against challenges from differing IBV strains. The efficacy of Mass-type vaccines administered at one day of age in light of variation in the

challenge IBV strain was raised and shown to be improved with a follow-up vaccination of an alternate strain (Meulemans *et al* 2001).

The importance of IgG maternal antibodies in progeny was very successfully correlated with broiler breeder flocks with low or erratic IBV titres, resulting in more significant economic and performance effects during IBV challenges of their progeny (De Herdt *et al* 2001).

Several studies have investigated the mechanism behind the role of maternal antibodies. Most notably, it was demonstrated that maternal IgG does not seem to promote immune-tolerance as sometimes experienced in mammals. It did however reveal that high levels of day-old IgG did result in poorer response to day-old vaccination as well subsequent revaccination titres (De Herdt *et al 2001*, Mondal & Naqi 2001). Cell-mediated immunity appears to be driven primarily through CD8 T cytotoxic cells and the role of cytokines in IBV immunity is still unclear (Dhinakar Raj & Jones 1997).

2.4 Diagnostic procedures and developments regarding Infectious bronchitis virus

The basis of IBV diagnosis had traditionally relied on clinical signs, viral isolation, antigen/antibody detection such as traditional serology and more recent molecular techniques such as reverse transcriptase polymerase chain reaction detection (Cavanagh & Naqi 1997).

Viral isolation (VI) has successfully been described in embryonated eggs, cell culture and tracheal organ ring culture. Isolation through SPF embryonated eggs was first attempted and viral titre reaches a maximum 1-2 days post inoculation, non-embryo adapted field strains may however be delayed in the maximum titre timing (Darbyshire *et al* 1975, De Wit 2000). Due to this reason, it is often common laboratory procedure to perform three to four successive passages through embryonated eggs before diagnosing a field sample as IBV negative (Gelb 1989). Diagnosis through this isolation technique is attained by characteristic embryo lesions of curling dwarfing, clubbing of the down feathers and embryo mortality (Gelb 1985, Cavanagh & Naqi 1997). It has also been shown that up one third of isolates will not cause embryo dwarfism. Due to this, several antigen detection techniques have been described for allantoic fluid between two and three passages post inoculation (De Wit 2000).

Infectious Bronchitis virus is an enveloped virus and therefore sensitive to chloroform inactivation. The principle of this assay has been a cornerstone in the classification process of viruses. Organic solvents such as ether or chloroform are added to a salt solution and the test sample. This is then left overnight and the $CHCl_3$ sediments. The supernatant is then used in infectivity studies (Gelb 1983). The mechanism of action is that the solvent destroys the lipid containing structures around the viral cell. In the case of enveloped cells these are then rendered partially for completely inactivated (Feinstone *et al* 1983).

Cell culture can be performed as a diagnostic technique but has shown to be of varying success due to the need for cell-adapted virus. Chicken kidney cells and chicken embryo kidney cells have shown the most success in this technique (de Wit 2000).

The use of chicken organ culture has proven an effective technique for isolation and in addition is a favourable *in vitro* technique to attempt to quantify isolate pathogenicity. The use of tracheal organ cultures from 20-day-old Specific pathogen free (SPF) chickens is advocated and viral action will induce cilliostasis which is visualized under low light microscopy (Dhinakar Raj & Jones 1996, Cavanagh & Naqi 1997, De Wit 2000). Due to the ability of other poultry viruses to produce cilliostasis, antigen confirmation from the culture would be necessary if this technique is used as a primary diagnostic technique (De Wit 2000).

Antigen detection has successfully been described within regards to agar gel precipitation assays as well and immunofluorescence assays (IFA) through the use of either monoclonal or polyclonal antibodies. With regards to the former, despite a perception of reduced sensitivity, Lohr (1981) demonstrated that agar gel precipitation detected antigen in 67% of positive isolates opposed to only 50% of isolates detected by virus isolation alone (De Wit 2000). IFA's are a relatively cheap and easy technique to confirm IBV infection, there are however limitations due to cross-reactions, especially with field samples where ammonia, dust and secondary *E.coli* infections damage the epithelium. The cross-reactions are less pronounced in allantoic or cell culture fluid (Cavanagh & Naqi 1997, De Wit 2000). Clarke *et al* demonstrated that use of IFA in conjunction with VI reduced the number of passages required to detect IBV, as well as reduce the passage length from 5 to 2 days per passage (De Wit 2000). Immunoperoxidase assays are also described for IBV detection and these compare favourably to IFA's but are somewhat more laborious.

Antigen and antibody detection Enzyme Linked Immunosorbant Assays (ELISA) are available and shown a great deal of sensitivity. A great deal of variability is present between IBV serotypes but there are however highly conserved antigenic epitopes within the M and N proteins, and these non-specific areas have allowed for broad IBV detection ELISA's to be developed and manufactured on a commercial scale (Cavanagh & Naqi 1997). The mechanics of antibody response and detection are well-described and follow typical avian viral dynamics. The onset of IgM and IgG immune responses to vaccination or field challenge have been found to start between 2-4 weeks post exposure and serial sera should be collected in order to use serology to confirm challenge (De Wit 2000). The detection of antibodies play an import role in vaccination management and the routine testing thereof can assist in the application and selection of vaccines. Several management or external factors

have been shown to have a strong negative correlation to IBV antibody response. These include younger age at the time of vaccination (Ignjatovic & Galli 1995), the presence of maternal derived antibodies (Cook *et al* 1991, Mondal & Naqi 2001), presence of immunity at the time of vaccination (Cook *et al* 1999), vaccine application route (Toro, 1997) and sample collection size. Another study demonstrated the negative correlations between the following variables: increased flock size – for every additional 1000 birds a 1% lower IgM response was seen, ventilation systems being on at the time of vaccination, house lights being off at the time of vaccination, longer timing between vaccine application and sampling. Temperature of the water as well as housing type was also shown to have direct correlative values (De Wit *et al* 2010).

Molecular detection of the IBV genome is a widely-used technique for field detection of IBV as well as for further genotyping. Various methods for reverse transcriptase PCR detection of IBV have been described with variation existing in the primer selection and the use of <550 bp products amplified directly from field samples or >1000bp products from virus isolation-amplified material (Lin *et al* 1991, Adhzar *et al* 1996, Falcone *et al* 1997, Govender 1997, Wang *et al* 1997, De Wit 2000, Meir *et al* 2004, Ladman *et al* 2006, Lui *et al* 2007, McKinley *et al* 2008, Sun *et al* 2011, Gallardo *et al* 2011, Ma *et al* 2012). Full genome sequences were first published in 1987 (Boursnell *et al* 1987). Simple concise detection methodologies have been described making use of the conserved nuclocapsid gene as the target for the primers. These techniques allow for the demonstration of a 200-320bp product and can provide results within 12 hours of a field sample collection (Falcone *et al* 1997). Alternative techniques have used the 3′ un-translated region which is also highly conserved as a regulatory area of replication. This area has also been well-utilized in the RT-PCR development for turkey Coronavirus (Cavanagh 2001). Adhzar *et al* and coworkers (1996) published various primer sets to amplify the N, M or S1 genes in various IBV isolates. Details on amplification techniques for the S1 and S2 portion of the spike protein are also detailed in this comprehensive selection of oligonucleotides. It was however demonstrated in this paper that certain S1 oligonucleotide pairs did not consistently produce RT-PCR products. The RNA yield was one identified variable with pelleted virus providing better results than allantoic fluid samples. Another possibility was the lack of conservation of sequence with the

S gene, added to the low G+C content of the area, made mis-priming more likely and resulted in smaller than expected products.

Nested RT-PCR's have been developed and have proven to be very sensitive in detecting IBV, but are not a popular choice as routine diagnostic assays since they are prone to produce false positive reactions, due to small non-specific sections of DNA in field samples (Cavanagh & Naqi 1997, De Wit 2000, Cavanagh 2001).

The replication of the highly variable S protein has also been documented and various oligonucleotide sets have been utilized in the amplification of the partial or full S1 gene (Adhzar & Gough 1997, Gelb *et al* 1997, Falcone *et al* 1997, Cavanagh *et al* 1998, Capua *et al* 1999, Lee & Jackwood 2001, Cavanagh *et al* 2005, Bochkov *et al* 2006, McKinley *et al* 2008, Liu *et al* 2009(a), Dolz *et al* 2011). The variable success in amplification of the S1 gene was improved by utilizing degenerate primers, thereby improving the primers attachment likelihood (Gelb *et al* 1997).The emergence of the DE072 strain of IBV necessitated the redesign of degenerate RT-PCR primers for the amplification of its S1 gene (Lee *et al* 2000). On the occasions where genotype- specific primers are not utilized, false negative results are possible (Jackwood *et al* 1997).

With the greater application of real time RT-PCR, various protocols have been developed to detect IBV through the use of TaqMan assays (Benyeda *et al* 2009, Meir *et al* 2010). The use of this technique showed a 30-40 fold greater sensitivity in N gene detection versus RT-PCR and a 17-75% greater sensitivity in detection of S1 genes (Meir *et al* 2010). Obvious advantages lie with this technique in that it increases the sensitivity as well as reduces the run time on a sample. Through the combined use of real time RT-PCR and melting curves, Jackwood *et al* (2003) demonstrated variation within the Beaudette strain. The development of a fluorescein hybridization probe real time PCR technique showed success in identifying several American IBV serotypes such as Ark, Conn, Beaudette and Ma41. The variation in melting curve profiles of these serotypes allowed for the differentiation of these strains.

Techniques to classify IBV isolates on a molecular basis have also made use of restriction fragment length polymorphisms (RFLP) or sequencing in order to demonstrate variation between IBV isolates (De Wit, 2000). RFLP's were first

described and used to classify 12 known different serotypes into five RFLP classes based on the cleavage pattern of a 400bp portion of the S gene encoding for the S2 protein (Lin *et al* 1991).

2.5 Determination and classification of Infectious bronchitis virus sero- and genotypes

Classification of variation with the IBV population was recorded as early as 1956 when the Connecticut isolate of 1951 was neither cross protected against nor neutralized by the Massachusetts (Mass) isolate of the 1940's (De Wit *et al* 2011).

Since this time, there have been numerous studies and reports of variations in serotype and genotypes within the IBV population.

Initial strain classification can be divided into two major groups being functional and non-functional tests. Functional tests indicate the biological function of the virus and the use of immunotypes or "protectotypes" fall into this category, along with serotyping and epitope typing. Non-functional tests indicate genotypes and include DNA sequencing, RFLP's or genotype-specific RT-PCR.

Serotypes identified via virus neutralization tests (VNT) are designated based on the reaction of an IBV strain and specific IBV serotype antibodies. Two strains are considered to be the same serotype when the two-way heterologous neutralization titres concur. In other words, the antisera of A with virus B and the antisera of B with the virus A, differ less than 20-fold than the homologous titre, i.e. antisera A with virus A and antisera B with virus B (Hesselink 1991). These assays have utilized various systems such as embryonated eggs, cell culture and organ culture (Cook 1984, Gelb *et al* 1991, Cavanagh *et al* 1992, Dhinakar Raj & Jones 1996, Adhzar & Gough 1997, Govender 1997). Lack of standardization is a disadvantage of this technique, however this technique does offer good quality information regarding the biological potential of certain vaccines (De Wit 2000). Despite this, VNTs are considered the gold standard in classification of IBV isolates. Interpreting VNT's can become complex once vaccines are utilized in the field, as exposure to varying serotypes has shown to provide broader cross-protection and thus false negative results in this assay (De Wit, 1997). Exposure to a single IBV strain only resulted in 100% serotype specific-sera, however, in the situation of vaccine exposure and heterogeneous IBV isolate exposure the sera contained antibodies against unexposed IBV strains.

The use of monoclonal antibodies to identify specific epitopes within the IBV isolate have also been described and make use of serotype-specific antigen ELISA or IFA's to demonstrate the presence of a specific epitope (Cavanagh *et al* 1992). This holds the advantage of being very specific but when the HVR of the S1 protein is the target area, the high degree of variability may result in false negatives and negative samples should be retested using another method before releasing results (De Wit 2000).

Sequencing offers relatively rapid results and has been well described in the literature (Boursnell *et al* 1984, Cavanagh *et al* 1988,1992,1998,Cavanagh & Naqi 1997, Wang *et al* 1997, Cavanagh *et al* 2005, McKinley *et al* 2008.). Based on sequence data, phylogenetic trees can be constructed to demonstrate genome relatedness (Capua *et al* 1998). The greatest criticism of the technique is the comparison of the primary structure of the virus only and secondary and tertiary structures of the protein are not considered in the classification. This has been demonstrated that small sequence variation of the S1 gene can result in differentiation of the serotype and greater variation of the gene can be found within the same serotype (Cavanagh *et al,* 1992). Despite this, it has been proven on at least one occasion that the correlation of genotypic variation is more strongly related to protective relationships then initially thought. Ladman *et al* (2006) proved that for American IBV field strains, a higher correlation was observed between sequence identity of the S1 gene and protective relatedness than was achieved between antigenic and protective relatedness,viz. 0.72 versus 0.61.

Alternate methods of non-biological classification utilize the use of genotype-specific RT-PCR techniques with general IBV primers as well as type-specific oligonucleotides in a nested RT-PCR (Cavanagh *et al* 1998). Sampling period was shown to be a variable to consider in this instance, with certain vaccine RNAs only being detected for 2 days to a week. Field virus RNA was detected up to 5 weeks post challenge but other instances the period of detection was only one day.

