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Infectious Bronchitis in Brazil- a Minor Field Study in Bastos Municipality, São Paulo

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SUMMARY

Infectious Bronchitis (IB) is a highly contagious viral respiratory and production depressant disease distributed world wide. The disease is caused by the Infectious Bronchitis Virus (IBV), a positive RNA strand Avian Coronavirus within the genus *Gammacoronavirus.* Due to a combination of high mutation rate and a tendency of recombination a constant emergence of new genotypes and serotypes is seen. In Brazil, a country with an extensive poultry production and major problems with disease control, the diversity of circulating strains are wide and outbreaks of clinical disease despite intensive use of vaccines are common. The aim of this study was to investigate the presence of IBV and to identify genotypes of isolated IBVs in a limited number of layer flocks in Bastos, São Paulo, as part of the screening for circulating genotypes in the country. As a minor part the Biosecurity measures (precautions) was observed. In addition, a literature study was performed on IB and IBV. Organ samples from four farms were collected as pools of trachea, lungs, kidneys, reproductive tract and enteric content and were analyzed using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Over fifty percent of the samples were positive for IBV and three samples were identified as 4/91-genotype using Genotype-specific PCR. Due to problems with the RT-PCR that was targeting part of the S1-gene, the genotypes of the rest of the positive samples could not be determined using DNA sequencing. They were instead identified as not belonging to Massachusetts, D274 or 4/91 genotypes using Genotype-specific RT-PCR. Considering the results of previous studies in Brazil stating that a national Brazilian Variant dominates, the possibility that the unidentified genotypes belonged to the national genotype were considered high.

Keywords: IBV, Coronavirus, Poultry, Brazil, Genotypes, Biosecurity

SAMMANFATTNING

Infektiös Bronkit är en mycket smittsam viral respiratorisk och produktionsnedsättande sjukdom som är spridd över hela världen. Sjukdomen orsakas av ett positivt RNA-virus tillhörande Aviära Coronavirus inom genus *Gammacoronavirus*, kallat Infektiöst Bronkit Virus (IBV). Virusets egenskaper såsom hög mutationsfrekvens i kombination med tendens till rekombination leder till konstant uppkomst av nya serotyper och genotyper. I Brasilien, ett land med både omfattande fjäderfäproduktion och stora problem med sjukdomskontroll, är variationen mellan cirkulerande stammar stor och utbrott av klinisk sjukdom trots utbredd användning av vaccin är vanligt. Syftet med den här studien var att undersöka förekomst av IBV och att identifiera genotyper av isolerade IBVs i ett begränsat antal värphönsflockar i Bastos, São Paulo, som del av den pågående screeningen med avseende på cirkulerande genotyper i landet. Som ett mindre delsyfte utfördes observationer med avseende på hur väl biosäkerheten var utvecklad. Vidare utfördes en litteraturstudie avseende IB och IBV. Organprover samlades in från fyra gårdar som pooler bestående av trachea, lungor, njurar, reproduktionsorgan och tarminnehåll och proverna analyserades med Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Mer än hälften av proverna var positiva för IBV och tre prover identifierades som genotyp 4/91 med Genotyp-Specifik PCR. På grund av problem med den RT-PCR som var riktad mot delar av S1-genen kunde resterande positiva provers genotyp inte identifieras via DNA

sekvensering. Med Genotypspecifik PCR kunde de dock identifieras som icke tillhörande någon av genotyperna Massachusetts, D274 eller 4/91. Med hänsyn till resultat från tidigare studier där en nationell Brasiliansk Variant visats dominera, bedömdes sannolikheten att de oidentifierade proverna skulle tillhöra den nationella genotypen som stor.

Nyckelord: IBV, Coronavirus, fjäderfä, Brasilien, Genotyp, Biosäkerhet

INTRODUCTION

Introduction

Infectious bronchitis (IB) is a highly contagious viral respiratory and production suppressant disease that causes major financial losses and decrease in animal well fare in poultry flocks all over the world (Cavanagh, 2008; Engström *et al,* 2010).

The disease was first described in North Dakota, USA, in the 1930s, as an acute and highly infectious respiratory disease in chicken (Cavanagh, 2008; Pattison *et al,* 2008). The etiology at this time was unknown. The causative agent, the Avian Coronavirus Infectious Bronchitis Virus (IBV), was discovered in 1936 by Beach and Schalm (Cavanagh, 2008). IB became very important due to the financial losses that outbreaks resulted in, the worldwide distribution and the various manifestations the disease exhibited. Apart from respiratory disease, poor weight gain in broilers, and prominent (often persistent) decreases in egg production and egg quality in layers, are results of IBV infection. Furthermore, kidney disease may develop. Complications with secondary bacterial infections are common and may result in an increase in mortality (Cavanagh, 2008).

Many factors contribute to the problems of IB control. The highly contagious nature of the disease, resulting in rapid and thorough transmission within a flock, and the tendency of the virus to constant emerge into new serotypes and genotypes are true challenges for the poultry industry (Cavanagh, 2008; Engström *et al,* 2010). There is a constant need for development of new vaccines since the cross protection between serotypes has shown to be low (Cavanagh, 2007) and outbreaks in vaccinated flocks occur (Cavanagh, 2008).

Virus neutralization (VN) and Hemagglutination inhibition (HI) test have traditionally been used to distinguish serotypes (Cavanagh, 2007). A lot of research has been aimed at the gene for the membrane bound glycoprotein called Spike, the S-gene, due to its importance in antigenic variation (Cavanagh, 2007). Nowadays nucleic acid analysis is thoroughly used when it comes to classification and molecular epidemiology studies, and the definition is then into genotypes instead of serotypes (Jones *et al,* 2009). Monoclonal Antibody analyses like ELISA are commonly used for prevalence screening for detection of anti-IBV antibodies (OIE Terrestrial Manual, 2008).

IBV was long considered to be limited to infect chicken (*Gallus gallus*). However, results from recent research indicate that the host range is much wider and Avian Coronaviruses have now been isolated in both gallinaceous and non-gallinaceous species (Cavanagh, 2008).

Incidence and distribution

Infectious bronchitis is distributed world wide (Cavanagh, 2008). The Massachusetts serotype is found globally and the presence of other genotypes varies. In the USA several types of major importance