Restriction fragment length polymorphisms (RFLP) have also been utilized and attain a high correlation with serotyping (Lin *et al* 1991, Jackwood *et al* 1997, Lee *et al* 2000). The high variability of the HVR of the S1 gene and susceptibility of restriction enzymes to single nucleotide changes may result in possible false classification, and it has thus been recommended that where RFLP results to not support field or

antigenic data, that conventional serotyping is performed (De Wit, 2000). Lin *et al* and coworkers (1991) demonstrated that despite having seven distinct serotypes in their study, RFLP's could only group them into five groups.

Chapter 3

Materials and Methods

3.1 Sample collection

Infectious bronchitis virus (IBV) samples were collected over a period of 18 months from January 2011 to June 2012. These isolates originated from post mortem samples collected on suspicion of IBV infection. Organs collected included either tracheal samples or pooled tracheal and kidney samples. These samples were collected at Rainbow veterinary laboratory and processed at the same facility, thus no transport media was required to preserve the organ samples. In the case where the virus isolation processing of the sample was delayed, the organs were stored at 70•C until processing.

3.2 Virus isolation

The standard Rainbow veterinary laboratory procedure was followed for virus isolation, which is based on the procedures published in the Laboratory manual for the isolation, identification and characterization of avian pathogens (Gelb, 1989). Samples were homogenized in a pestle and mortar using nutrient broth treated with a combination of penicillin and streptomycin. Once sufficiently homogenized, the nutrient broth was collected, centrifuged at 7500g at for 10 minutes, and the supernatant collected. This was then processed through a 0.22µm filter to remove any possible bacterial contaminants. 0,2mls of filtrate was inoculated into 9-10 day old chicken embryonated eggs via the allantoic sac inoculation route. Four eggs were inoculated per sample tested. The eggs were then incubated at 37.3° C for five days and candled daily to assess viability of the embryo. 37.3 °C was used under the recommendation of Cobb-Vantress as the optimal incubation temperature for a multistage setter. Any dead embryos were opened after candling; diagnosis of the cause of death conducted and in the case of evidence that the embryo was infected with IBV, the chorioallantoic fluid was collected. At the end of the five day incubation period, all viable embryos were placed at 4•C for 12-24 hours to terminate life. The embryonated eggs were then opened, diagnostics performed and chorioallantoic

fluid collected. Typical embryo signs of IBV infection were observed including stunting of the embryo size, curling of the embryo, urate deposits in the mesonephros as well as clubbing of the down feathers. In the case of no pathological signs in the embryo, the allantoic fluid was still collected and re-inoculated for an additional passage. A total of three passages were performed until the sample was declared negative. Inoculations with supporting IBV clinical signs were confirmed for the presence of IBV through a chloroform inactivation test as well as diagnostic polymerase chain reaction assay. The diagnostic criteria for confirmation of IBV infection was stipulated below:

- Characteristic IBV embryo pathology
- Haemagglutination negative
- Chloroform sensitive

Alternatively

- Non-specific IBV embryo pathology
- Haemagglutination negative
- Ether sensitive
- Confirmed with diagnostic IBV RT-PCR

Confirmed IBV-positive chorioallantoic fluid isolates were then stored at -70•C until further processing. Over 100 IBV isolates were collected during the 18 month period, but 46 isolates were selected for the present study based on clinical pathology caused by the strain, geographic distribution, and serum neutralization test results. In addition eight prior isolates were also included in the study. These isolates included six RVF IBV reference strains and two historical isolates. These isolates were collected by the preceding diagnostician at the time and processed in a similar manner as outlined above. In total 54 isolates were presented for the next stage of the study.

3.3 Serum inhibition test

Viral antigen was treated in serial dilution with anti-sera collected against each reference isolate. These antisera were produced through the hyper-immunization of SPF leghorn chickens in a filtered air positive pressure (FAPP) housing facility.

3.4 RNA extraction

RNA was extracted using Trisure reagent (Bioline) according to the procedure recommended by the manufacturer. In brief, 200µl of allantoic fluid was used per extraction this was added to 800µl of Trisure reagent. Following an incubation step on the bench for 5 minutes, 200µl of chloroform was added and shaken to emulsify. Reactions were incubated on the bench for a further 10 minutes and the centrifuged at 12 000g for 15 minutes at 4∘C. The aqueous phase was then transferred to a new marked tube and 500 µl of isopropanol was added. This was incubated at room temperature for 10 minutes followed by centrifugation at 12 000g for 10 minutes at 4∘C. The supernatant was carefully drawn off and the pellet washed with 1ml 70% ethanol by vortexing. Samples were then centrifuged for a final time at 7 500g at 4∘C. Supernatant was discarded and samples were air- dried before being reconstituted in 50µl ddH_20. The RNA was stored at -70∘C.

RNA concentrations of each sample were spectrophotometrically determined using a NanoDrop device (Inqaba Biotech, Pretoria).

3.5 RT-PCR amplification of the S1 gene

5µg of RNA extraction was added to 4µl 5X incubation buffer (Roche), 1µl random hexamers (Roche), 2µl dNTP's (Bioline), 0.3µl RNAase inhibitor (Affymetrix USB, 40U/ul), 0.5µl M-MuLV reverse transcriptase (Roche) and DEPC H_20 to a final reaction volume of 20ul. The reaction was then incubated at 37∘C for 60 minutes. Reactions were then diluted using 20µl PCR-grade water (Roche). DNA concentration were determined using a NanoDrop device (Inqaba Biotech, Pretoria) and are represented in Table 2.

10µl 2X PCR master mix (Thermo Scientific Phusion Flash High-Fidelity PCR master mix (Inqaba Biotech, Pretoria)

2µl OP IBV S1 forward primer

2µl OP IBV S1 reverse primer

1µl PCR grade H_20

5µl cDNA

The reactions were run on a Veriti thermal cycler (Life Technologies) using a cycling profile of 98•C for 10 seconds, 30 cycles of 98•C for 5 seconds, 50•C for 15 seconds and an elongation phase of 72•C for 2 minutes. A final elongation step of 72•C for 4 minutes was used.

The selection of primers proved to be a constraint. The initial use of published primers of IBP1+ and IBRP2 (Lin *et al* 1991) as well as S1Uni1- and S1 Uni2+ (Adhzar & Shaw 1996) proved to yield very poor results. Primers that successfully amplified the S1 genes of South African IBV strains were designed by C Abolnik, based on full genomic sequences obtained during a previous study. The addition of RNAse inhibitor was also critical to obtaining PCR products.

OP IBV S1 FOR:

5′-GAACAAAASACNGACTTAG-3′

OP IBV S1 REV

5′-CCATAACTAACATAAGGRCAA-3′

These primers had a melting temperature of 53.6•C and 54.76•C respectively.

PCR reactions were mixed with 3.3ul 6X loading dye (KAPA™) and electrophoretically separated on a 1% agarose gel strained with ethidium bromide at 130 V. A KAPA™ universal ladder was included to identify the and 1700bp S1 gene amplicon. Bands of the correct size were excised from the gel using a scalpel blade and DNA purified using QiaQuick PCR purification kit (Qiagen) following standard protocol.

3.6 DNA sequencing and bioinformatics analysis

Purified PCR reactions were quantified as previously described and submitted to Inquaba Biotech for Sanger sequencing. Results were edited using Chromas Lite and then aligned using Bio Edit ver. 7.1.11 (Hall, 1999). Sequences were subjected to Basic Local Alignment Search Tool (BLAST) server and similar sequences selected from Genbank as for comparative analysis, along with important reference

viruses. Pairwise similarity was calculated using Bio Edit (Hall 1999) and phylogenetic trees was constructed in MEGA 5.2 (Tamura *et al*, 2011). Pairwise similarities were calculated between study isolates as well as against selected reference strains representing important IBV serotypes (Massachusetts, QX-like, TC07-2, Italy-02, Q1 and 4/91). All reference strains and accession numbers are listed in Annexure 5. Full 1665 bp assembled sequences were submitted to Genbank and accession numbers are listed in Annexure 5.

Phylogenetic relationships were inferred using the Neighbour-Joining tree inference method with a Jukes-Cantor model of sequence evolution using MEGA 5.2 (Tamura *et al* 2011). 1000 bootstrap samplings were performed to assign confidence values to branching orders. The trees were edited using Corel draw X6 (Zukerman 2012).

Chapter 4

Results

4.1 Virus collection and isolation

A total of 54 isolates confirmed as IBV positive by serological techniques were analyzed by RT-PCR.

The isolates were named in accordance to the system proposed by Cavanagh (2001(a)). Forty-two of the proposed isolates were collected from KwaZulu-Natal but some isolates from other provinces were included for comparative purposes. The Eastern Cape, Western Cape and Gauteng Provinces had 2 isolates respectively and 6 isolates were included from the North West Province.

Table 1. Isolates used in this study, with virus neutralization serotype results

Isolate reference number	Study reference code	Province of origin	Month of collection	Year of collection	Serotype
ckZA/2287/11	A1	KwaZulu-Natal	April	2011	Mass
ckZA/2953/11	A2	KwaZulu-Natal	May	2011	Mass
ckZA/3190/11	A3	KwaZulu-Natal	May	2011	Mass
ckZA/3265/11	A4	KwaZulu-Natal	May	2011	Unclassified variant
ckZA/2754a/11	A5	North West Province	April	2011	QX-like
ckZA/2754b/11	A6	North West Province	April	2011	QX-like
ckZA/2754c/11	A7	North West Province	April	2011	QX-like
ckZA/2312/11	A8	KwaZulu-Natal	April	2011	Unclassified variant
ckZA/3466/11	A9	Western Cape	June	2011	QX-like
ckZA/4015/11	B1	KwaZulu-Natal	June	2011	Mass
ckZA/3809/11	B2	KwaZulu-Natal	June	2011	Mass
ckZA/3665/11	B3	KwaZulu-Natal	June	2011	QX-like
ckZA/4421/11	B4	KwaZulu-Natal	July	2011	QX-like
ckZA/3418/11	B5	KwaZulu-Natal	May	2011	QX-like
ckZA/4663/11	B6	KwaZulu-Natal	July	2011	Mass
ckZA/4914/11	B7	KwaZulu-Natal	July	2011	Mass
ckZA/4916/11	B8	KwaZulu-Natal	July	2011	QX-like
ckZA/5250/11	B9	North West Province	July	2011	QX-like
ckZA/5315/11	C1	KwaZulu-Natal	July	2011	Mass
ckZA/5180/11	C2	KwaZulu-Natal	July	2011	QX-like
ckZA/5821/11	C3	KwaZulu-Natal	August	2011	Mass
ckZA/5741/11	C4	KwaZulu-Natal	September	2011	Mass
ckZA/5815/11	C5	KwaZulu-Natal	August	2011	Mass
ckZA/6056/11	C6	KwaZulu-Natal	August	2011	Mass
ckZA/6776/11	C7	KwaZulu-Natal	September	2011	QX-like
ckZA/6681/11	C8	KwaZulu-Natal	September	2011	Mass
ckZA/6621/11	C9	KwaZulu-Natal	September	2011	Mass
ckZA/6516/11	D1	KwaZulu-Natal	September	2011	Mass
ckZA/6743a/11	D2	Western Cape	October	2011	QX-like
ckZA/6743b/11	D3	Western Cape	October	2011	QX-like
ckZA/7390/11	D4	KwaZulu-Natal	November	2011	QX-like

ckZA/7388/11	D5	KwaZulu-Natal	November	2011	QX-like
ckZA/6917/11	D6	KwaZulu-Natal	November	2011	QX-like
ckZA/8306/11	D7	North West Province	December	2011	QX-like
ckZA/mass/	D8	KwaZulu-Natal	Unknown	Unknown	Mass
ckZA/0269/01	D9	KwaZulu-Natal	Unknown	2001	Israel 1496 like
ckZA/0120/00	E1	North West Province	Unknown	2000	Israel 1496 like
ckZA/1275/06	E2	KwaZulu-Natal	Unknown	2006	TC07 like
ckZA/2034/99	E3	KwaZulu-Natal	Unknown	1999	793/B
ckZA/2281/01	E4	KwaZulu-Natal	Unknown	2001	793/B
ckZA/0772/12	E5	KwaZulu-Natal	February	2012	Unclassified variant
ckZA/1272/12	E6	Gauteng	February	2012	Mass
ckZA/1264/12	E7	Gauteng	February	2012	Mass
ckZA/1863/12	E8	KwaZulu-Natal	April	2012	TC07 like
ckZA/1490/12	E9	KwaZulu-Natal	April	2012	Mass
ckZA/1458/12	F1	KwaZulu-Natal	April	2012	QX-Like
ckZA/4869/12	F2	Eastern Cape	October	2012	QX-Like
ckZA/4871/12	F3	Eastern Cape	October	2012	QX-Like
ckZA/6089/12	F4	KwaZulu-Natal	October	2012	QX-Like
ckZA/6573/12	F5	KwaZulu-Natal	October	2012	Unclassified variant
ckZA/6720/12	F6	KwaZulu-Natal	October	2012	QX-Like
ckZA/6689/12	F7	KwaZulu-Natal	October	2012	QX-Like
ckZA/0890/81	F8	KwaZulu-Natal	May	1981	Unknown
ckZA/0415/85	F9	KwaZulu-Natal	October	1985	Unknown

RNA and cDNA concentrations were measured to verify the extraction and cDNA steps. The results of the DNA concentrations after extraction and RT-PCR results are listed in Table 2.

4.2 RT - PCR results

Table 2. cDNA concentrations (ng/µl) and RT-PCR results

Isolate reference number	Study reference code	DNA concentration (ng/µl)	1700bp band present	Repeat PCR 1700bp band present
ckZA/2287/11	A1	7.0	Yes	Not performed
ckZA/2953/11	A2	5.7	Yes	Yes
ckZA/3190/11	A3	8.1	Yes	Yes
ckZA/3265/11	A4	13.8	Yes	Yes
ckZA/2754a/11	A5	0.5	Yes	No
ckZA/2754b/11	A6	6.9	Yes	Yes
ckZA/2754c/11	A7	6.7	Yes	Yes
ckZA/2312/11	A8	8.4	Yes	Yes
ckZA/3466/11	A9	5.3	Yes	Yes
ckZA/4015/11	B1	13.3	Yes	Yes
ckZA/3809/11	B2	12.9	Yes	Yes
ckZA/3665/11	B3	2.6	Yes	Not performed
ckZA/4421/11	B4	3.7	Yes	Yes
ckZA/3418/11	B5	14.1	Yes	Yes
ckZA/4663/11	B6	0.7	Yes	Yes
ckZA/4914/11	B7	6.0	Yes	Yes
ckZA/4916/11	B8	0.2	Yes	Not performed
ckZA/5250/11	B9	5.8	Yes	Not performed
ckZA/5315/11	C1	7.1	Yes	Not performed
ckZA/5180/11	C2	8.3	Yes	Not performed
ckZA/5821/11	C3	6.9	No	No
ckZA/5741/11	C4	3.8	Yes	Yes
ckZA/5815/11	C5	8.1	Yes	Yes
ckZA/6056/11	C6	10.8	Yes	Yes
ckZA/6776/11	C7	1.9	Yes	Yes
ckZA/6681/11	C8	5.0	Yes	Yes
ckZA/6621/11	C9	4.1	Yes	Not performed
ckZA/6516/11	D1	5.5	Yes	Yes
ckZA/6743a/11	D2	3.4	Yes	Not performed
ckZA/6743b/11	D3	4.6	Yes	Not performed

ckZA/7390/11	D4	6.2	Yes	Yes
ckZA/7388/11	D5	6.1	Yes	No
ckZA/6917/11	D6	5.1	Yes	Yes
ckZA/8306/11	D7	4.4	Yes	Yes
ckZA/mass/	D8	4.6	Yes	No
ckZA/0269/	D9	7.6	Yes	No
ckZA/0120/	E1	9.0	Yes	No
ckZA/1275/	E2	5.5	Yes	Yes
ckZA/2034/	E3	4.8	Yes	Yes
ckZA/2281/	E4	16.7	Yes	No
ckZA/0772/12	E5	267.1	Yes	Yes
ckZA/1272/12	E6	32.1	Yes	Yes
ckZA/1264/12	E7	7.6	Yes	No
ckZA/1863/12	E8	7.8	Yes	No
ckZA/1490/12	E9	4.6	Yes	not performed
ckZA/1458/12	F1	8.8	Yes	Yes
ckZA/4869/12	F2	6.0	Yes	No
ckZA/4871/12	F3	6.7	Yes	Yes
ckZA/6089/12	F4	7.9	Yes	No
ckZA/6573/12	F5	6.9	Yes	No
ckZA/6720/12	F6	85.4	Yes	Yes
ckZA/6689/12	F7	3.3	Yes	Not performed
ckZA/0890/81	F8	115.8	Yes	Not performed
ckZA/0415/85	F9	2.9	Yes	No

41 samples produced a detectable band of approximately 1700 base pairs on either the initial or repeat run (Table 2). Some results of the agarose gel electrophoretic separation of RT-PCR products are presented in figure 5 and 6.

PCR products of correct size were extracted and purified and then submitted for sequencing. The sequencing results produced full S1 gene sequences for 20 isolates. 30 isolates yielded partial sequences large enough to submit for a BLAST analysis. 19 of these were for the forward reaction only, and 11 were in the reverse direction only. The results of all sequences were compared to selected reference strains, the results are presented in Annexures 1 to 3.

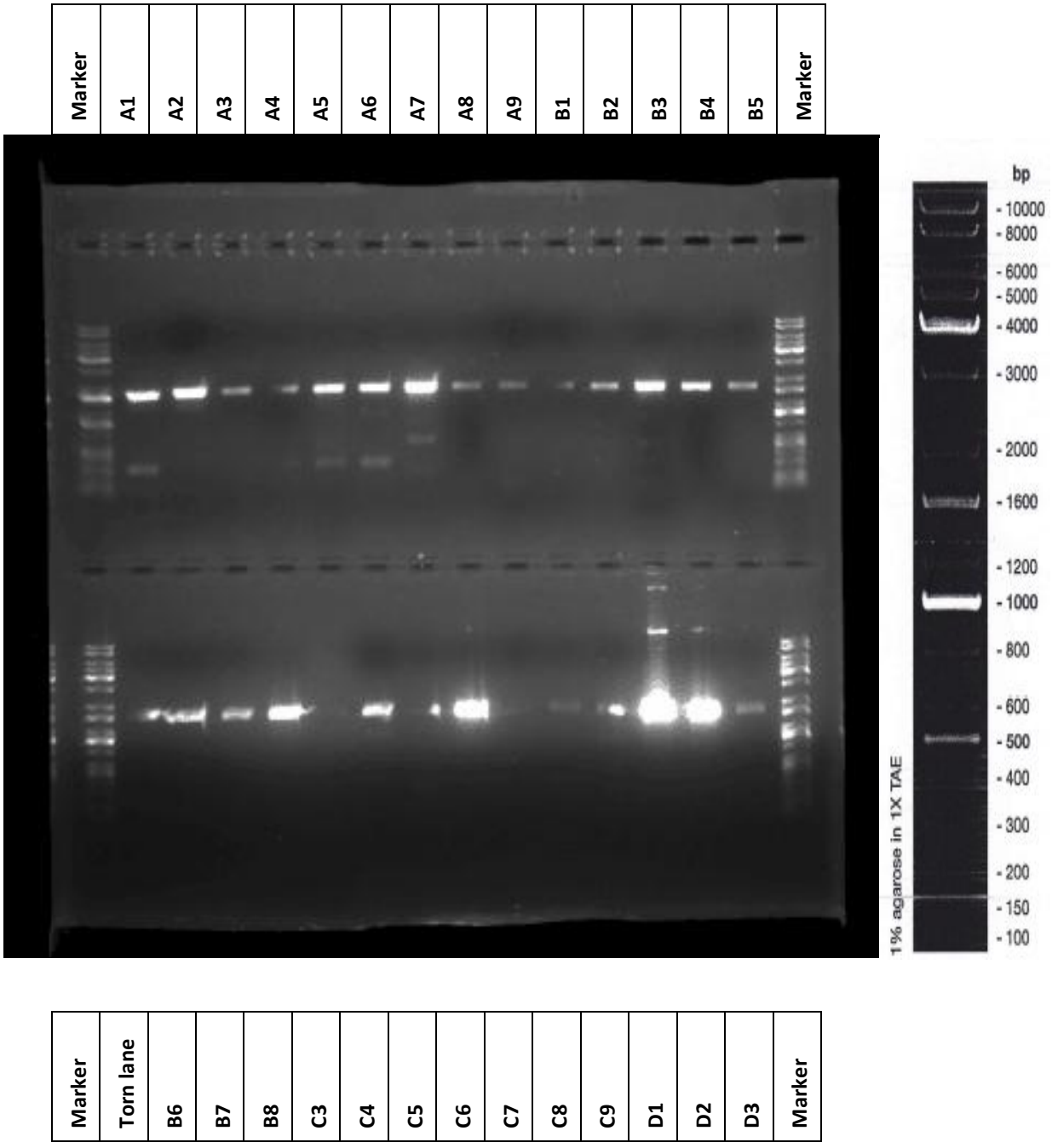

Figure 6. Agarose gel of S1 gene RT-PCR reactions.1% agarose gel ran at 130V over 25 minutes.

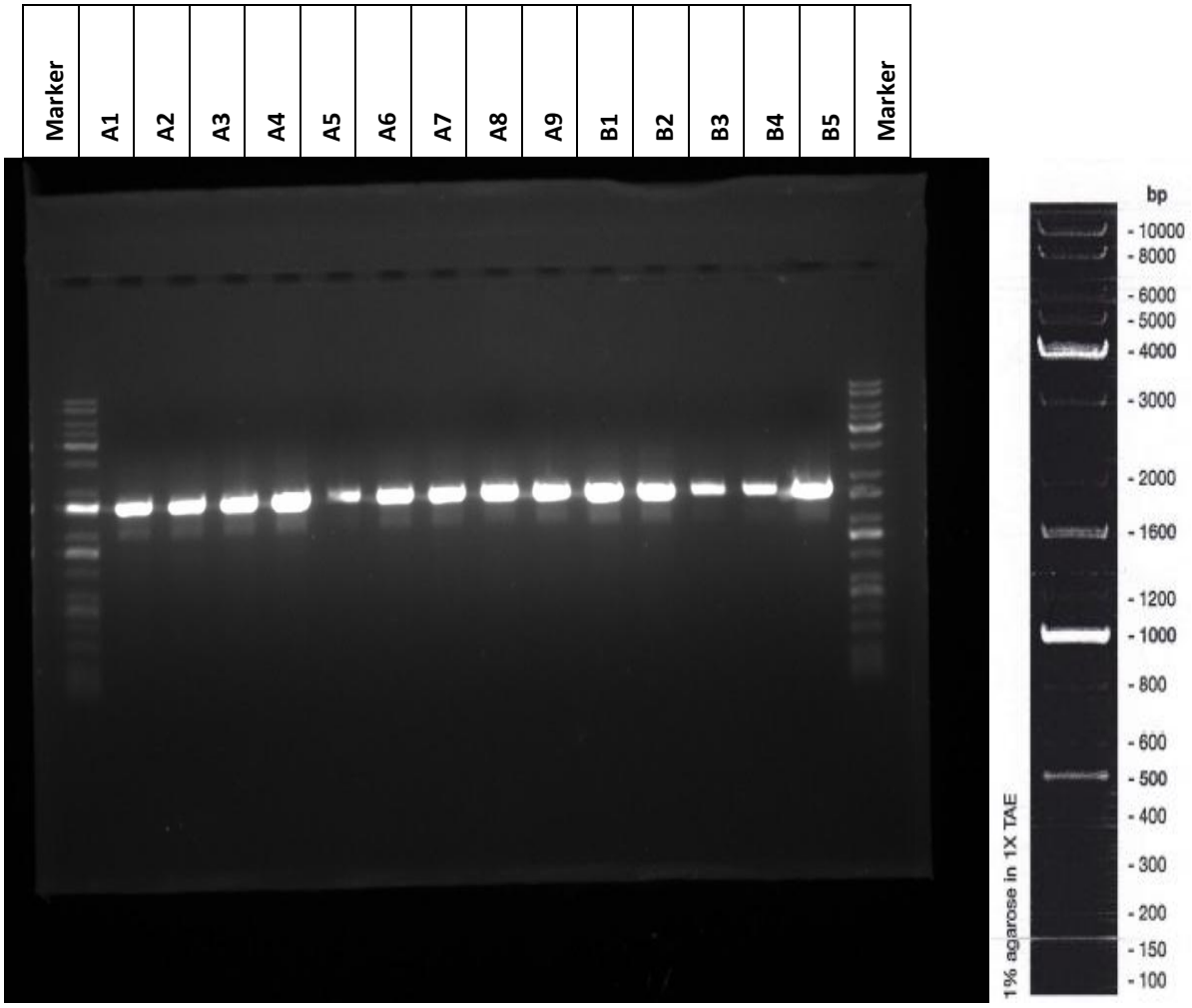

Figure 7. Agarose gel of S1 gene RT-PCR reactions performed under optimised conditions. 1% agarose gel ran at 130V over 25 minutes.

4.3 Sequencing results

Phylogenetic trees were constructed using the Neighbor Joining method, three comparisons were drawn using the full S1 gene sequences (1665 bp) alone (Figure 8), combined with partial sequences for the 5' end of the S1 gene (forward reactions; Figure 9), and 3' end of the S1 gene (reverse reactions, Figure 10). Reference sequences from Genbank were included in each tree for classification purposes.

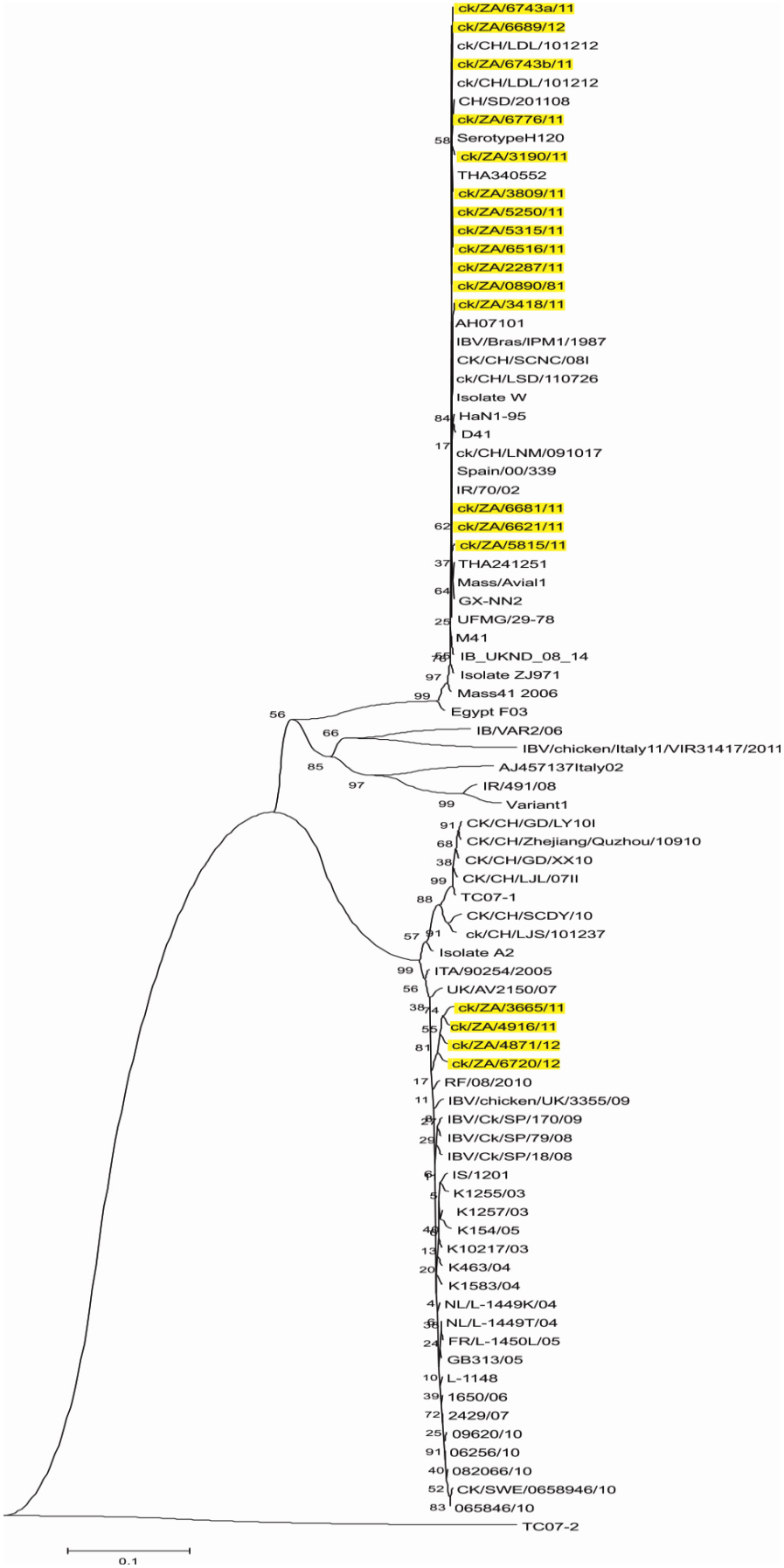

Figure 8 Phylogenetic tree of full S1 gene nucleotide sequences (1665bp) with isolates sequenced in this study highlighted.

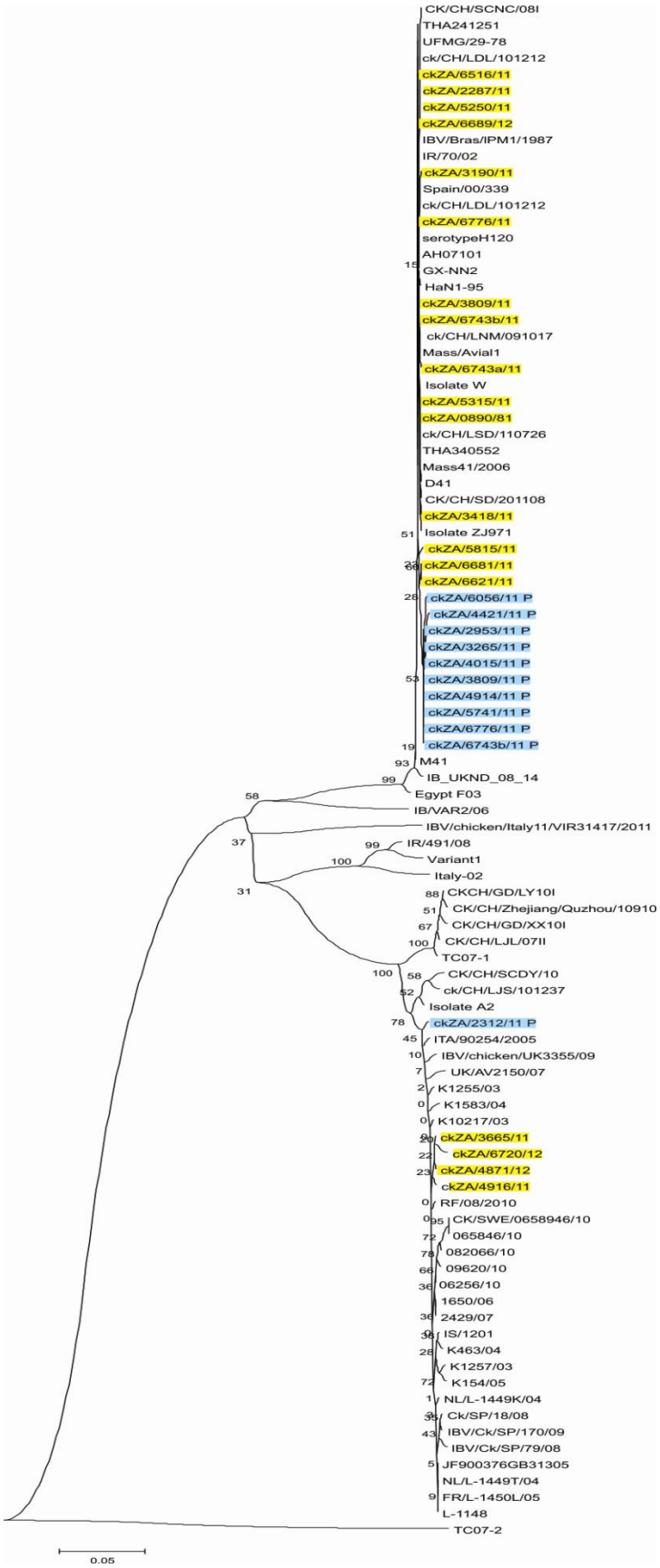

Figure 9. Phylogenetic tree of full S1 gene sequences combined with partial 617 bp nucleotide sequences from the reverse sequencing reaction. Partial sequences are indicated with suffix P.

Figure 10. Phylogenetic tree of full S1 gene sequences combined with partial 281 bp nucleotide sequences from the forward sequencing reaction. Partial sequences are indicated with suffix P.

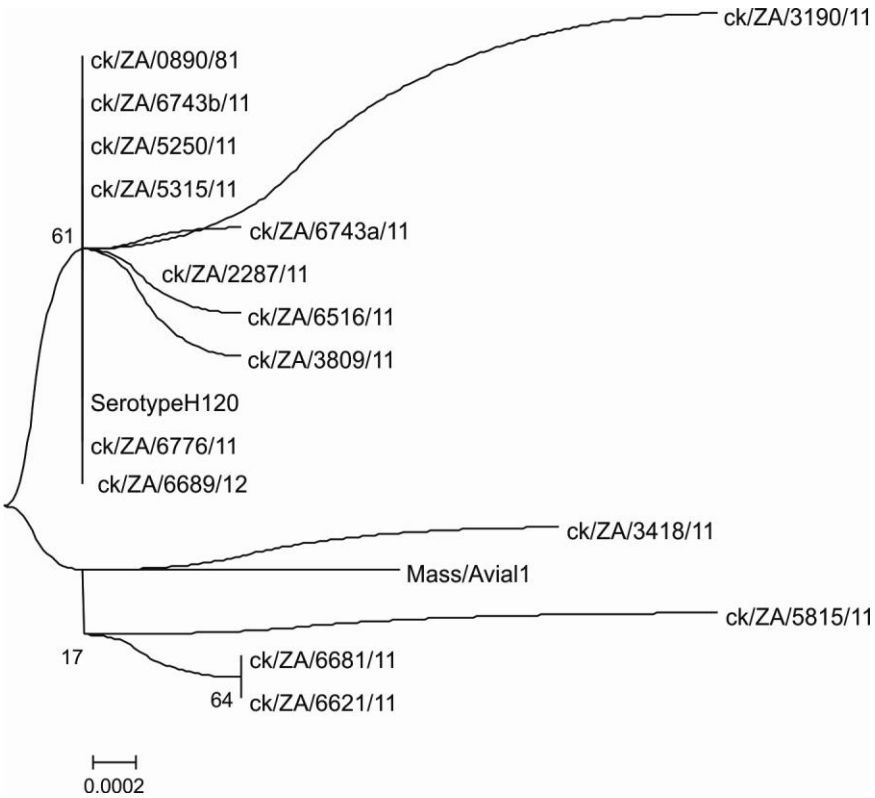

Figure 11. Phylogenetic tree of full S1 gene sequences of Mass-type stains

Full S1 gene nucleotide sequence comparisons revealed variability in isolates CK/ZA/3665/11, CK/ZA/4916/11, CK/ZA/4871/12 and CK/ZA/6720/12 when compared to a standard H120 Mass serotype, and these were subsequently classified as QX-like strains (Figure 8). This variation was located areas across the gene with a high number of base changes observed at positions 95-119, 142-148,165-172 and 695-708 (Annex 1). This was evident in the translated amino acid sequences too, and notably between residues 58 and 62 of these isolates where ISSES is replaced with STNHT.

In the QX-like group (Annex 4), residues 77-85 contained the motif KDVYNQSVA compared to the Mass grouping with a motif of HGGRVVNA. A large insertion was also detected between residues 122-129 with a motif of SSGSGSCP, this is not present in any of the Mass type isolates. Within this group, point mutations are also evident as an insertion at position 639 on isolate Ck/ZA/3665/11, this resulted in the incorporation of cysteine instead of glycine. Two other point mutations within Ck/ZA/3665/11 resulted in the replacement of asparagine with histidine at position 24 and then aspartic acid with tyrosine at position 243. Variation was observed between the two 2011 and the 2012 isolates within the QX-like group where the protein

sequence at position 18-22 of the 2012 isolates was FALWK compared to the 2011 isolates with motif CALCS at the same position. The most variable portion of the amino acid sequence for this group occurred at residue 400 where no consensus was achieved and variation between asparagine, histidine and tyrosine was seen.

The remaining isolates were closely related to reference sequence H120 (Mass serotype) (Figure 8 and Annex 1). Single point mutations were most commonly observed at position 229, with 4 of the isolates (ckZA/3809/11, ckZA/5815/11, ckZA/6681/11, ckZA/6621) replacing cysteine for thymine. No variation in sequence is seen after base pair 792, demonstrating greater conservation in the second half of the gene. This is in line with the fact that this area falls outside of the hypervariable regions (as indicated in Annexure 1). A phylogenetic tree was compiled using the Mass-type field isolates to compare them to Mass serotype strains commonly used in the vaccination of flocks (Figure 11). A high degree of amino acid similarity was seen within the group (Table 3). Similarities within this group ranged between 98.8% and 100%.

Phylogenetic analysis of the partial 5' end of the S1 gene sequences (forward reactions of 281 bp in length) revealed the majority of the sequences to be closely-related to reference serotype H120 (Massachusetts) (Figure 10). Three sequences in this grouping had 2, 6 and 2 point mutations detected in Ck/ZA/4915/11, Ck/ZA/5741/11 and Ck/ZA/6056/11 respectively (Annex 2). Sequence Ck/ZA/4663/11 was the only sequence with a significant variation and differed by 27% from the main group. It did however share 96% similarity with reference strain IBV_UK_3355/09 (QX-like).

Phylogenetic analysis of the 3' end of the S1 gene (617bp partial sequences generated by reverse sequencing reactions) showed a great deal of stability amongst the isolates as well as against a H120 (Mass) reference strain. This is to be expected since this portion of the S1 gene contains no reported hypervariable regions. The similarity in the Massachusetts grouped reverse sequences was 99.7% -100% whilst in the Mass group of the forward sequences the similarity was 97.9%-100%. Ck/ZA/2312/11 showed the greatest amount of variation with a calculated similarity of 81.8% with its nearest study sequence and 82% with the reference strain (Table 10). This strain was determined to share 98.7% identity to IBV/UK/3355/09 (QX-like).

Pairwise similarity was also calculated between the study isolates and selected sequences obtained from Genbank representing selected serotypes, results are displayed in Table 4.

Table 3. Pairwise similarity values based on nucleotide sequence comparison of 1460 bp aligned sequences

	CK/ZA/3665/11	CK/ZA/4916/11	CK/ZA/4871/12	CK/ZA/6720/12	CK/ZA/2287/11	CK/ZA/3190/11	CK/ZA/3809/11	CK/ZA/3418/11	CK/ZA/5250/11	CK/ZA/5315/11	CK/ZA/5815/11	CK/ZA/6776/11	CK/ZA/6681/11	CK/ZA/6621/11	CK/ZA/6516/11	CK/ZA/6742a/11	CK/ZA/6743b/11	CK/ZA/6689/12	CK/ZA/0890/81
CK/ZA/3665/11																			
CK/ZA/4916/11	98.4																		
CK/ZA/4871/12	98.0	98.3																	
CK/ZA/6720/12	97.5	97.7	98.2																
CK/ZA/2287/11	78.2	78.3	78.2	79.7															
CK/ZA/3190/11	77.8	78.0	77.9	79.5	99.3														
CK/ZA/3809/11	78.0	78.2	78.2	79.7	99.7	99.1													
CK/ZA/3418/11	78.2	78.3	78.2	79.6	99.5	98.9	99.4												
CK/ZA/5250/11	78.2	78.3	78.2	79.7	99.9	99.3	99.8	99.6											
CK/ZA/5315/11	78.2	78.3	78.2	79.7	100	99.3	99.7	99.5	99.9										
CK/ZA/5815/11	78.0	78.2	78.2	79.8	99.4	98.8	99.4	99.3	99.4	99.4									
CK/ZA/6776/11	78.1	78.2	78.3	79.7	99.9	99.3	99.9	99.5	99.9	99.9	99.5								
CK/ZA/6681/11	78.2	78.3	78.4	79.8	99.7	99.0	99.7	99.5	99.7	99.7	99.6	99.8							
CK/ZA/6621/11	78.2	78.4	78.3	79.8	99.7	99.1	99.6	99.6	99.8	99.7	99.5	99.7	99.9						
CK/ZA/6516/11	78.0	78.2	78.3	79.8	99.7	99.1	99.7	99.4	99.8	99.7	99.4	99.9	99.7	99.6					
CK/ZA/6742a/11	78.0	78.2	78.2	79.6	99.9	99.2	99.7	99.5	98.9	99.9	99.3	99.8	99.6	99.7	99.7				
CK/ZA/6743b/11	77.7	77.9	78.0	79.4	99.6	98.8	99.5	99.1	99.5	99.6	99.1	99.6	99.4	99.3	99.5	99.5			
CK/ZA/6689/12	78.2	78.3	78.2	79.7	99.9	99.3	99.8	99.6	100	99.9	99.5	99.9	99.7	99.8	99.8	99.9	99.5		
CK/ZA/0890/81	78.2	78.3	78.2	79.7	99.9	99.3	99.8	99.6	100	99.9	99.5	99.9	99.7	99.8	99.8	99.9	99.5	100	

Two groupings of isolates can be inferred from the pairwise similarity with four isolates namely CK/ZA/3665/11, CK/ZA/4916/11, CK/ZA/4871/12 and CK/ZA/6720/12 that shared between 98 -98.4% similarity. The remainder of the full

sequences compared fall into a second grouping of between 98.9 – 100% similarities.

Table 4. Pairwise similarity of 1460 bp nucleotide sequence of study isolates and selected reference sequences. Highest similarity is emphasized. Genbank description and accession numbers listed in Annexure 4

	QX-like	Qx-like	793/B like	IS/1496/06	TC07-2	Q1	ITALY 02	Mass	Mass
	Chicken UK 3355/09	TC07-1	IBV Variant 1	IBV Variant 2	TC07-2	Italy/11	Italy 02	Mass Avial	Egypt F03
CK/ZA/3665/11	97.3	**98.0**	77.6	76.5	66.1	76.3	77.8	78.1	78.4
CK/ZA/4916/11	**97.5**	95.1	78.0	76.8	66.1	76.6	78.2	78.2	78.6
CK/ZA/4871/12	**97.3**	95.1	78.1	76.8	66.4	76.6	78.0	78.2	78.6
CK/ZA/6720/12	**97.1**	94.8	78.4	78.2	65.6	77.5	78.8	79.2	79.9
CK/ZA/2287/11	78.5	78.3	77.7	80.6	66.2	77.9	78.2	**99.7**	97.8
CK/ZA/3190/11	78.1	78.0	77.5	80.4	65.5	77.6	77.9	**99.0**	97.2
CK/ZA/3809/11	78.3	78.1	77.7	80.5	65.4	77.8	78.1	**99.5**	97.7
CK/ZA/3418/11	78.5	78.3	77.9	80.4	65.4	78.0	78.4	**99.6**	97.7
CK/ZA/5250/11	78.5	78.3	77.8	80.7	65.6	77.9	78.3	**99.7**	97.9
CK/ZA/5315/11	78.5	78.3	77.7	80.6	65.5	77.9	78.2	**99.7**	97.8
CK/ZA/5815/11	78.4	78.2	77.6	80.3	65.5	77.9	78.2	**99.3**	97.5
CK/ZA/6776/11	78.4	78.2	77.7	80.6	65.3	77.9	78.2	**99.7**	97.8
CK/ZA/6681/11	78.5	78.3	77.9	80.6	65.5	77.9	78.3	**99.7**	97.8
CK/ZA/6621/11	78.6	78.4	77.9	80.7	65.5	77.9	78.4	**99.7**	97.9
CK/ZA/6516/11	78.4	78.2	77.6	80.6	65.5	77.9	78.2	**99.5**	97.7
CK/ZA/6742a/11	78.4	78.2	77.7	80.5	65.5	77.9	78.2	**99.6**	97.7
CK/ZA/6743b/11	78.1	77.9	77.4	80.2	65.1	77.5	77.9	**99.2**	97.4
CK/ZA/6689/12	78.5	78.3	77.8	80.7	65.5	77.9	78.3	**99.7**	97.9
CK/ZA/0890/81	78.5	78.3	77.8	80.7	65.5	77.9	78.3	**99.7**	97.9

Pairwise comparisons against reference sequences support the description of two distinct groups of isolates. The grouping of CK/ZA/3665/11, CK/ZA/4916/11 and CK/ZA/4871/12 being closest to IBV QX-like strains but being split between Chinese origin TC07-1 and UK origin 3355/09. The other group shared 99-99.7% similarity with isolate Mass Avail.

The results of nucleotide sequence pairwise similarity are represented in Tables 6 and 7.

Table 5. Pairwise similarity of 427 residue amino acid sequence of study isolates and selected reference sequences. Highest similarity emphasized. Genbank description and accession numbers are listed in Annexure 4.

	QX-like	Qx-like	793/B like	IS/1496/06	TC07-2	Q1	ITALY 02	Mass	Mass
	ChickenUK 3355/09	TC07-1	IBV Variant 1	IBV Variant 2	TC07-2	Italy/11	Italy 02	Mass Avial	Egypt F03
CK/ZA/3665/11	**99.0**	97.9	89.0	87.3	72.1	86.4	87.6	88.2	88.0
CK/ZA/4916/11	**98.8**	97.6	89.0	87.8	72.6	86.4	88.0	88.3	88.1
CK/ZA/4871/12	**98.8**	97.6	89.2	87.6	72.1	86.7	87.1	87.8	87.6
CK/ZA/6720/12	**98.1**	96.9	88.5	86.6	71.8	86.1	87.1	97.5	87.3
CK/ZA/2287/11	87.8	88.2	85.2	86.4	72.8	84.3	84.7	**96.7**	95.0
CK/ZA/3190/11	87.5	88.1	85.0	86.1	72.3	84.1	84.5	**96.5**	95.1
CK/ZA/3809/11	87.8	88.2	85.2	86.4	72.8	84.3	84.8	**96.5**	94.8
CK/ZA/3418/11	87.6	88.1	85.0	86.2	72.6	84.0	84.5	**95.5**	94.9
CK/ZA/5250/11	87.8	88.2	85.2	86.4	72.8	84.3	84.8	**96.7**	95.1
CK/ZA/5315/11	87.8	88.2	85.2	86.4	72.8	84.1	84.8	**96.7**	95.1
CK/ZA/5815/11	87.4	87.8	84.8	85.2	72.6	84.1	84.3	**96.5**	94.8
CK/ZA/6776/11	87.8	88.3	85.2	86.4	72.8	84.3	84.8	**96.7**	95.0
CK/ZA/6681/11	87.8	88.3	85.2	86.4	72.8	84.3	84.8	**96.7**	95.0
CK/ZA/6621/11	87.8	88.3	85.2	86.4	72.8	84.3	84.8	**96.7**	95.0
CK/ZA/6516/11	87.8	88.3	85.2	86.4	72.8	84.3	84.8	**96.7**	95.0
CK/ZA/6742a/11	87.6	88.1	85.0	86.2	72.8	84.3	84.5	**96.5**	94.8
CK/ZA/6743b/11	87.8	88.3	85.2	86.4	72.8	84.3	84.7	**96.7**	95.0
CK/ZA/6689/12	87.8	88.3	85.2	86.4	72.8	84.3	84.7	**96.7**	95.0
CK/ZA/0890/81	87.8	88.3	85.2	86.4	72.8	84.3	84.7	**96.7**	95.0

Table 6. Pairwise similarity values based on comparison of 427 amino acid residue aligned sequences.

	CK/ZA/3665/11	CK/ZA/4916/11	CK/ZA/4871/12	CK/ZA/6720/12	CK/ZA/2287/11	CK/ZA/3190/11	CK/ZA/3809/11	CK/ZA/3418/11	CK/ZA/5250/11	CK/ZA/5315/11	CK/ZA/5815/11	CK/ZA/6776/11	CK/ZA/6681/11	CK/ZA/6621/11	CK/ZA/6516/11	CK/ZA/6742a/11	CK/ZA/6743b/11	CK/ZA/6689/12	CK/ZA/0890/81
CK/ZA/3665/11																			
CK/ZA/4916/11	98.8																		
CK/ZA/4871/12	98.6	98.4																	
CK/ZA/6720/12	97.9	97.6	98.4																
CK/ZA/2287/11	86.9	86.9	86.6	86.4															
CK/ZA/3190/11	86.7	86.6	86.4	86.2	99.5														
CK/ZA/3809/11	86.9	86.9	86.6	86.4	99.8	99.3													
CK/ZA/3418/11	86.7	86.9	86.6	86.2	99.8	99.3	99.5												
CK/ZA/5250/11	86.9	86.9	86.6	86.4	100	99.5	99.8	99.8											
CK/ZA/5315/11	86.9	86.9	86.6	86.4	100	99.5	99.8	99.8	100										
CK/ZA/5815/11	86.4	86.4	86.1	85.9	99.5	99.0	99.3	99.3	99.5	99.5									
CK/ZA/6776/11	86.9	86.9	86.6	86.4	100	99.5	99.8	99.8	100	100	99.5								
CK/ZA/6681/11	86.9	86.9	86.6	86.4	100	99.5	99.8	99.8	100	100	99.5	100							
CK/ZA/6621/11	86.9	86.9	86.6	86.4	100	99.5	99.8	99.8	100	100	99.5	100	100						
CK/ZA/6516/11	86.9	86.9	86.6	86.4	100	99.5	99.8	99.8	100	100	99.5	100	100	100					
CK/ZA/6742a/11	86.6	86.6	86.4	86.2	99.8	99.3	99.5	99.5	99.8	99.8	99.3	99.8	99.8	99.8	99.8				
CK/ZA/6743b/11	86.9	86.9	86.6	86.4	100	99.5	99.8	99.8	100	100	99.5	100	100	100	100	100			
CK/ZA/6689/12	86.9	86.9	86.6	86.4	100	99.5	99.8	99.8	100	100	99.5	100	100	100	100	100	100		
CK/ZA/0890/81	86.9	86.9	86.6	86.4	100	99.5	99.8	99.8	100	100	99.5	100	100	100	100	100	100	100	

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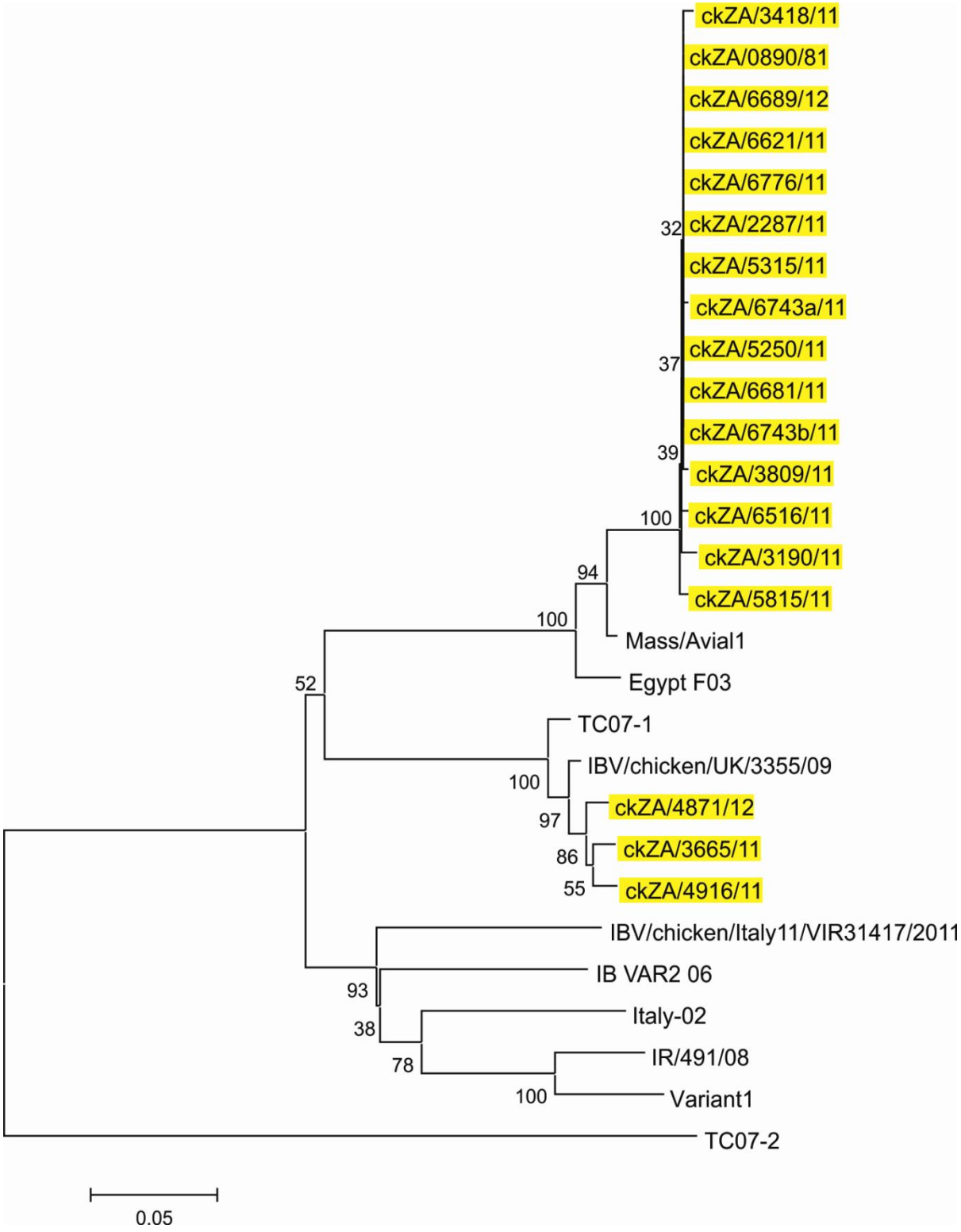

Figure 12. Phylogenetic tree constructed with 427 amino acid residue IBV sequences (study isolates emphasized)

The results presented in Figure 12 supported the sequence and similarity comparisons with the identification of two significant groups (Mass-like and QX-Like) within all three of the comparisons. Within the Mass grouping, minor variation was

detected, but three sub-groups were discerned. All groups shared more than 99% similarity with H120.

Table7. Pairwise similarity values based on nucleotide sequence comparison of 281 bp (forward) aligned sequences.

	CK/ZA/2953/11	CK/ZA/3265/11	CK/ZA/2754a/11	CK/ZA/2754b/11	CK/ZA/2754c/11	CK/ZA/3809/11	CK/ZA/4421/11	CK/ZA/4663/11	CK/ZA/4914/11	CK/ZA/5741/11	CK/ZA/6056/11
CK/ZA/2953/11											
CK/ZA/3265/11	100										
CK/ZA/2754a/11	100	100									
CK/ZA/2754b/11	100	100	100								
CK/ZA/2754c/11	100	100	100	100							
CK/ZA/3809/11	100	100	100	100	100						
CK/ZA/4421/11	100	100	100	100	100	100					
CK/ZA/4663/11	73.0	73.0	73.0	73.0	73.0	73.0	73.0				
CK/ZA/4914/11	99.3	99.3	99.3	99.3	99.3	99.3	99.3	73.4			
CK/ZA/5741/11	97.9	97.9	97.9	97.9	97.9	97.9	97.9	71.6	97.1		
CK/ZA/6056/11	99.3	99.3	99.3	99.3	99.3	99.3	99.3	73.0	98.9	97.9	

Table 8. Pairwise similarity of 281 bp (forward) nucleotide sequence of study isolates and selected reference sequences. Highest similarity is emphasized

	QX-like	Qx-like	793/B like	IS/1496/06	TC07-2	Q1	ITALY 02	Mass	Mass
	Chicken UK 3355/09	TC07-1	IBV Variant 1	IBV Variant 2	TC07-2	Italy/11	Italy 02	Mass Avial	Egypt F03
CK/ZA/2953/11	74.1	73.0	69.5	77.3	57.8	69.1	70.2	**99.6**	96.8
CK/ZA/3265/11	74.1	73.0	69.5	77.3	57.8	69.1	70.2	**99.6**	96.8
CK/ZA/2754a/11	74.1	73.0	69.5	77.3	57.8	69.1	70.2	**99.6**	96.8
CK/ZA/2754b/11	74.1	73.0	69.5	77.3	57.8	69.1	70.2	**99.6**	96.8
CK/ZA/2754c/11	74.1	73.0	69.5	77.3	57.8	69.1	70.2	**99.6**	96.8
CK/ZA/3809/11	74.1	73.0	69.5	77.3	57.8	69.1	70.2	**99.6**	96.8
CK/ZA/4421/11	74.1	73.0	69.5	77.3	57.8	69.1	70.2	**99.6**	96.8
CK/ZA/4663/11	**96.1**	93.6	68.4	69.5	59.2	65.2	68.8	73.4	74.1
CK/ZA/4914/11	74.5	73.0	68.8	75.2	58.5	69.9	69.9	**98.9**	96.1
CK/ZA/5741/11	72.7	71.6	69.9	73.8	57.8	68.4	70.2	**98.2**	95.4
CK/ZA/6056/11	74.1	73.0	69.5	74.8	58.5	69.5	70.2	**99.6**	96.8

Table 9. Pairwise similarity values based on nucleotide sequence comparison of 617 bp (reverse) aligned sequences

	CK/ZA/2953/11	CK/ZA/3265/11	CK/ZA/2312/11	CK/ZA/4015/11	CK/ZA/3809/11	CK/ZA/4421/11	CK/ZA/4914/11	CK/ZA/5741/11	CK/ZA/6056/11	CK/ZA/6776/11	CK/ZA/6743b/11
CK/ZA/2953/11											
CK/ZA/3265/11	99.7										
CK/ZA/2312/11	81.8	81.5									
CK/ZA/4015/11	100	99.7	81.8								
CK/ZA/3809/11	100	99.7	81.8	100							
CK/ZA/4421/11	99.7	99.4	82.0	99.7	99.7						
CK/ZA/4914/11	100	99.7	81.8	100	100	99.7					
CK/ZA/5741/11	100	99.7	81.8	100	100	99.7	100				
CK/ZA/6056/11	99.8	99.5	81.8	99.8	99.8	99.6	99.8	99.7			
CK/ZA/6776/11	100	99.7	81.8	100	100	99.7	100	100	99.8		
CK/ZA/6743b/11	100	99.7	81.8	100	100	99.7	100	100	99.8	100	

Table 10. Pairwise similarity of 617 bp (reverse) nucleotide sequence of study isolates and selected reference sequences. Highest similarity is emphasized

	QX-like	Qx-like	793/B like	IS/1496 /06	TC07-2	Q1	ITALY 02	Mass	Mass
	Chicken UK 3355/09	TC07-1	IBV Variant 1	IBV Variant 2	TC07-2	Italy/11	Italy 02	Mass Avial	Egypt F03
CK/ZA/2953/11	81.5	81.7	82.7	84.4	65.6	81.2	82.0	**99.7**	98.2
CK/ZA/3265/11	81.2	81.3	82.3	84.1	65.3	81.8	81.7	**99.3**	97.9
CK/ZA/2312/11	**96.7**	95.6	83.0	81.2	66.6	82.8	83.4	82.0	82.3
CK/ZA/4015/11	81.5	81.7	82.7	84.4	65.6	81.2	82.0	**99.7**	98.2
CK/ZA/3809/11	81.5	81.7	82.7	84.4	65.6	81.2	82.0	**99.7**	98.2
CK/ZA/4421/11	81.7	81.8	82.2	84.8	65.3	82.3	82.2	**99.4**	97.9
CK/ZA/4914/11	81.5	81.7	82.7	84.4	65.6	81.2	82.0	**99.7**	98.2
CK/ZA/5741/11	81.5	81.7	82.7	84.4	65.6	81.2	82.0	**99.7**	98.2
CK/ZA/6056/11	81.5	81.7	82.8	84.6	65.5	82.0	82.0	**99.5**	98.0
CK/ZA/6776/11	81.5	81.7	82.7	84.4	65.6	81.2	82.0	**99.7**	98.2
CK/ZA/6743b/11	81.5	81.7	82.7	84.4	65.6	81.2	82.0	**99.7**	98.2

Chapter 5

Discussion

Full S1 genes were successfully amplified for 54 South African IBV isolates collected mainly from broiler flocks in KZN, but some other provinces were also included. Despite repeated attempts, 20 full S1 gene sequences and 30 partial sequences were obtained. Twenty two isolates yielded no usable sequence data.

Phylogenetic analysis revealed two distinct groupings of IBV within the study isolates. The larger group contained 15 of the initial 20 full sequences and shared 99.0 – 99.7% amino acid sequence similarity with the H120 serotype. Within this group there was 98.8 – 100 % similarity at the nucleotide sequence level. Four isolates (CK/ZA/0890/81, CK/ZA/6689/12, CK/ZA/5315/11 and CK/ZA/2287/11) were very closely related. These sequences differed by 0.3% or four point mutations at base pairs 294, 302, 374 and 483. The replacement of alanine with cysteine at residue 296 was the only variation between the two 2011 isolates, and the 2012 and 1981 isolate. Reported S1 gene mutation rates under vaccine selective pressure are 2.5% (Lee & Jackwood 2001) and reports suggest that in the absence of a selective vaccine, mutation rates are twice as high than when under selective pressure (McKinley *et al* 2011). Therefore, detecting two field isolates identical in sequence, 12 years apart, is remarkable. Cook *et al* (1999) previously identified the Ck/ZA/0890/81 isolate to be variant and not neutralized by Mass or 4/91 antisera. It is more likely that the Ck/ZA/0890/81represents a laboratory contamination or mislabeling in this case.

The remainder of the Mass-type South African isolates shared high degrees of similarity with two point mutations common to all isolates when compared to the reference strain. The replacement of thymine with cysteine at residue 11 and thymine with adenine at residue 138 were found amongst all Mass isolates. However, the study isolates did share these changes with Mass Avail reference strain. This strain was sequenced in the study by McKinley *et al* (2006) and originated as a first passage from an undisclosed vaccine company IBV vaccine. Isolate Ck/ZA/3190/11 was the most divergent of the Mass grouping with only 90% nucleotide similarity to the reference strain H120, the highest frequency of variation in this case was found between base pairs 706 and 818, with 10 point mutations

found in this region. No variation was noted after base pair 818. When compared against the year of isolation or the area of isolation it was found that the highly similar Mass grouping originated from KZN between April-September 2011 and then again October 2012, one isolate from the North West province from July 2011 and two from the Western Cape collected in October 2011 also fell into this grouping. No distinct pattern of change is identified in the isolates that originated outside of KwaZulu-Natal. Despite the geographic areas being distinct there is movement of eggs and embryos between provinces reported during the study period. This should not be overlooked as a potential reason for similarity seen in isolates between provinces. There was however the variability of 0.4% discovered between Ck/ZA/6743a/11 and Ck/ZA/6743b/11, these samples were collected from the same farm on the same day but originated from separate chicken houses/units. The existence of an insertion at 673 and point mutations at 4, 6, 296 and 879 emphasize the existence of variability and selection of subpopulations on a single farm. The selective criteria for these subpopulations are unknown, but variables such as maternal-derived antibody levels and timing of vaccination could apply greater selective pressure on vaccine similar epitopes (De Herdt *et al* 2001, Meulemans *et al* 2001, Mondal & Naqi 2001). An alternate hypothesis is that the individual house condition with regards to air quality and the degree of damage to the chickens tracheal epithelium and cilia affect the selection of S1 protein subpopulations.

The split of farms was observed as all the Mass genotyped isolates originated from broiler chicken farms. It may be significant that all isolates in the study where subject to at least 3 passages, with a maximum of five passages, in an attempt to improve RNA yield. It has been suggested that intra-passage mutation and host-driven selection may play a role in reducing variation within the Mass grouping of isolates (Cavanagh 2005, McKinley *et al* 2008, Liu *et al* 2009). With this statement in mind it could be postulated that the similarity seen between CK/ZA/0890/81 and CK/ZA/6689/12 is due to *in vitro* selection of an embryo dominant quasi-species through many passages of the virus. This is supported by the fact that Ck/ZA/0890/81 was subjected to five passages from a freeze dried sample in preparation for this study. All farms that submitted samples for this study made use of a live IBV vaccination program containing serotype H120. Broiler sites used a single vaccine application at 1 day of age whilst breeder samples made use of a combined live and inactivated program. The inactivated program made use of

commercial Mas- type strains as well as an autogenous IBV component of regionally-significant serotypes. These include Egypt/Beniusef-like, TC07-02 like, QX-Like and 4/91-like. The use of especially the live day old H120 vaccine and the close similarity of Mass-type isolates to H120 must not be overlooked as the most likely influence on the prevalence of the H120 group. This does mean that the most likely contributor to IBV serotypes within these flocks is Mass-type live vaccines. However, there is the possibility that either through genetic drift or quasi-species selection field adapted virulent Mass type IBV was able to proliferate in suitable hosts. The basis of this statement originates in that all the samples collected were done so from clinical cases of respiratory disease and the presence of suggestive pathology on post mortem.

Variant IBV live vaccines were recently registered in South Africa (August 2013) and through the registration process extensive safety tests, laboratory and field trials have been performed. The H120 vaccine of European origin was subjected to 120 passages and has been used globally for over 60 years (Bijlenga *et al* 2004). However, it is reported that the use of IBV vaccines does render the chicken more susceptible to secondary collibaccilosis, even under controlled conditions. The most conclusive evidence of this is provided in a publication from Cavanagh *et al* in 1992 that found field Mass type isolates that differed by 2-3% in the S1 gene. Their conclusion, as is here, is that enough diversity exists between field and vaccine strain to not constitute a re-isolation but rather a selected mutant of the vaccine strain.

The role of maternal antibodies to other IBV serotypes and its effect on the selection or prevalence of those serotypes has not been determined and therefore provides an additional potential selective pressure against serotypes such as 4/91.

Variability within the QX-like was greater than that seen in the Mass grouping, with 97.7-98.5% similarity observed. A temporal trend in genetic drift was observed. This observation contrasts to evolutionary patterns within the Mass group and supports the existence of genetic drift within this IBV subpopulation. This adds support to the earlier conclusion that the Mass grouped isolates are vaccine derivatives or mutants. The greatest similarity was noted when compared to the European origin QX-Like strains (97%) compared to the Asian QX-Like strains (94%), this provides

circumstantial evidence to the spread of QX-like IBV from Asia into Europe and then downward into Africa.

Geographic splitting demonstrated that of the four full sequence and two partial sequence QX-Like IBV isolates, five of them originated from farms within KZN and the remainder from the Eastern Cape. The split of farms was observed the QX-like isolates originated from a spilt of 60% broiler farms and 40% breeder chicken farms. A similar ratio split was been was comparing the amount QX positive isolates collected in 2011 and 2012. Worthington *et al* (2008) described that if the available vaccine serotypes are removed for survey results QX-Like IBV rose to be the leading IBV variant in Western Europe between 2002 and 2006. The results of the KZN isolates would support the high host adaptability of the QX-Like variant and identify it as the leading “variant” within KZN poultry flocks.

A comparison of the genotyping and serotyping results listed in Table 6.

Table 11. Comparative analysis of 1665bp sequence IBV isolates geno- and serotype.

Isolate	Serotype	Genotype
CK/ZA/3665/11	QX-like	QX-like
CK/ZA/4916/11	QX-like	QX-like
CK/ZA/4871/12	QX-like	QX-like
CK/ZA/6720/12	QX-like	QX-like
CK/ZA/2287/11	Mass	Mass
CK/ZA/3190/11	Mass	Mass
CK/ZA/3809/11	Mass	Mass
CK/ZA/3418/11	QX-like	Mass
CK/ZA/5250/11	QX-like	Mass
CK/ZA/5315/11	Mass	Mass
CK/ZA/5815/11	Mass	Mass
CK/ZA/6776/11	QX-like	Mass
CK/ZA/6681/11	Mass	Mass
CK/ZA/6621/11	QX-like	Mass
CK/ZA/6516/11	Mass	Mass
CK/ZA/6742a/11	QX-like	Mass
CK/ZA/6742b/11	QX-like	Mass
CK/ZA/6689/12	QX-like	Mass
CK/ZA/0890/81	Unknown Variant	Mass

The correlation between QX-like serotypes and the actual sequence was inconsistent with only 4 of the 11 serotype QX-Like isolates being classified within the QX-like genotype. All 7 Mass serotypes remaining genotyped into the same group.

Comparatively when examining partial S1 gene sequence data, neither of the QX-like partial sequences where serotyped as QX-like. Seven serotype QX-Like genotyped as Mass whilst the two isolates that did not fall into a known serotype at the time where split between the Mass and QX-Like genotype.

Table 12. Comparative analysis of 281(3') and 716(5') bp sequence IBV isolate geno- and serotype

Isolate	Partial	Serotype	Genotype
Ck/ZA/2312/11	5'	Unknown variant	QX-Like
Ck/ZA/4663/11	3'	Mass	QX-Like
Ck/ZA/6056/11	5' & 3'	Mass	Mass
Ck/ZA/4421/11	5' & 3'	QX-Like	Mass
Ck/ZA/2953/11	5' & 3'	Mass	Mass
Ck/ZA/3265/11	5' & 3'	Unknown Variant	Mass
Ck/ZA/4015/11	5'	Mass	Mass
Ck/ZA/3809/11	5' & 3'	Mass	Mass
Ck/ZA/4914/11	5' & 3'	Mass	Mass
Ck/ZA/5741/11	5' & 3'	Mass	Mass
Ck/ZA/6776/11	5'	QX-Like	Mass
Ck/ZA/6745b/11	5'	QX-Like	Mass
Ck/ZA/2754c/11	3'	QX-Like	Mass
Ck/ZA/2754b/11	3'	QX-Like	Mass
Ck/ZA/2754a/11	3'	QX-Like	Mass
Ck/ZA/2953/11	3'	Mass	Mass

The above results do cast doubt on the correlation between serotype and the genotype results. It is described that small sequence variation may alter the virus neutralizing epitope and thereby the serotype of the virus (Cavanagh *et al*, 1992). An alternative hypothesis could be that co-infection with multiple strains of IBV is responsible for clinical disease and between the culture/serum neutralization test and followed by successive passages and RT-PCR selection for host specific strains select for an alternate dominant strains (Xu *et al* 2007, Liu *et al* 2009).

The stability throughout all isolates of the latter half of the S1 gene supports the existence of two hypervariable regions located between base pairs 150 – 477 (Cavanagh *et al* 1988, Cavanagh *et al* 1992, Wang *et al* 1999). This study did note variability up until 900 base pair or 300 amino acid residues, five amino acids more than the accepted HVR 2. However Cavanagh *et al* (1992) reported variation in amino acid residues that would correspond to base pairs 753-1041. The study also

proposed that the virus neutralizing epitopes originate in the 1st and 3rd quarter of the S1 gene.

When comparing the correlation between serotype and genotype grouping it was noted that seven isolates serotyped as QX-like genotyped into the Massachusetts group. Comparing these protein sequences to the isolates that both geno- and serotypes as Massachusetts there is no common mutation within the isolates that serotyped as QX-like in contrast to the group that serotyped as Massachusetts-like.

Disappointingly, no sequence was obtained from any 4/91 (793B) or TC07-serotyped reference or field strains included in this study. This is despite obtaining a clear 1700bp band after RT-PCR, and use of the exact same forward and reverse primers for the sequencing reactions. The reasons for this are unknown. Adhzar *et al* (1996) and Lee *et al* (2000) described poor success when attempting to use one set of oligonucleotides for the amplification of various IBV isolates of varying serotypes. This is however not the situation in this case as 53 of 54 isolates produced PCR products where a 1.7kbp band was visualized after electrophoresis and the failure was experienced during the sequencing phase of the study.

Chapter 6

Summary and conclusions

This study describes the isolation, identification and serotyping of a representative selection of infectious bronchitis virus cases within KwaZulu-Natal and other South African provinces between 2011 and 2012. This study provides the first published investigation in South African IBV isolates and provides more comprehensive insights into circulation IBV in SA poultry flocks. The results of isolates VNT, RT-PCR and subsequent sequence data demonstrated that serotype as well as genotype variation exists within IBV infections of the KZN poultry flock. It also identified data that minor genotypic variation can drive alternate serotype formation. The study identified Massachusetts - like serotypes as the most prevalent and QX-like as the leading alternate sero- and genotype or “variant”.

Conclusions

- The Massachusetts (H120) serotype is the most prevalent serotype within the KZN poultry flock according to the sample analyzed in this study.
- Evidence suggests that field isolated Massachusetts types primarily are derived from variation or mutation of vaccine strains administered to flocks.
- The QX-like serotype is the leading non-vaccine serotype identified in the sample pool.
- Greater genotypic variation is seen amongst serotypes that aren’t exposed to vaccine selective pressure

Further areas of investigation

- A follow up study using alternate oligonucleotides may benefit the identification of additional genotypes
- The registered use of live 4/91 and QX vaccines within South Africa as of July 2013 will provide an interesting reference point in reviewing the prevalence of variation in years to come.
- *In vitro* pathogenicity studies may provide information correlating amino acid substitutions to increased clinical disease within the Mass or QX-like serotype.

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Annexure 1

Nucleotide multiple sequence alignment of full S1 genes (1665 bp)

Hypervariable regions are indicated in yellow.

```
                       410       420       430       440       450       460       470       480       490       500
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ckZA/2287/11  TGGCATGCTTCAACAGCATTCTATACGTGTTTCTGCTATGAAAAATGGCCAGCTTTTTTATAATTTAACAGTTAGTGTAGCTAAGTACCCTACTTTTAAA
ckZA/3190/11  ....................................................................................................
ckZA/3809/11  ............................................................................................T.......
ckZA/3665/11  A......A...C..GTG..CA...T...A.......A...........TTCTT.A....................C...T....A.......A.......
ckZA/3418/11  ..................................................................................G.................
ckZA/4916/11  A......A...C..GTG..CA...T...A.......A...........TTCTT.A....................C...T....A.......A.......
ckZA/5250/11  ....................................................................................................
ckZA/5315/11  ....................................................................................................
ckZA/5180/11  ....................................................................................................
ckZA/5815/11  ....................................................................................................
ckZA/6776/11  ....................................................................................................
ckZA/6681/11  ....................................................................................................
ckZA/6621/11  ....................................................................................................
ckZA/6516/11  ....................................................................................................
ckZA/6743a/11 ....................................................................................................
ckZA/6743b/11 ....................................................................................................
ckZA/4871/12  G......A...C..GTG..CA...T...A.......A...........TTCTT.A....................C...A....A.......A.......
ckZA/6720/12  A......A...C..GTT..CA...T...A.......A...........TTCTT.A....................C...A....A.......A.......
ckZA/6689/12  ....................................................................................................
ckZA/0890/81  ....................................................................................................

                       510       520       530       540       550       560       570       580       590       600
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ckZA/2287/11  TCATTTCAGTGTGTTAATAATTTAACATCCGTATATTTAAATGGTGATCTTGTTTACACCTCTAATGAGACCACAGATGTTACATCTGCAGGTGTTTATT
ckZA/3190/11  ....................................................................................................
ckZA/3809/11  ....................................................................................................
ckZA/3665/11  ..T.....A........C..C..C..C.....T..T...................TT..A.....CA.A..T..T........G..A...T.........
ckZA/3418/11  .........................................................G..........................................
ckZA/4916/11  ..T.....A........C..C..C..C.....T..T...................TT..A......A.A..T..T........G..A........G....
ckZA/5250/11  ....................................................................................................
ckZA/5315/11  ....................................................................................................
ckZA/5180/11  ....................................................................................................
ckZA/5815/11  ....................................................................................................
ckZA/6776/11  ....................................................................................................
ckZA/6681/11  ....................................................................................................
ckZA/6621/11  ....................................................................................................
ckZA/6516/11  ....................................................................................................
ckZA/6743a/11 ....................................................................................................
ckZA/6743b/11 ....................................................................................................
ckZA/4871/12  ..T.....A........C..C..C..C.....T..T...................TT..A.....CA.A..T..T........G..A........G....
ckZA/6720/12  ..T.....A........C..C..C..C.....T.KT...................TT..A.....CA.A..T..T...........A........G....
ckZA/6689/12  ....................................................................................................
ckZA/0890/81  ....................................................................................................

                       610       620       630       640       650       660       670       680       690       700
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ckZA/2287/11  TTAAAGCTGGTGGACCTATAACTTATAAAGTTATGAGAGAAGTTAGAGCCCTGGCTTATTTTGTTAATGGTACTGCACAAGATGTTATTTTGTGTGATGG
ckZA/3190/11  ....................................................................................................
ckZA/3809/11  ....................................................................................................
ckZA/3665/11  .......A.-.......G...A.....GTA......A....T...AG.TT..T.....C..............A...........A.........C..CAA
ckZA/3418/11  ....................................................................................................
ckZA/4916/11  .......A.........G...A...C.GTA......A....T...AG.TT..T.....C..............A...........A.........C..CAA
ckZA/5250/11  ....................................................................................................
ckZA/5315/11  ....................................................................................................
ckZA/5180/11  ....................................................................................................
ckZA/5815/11  ....................................................................................................
ckZA/6776/11  ....................................................................................................
ckZA/6681/11  ....................................................................................................
ckZA/6621/11  ....................................................................................................
ckZA/6516/11  ....................................................................................................
ckZA/6743a/11 ....................................................................................................
ckZA/6743b/11 ....................................................................................................
ckZA/4871/12  .......A.........G...A.....GTA......A....T...AG.TT..T.....C..............A...........A.........C..CAA
ckZA/6720/12  .......A.........G...A.....GTA......A....T...AG.TT..T.....................A...........A.........C..CAA
ckZA/6689/12  ....................................................................................................
ckZA/0890/81  ....................................................................................................

                       710       720       730       740       750       760       770       780       790       800
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ckZA/2287/11  GTCACCTAGAGGCTTGTTAGCATGCCAGTATAATACTGGCAATTTTTCAGATGGCTTTT-ATCCTTTT-ACTAATAGTAGTTTAGTTAAGCAGAAG-TTT
ckZA/3190/11  ...........................................................T........T.......................A...G...
ckZA/3809/11  ...........................................................-........-...........................-...
ckZA/3665/11  T.....C.AG..T...C....T..T..A..C..C...............T.........-........-..........C...G....G.G.A...T..C
ckZA/3418/11  ...........................................................-........-...........................-...
ckZA/4916/11  T.....C.AG..T...C....T..T..A..C..C.........................-........-..........C...G....G.G.A...T.-C
ckZA/5250/11  ...........................................................-........-...........................-...
ckZA/5315/11  ...........................................................-........-...........................-...
ckZA/5180/11  ...........................................................-........-...........................-...
ckZA/5815/11  ...........................................................-........-...........................-...
ckZA/6776/11  ...........................................................-........-...........................-...
ckZA/6681/11  ...........................................................-........-...........................-...
ckZA/6621/11  ...........................................................-........-...........................-...
ckZA/6516/11  ...........................................................-........-...........................T...
ckZA/6743a/11 ...........................................................-........-...........................-...
ckZA/6743b/11 ...........................................................-TAT.C...-...........................G...
ckZA/4871/12  T.....C.AG..T...C....T..T..A..C..C.........................-........-..........C...G....G.G.A...T.-C
ckZA/6720/12  T.....C.AG..T...C....T..T..A..C..C...........C.............-........-..........C...G....G.G.A...T.-C
ckZA/6689/12  ...........................................................-........-...........................-...
ckZA/0890/81  ...........................................................-........-...........................-...

                       810       820       830       840       850       860       870       880       890       900
              ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ckZA/2287/11  ATTGTCTATCGTGAAAATAGTGTTAATAC-TACTTTT-ACGTTACACAA--TTTCACTTTT-CATAA-TGAG-ACTGG-CGCCAACCCAAATCCTAGTGG
ckZA/3190/11  .............................C.......T.........C.AT..C.......T.....A...AC.....GGC...........T.CT....
ckZA/3809/11  .............................-.......-...........--..........-.....-....-.....-.....................
ckZA/3665/11  ..C........C.....GTAGT.......-....C.G-G.T..TA..T.A-..........A.TA.TG.A..T.A..----.AC.G..T...AG.G....
ckZA/3418/11  .............................-.......-...........--..........-.....-....-.....-.....................
ckZA/4916/11  ..C........C....G....T.......-....C.G-G...-A..T.A-..........A.TA.TG.A..T.A..----.AC.G..T...AG.G....
ckZA/5250/11  .............................-.......-...........--..........-.....-....-.....CGC.-.................
ckZA/5315/11  .............................-.......-...........--..........-.....-....-.....CGC.-.................
ckZA/5180/11  .............................-.......-...........--..........-.....-....-.....-.....................
ckZA/5815/11  ...............G.............-.......-...........--..........-.....-....-.....-.....................
ckZA/6776/11  ..-..........................-.......-...........--..........-.....-....-.....CGC.-.................
ckZA/6681/11  .............................-.......-...........--..........-.....-....-.....-.....................
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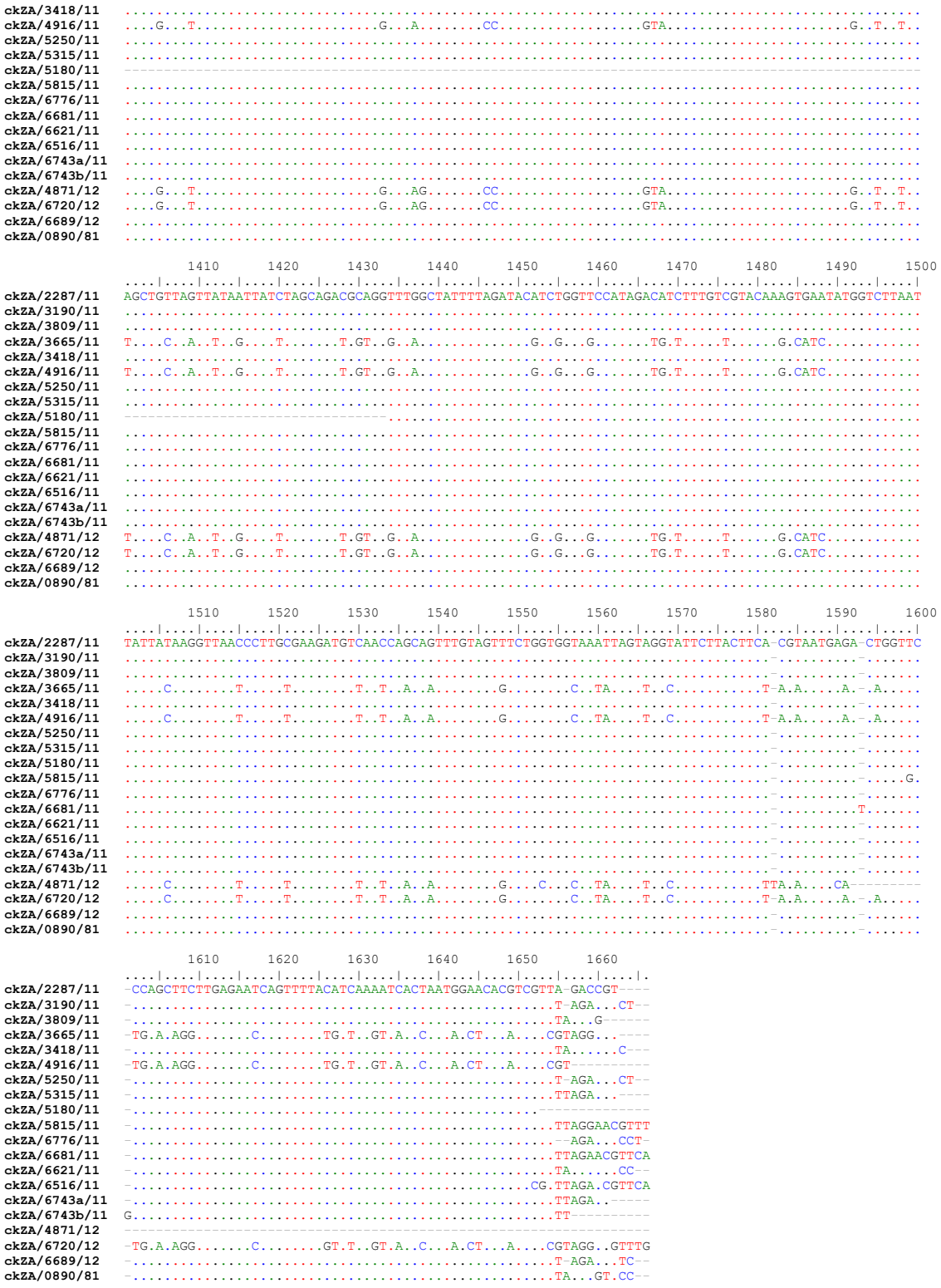


Annexure 2

Nucleotide multiple sequence alignment of a partial 281bp pair region of the S1 gene (forward sequencing reactions).

```
ckZA/2287/11    ....................................................................................................
ckZA/3190/11    ....................................................................................................
ckZA/3809/11    ....................................................................................................
ckZA/3418/11    ........................T...........................................................................
ckZA/5250/11    ....................................................................................................
ckZA/5315/11    ....................................................................................................
ckZA/5815/11    ........................T...........................................................................
ckZA/6776/11    ....................................................................................................
ckZA/6681/11    ........................T...........................................................................
ckZA/6621/11    ........................T...........................................................................
ckZA/6516/11    ....................................................................................................
ckZA/6743a/11   ....................................................................................................
ckZA/6743b/11   ....................................................................................................
ckZA/6689/12    ....................................................................................................
ckZA/0890/81    ....................................................................................................
```

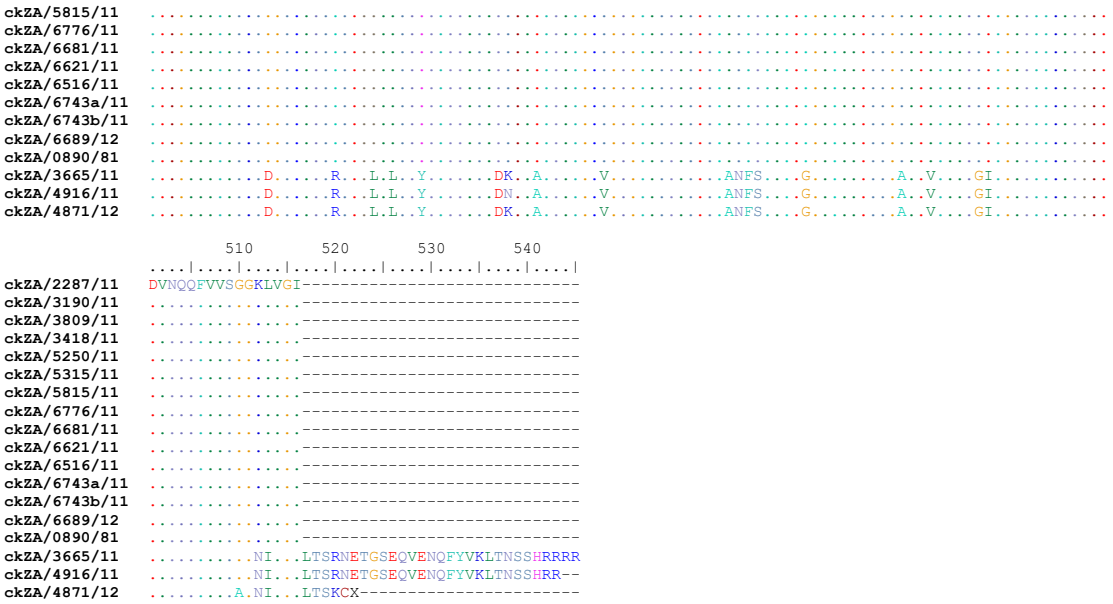
Annexure 3

Nucleotide multiple sequence alignment of a partial 617 base pair region of S1 gene (reverse sequencing reactions)

```
                ....|....|....|..
ckZA/2953/11    GTAAATTAGTAGGTATT
ckZA/3265/11    .................
ckZA/2312/11    .C..TA....T..C...
ckZA/4015/11    .................
ckZA/3809/11    .................
ckZA/4421/11    .................
ckZA/4914/11    .................
ckZA/5741/11    .................
ckZA/6056/11    .................
ckZA/6776/11    .................
ckZA/6743b/11   .................
ckZA/3665/11    .C..TA....T..C...
ckZA/4916/11    .C..TA....T..C...
ckZA/4871/12    .C..TA....T..C...
ckZA/6720/12    .C..TA....T..C...
ckZA/2287/11    .................
ckZA/3190/11    .................
ckZA/3809/11    .................
ckZA/3418/11    .................
ckZA/5250/11    .................
ckZA/5315/11    .................
ckZA/5815/11    .................
ckZA/6776/11    .................
ckZA/6681/11    .................
ckZA/6621/11    .................
ckZA/6516/11    .................
ckZA/6743a/11   .................
ckZA/6743b/11   .................
ckZA/6689/12    .................
ckZA/0890/81    .................
```

Annexure 4

Multiple amino acid sequence alignment for full S1 proteins. Hypervariable regions are indicated in yellow.



Annexure 5

Description of study isolate numbers and GenBank Accession numbers

Isolate description	**GenBank Accession number**
CK/ZA/2287/11	KJ200287
CK/ZA/3190/11	KJ200286
CK/ZA/3809/11	KJ200283
CK/ZA/3665/11	KJ200284
CK/ZA/3418/11	KJ200285
CK/ZA/4916/11	KJ200281
CK/ZA/5250/11	KJ200280
CK/ZA/5315/11	KJ200279
CK/ZA/5815/11	KJ200278
CK/ZA/6776/11	KJ200291
CK/ZA/6681/11	KJ200275
CK/ZA/6621/11	KJ200276
CK/ZA/6516/11	KJ200277
CK/ZA/6743a/11	KJ200290
CK/ZA/6743b/11	KJ200289
CK/ZA/4871/12	KJ200282
CK/ZA/6720/12	KJ200273
CK/ZA/6689/12	KJ200274
CK/ZA/0890/81	KJ200288

Description and Accession numbers of Genbank sequences used.

Accession number	Isolate reference		Accession number	Isolate reference
JN022548	062545/09		GQ253486	IBV/Ck/SP/170/09
JN022549	062561/09		GQ253483	IBV/Ck/SP/18/08
JN022550	065846/10		GQ253484	IBV/Ck/SP/79/08
JN022553	082066/10		GQ253485	IBV/La/SP/116/09
JN022545	09620/10		GQ253482	IBV/La/SP/17/08
JN022546	09621/10		HQ842710	IR/70/02
JN022547	09622/10		DQ400359	IS/1201
KC577404	75GX-07I		AY043312	Isolate A2
KC577416	93GD-09II		GQ149080	Isolate W
FJ829874	AH07101		AF352311	Isolate ZJ971
KC414154	chicken/CH/SD/2011/08		AF352313	Isolate ZJ971
HQ018894	CK/CH/GD/LY10		FN430414	ITA/90254/2005
HQ018906	CK/CH/GD/XX10		FN182281	ITA/90254/2005
JF828981	ck/CH/LDL/101212		FJ807927	K1019/03

JF828981	ck/CH/LDL/101212		AY790363	K10217-03
JQ739315	ck/CH/LHLJ/111050		FJ807928	K1255/03
FJ345374	CK/CH/LJL/07II		AY790364	K1255-03
JF330878	ck/CH/LJS/101237		FJ807929	K1257/03
JF330899	ck/CH/LNM/091017		FJ807930	K1277/03
JQ739367	ck/CH/LSD/110726		FJ807922	K154/05
HM106336	CK/CH/SCDY/10I		FJ807931	K1583/04
HM363025	CK/CH/SCNC/08I		FJ807923	K283/04
GU938431	CK/CH/Zhejiang/Quzhou1/0910		FJ807924	K463/04
JQ088078	CK/SWE/0658946/10		DQ431199	L-1148
AF036937	D41		GQ219712	M41
EF079117	FR/L-1450L/05		EU283075	Mass/Areisolated
EF079118	FR/L-1450T/05		EU283073	Mass/Avial1
JF900375	GB 1011/04		FJ904713	Mass41 2006
JF900379	GB 210/06		EF079115	NL/L-1449K/04
JF900376	GB 313/05		EF079116	NL/L-1449T/04
JF900376	GB 313/05		JQ991523	RF/08/2010
JF900382	GB 429/07		GU393335	Serotype H120
JF900374	GB 552/04		DQ064815	Spain/00/339
JF900381	GB 650/06		GQ885131	THA241251
HM540074	GX-NN2		GQ885140	THA340552
AY251817	HaN1-95		FR856855	Turkey coronavirus
GU967392	IB_UKND_08_14		JX182790	UFMG/29-78
GU393340	IBV/Brasil/PM1/1987		JX182773	UFMG/PM1
HM132098	IBV/chicken/UK/3355/09		EU914939	UK/AV2150/07
JX027070	IB Var2 06		GQ269448	TC07-2
HQ842716	IR/491/08		DQ487075	Egypt F03
GQ269447	TC07-1		AF093795	Variant 1
JQ290229	Chicken/Italy11/VIR31417/2011			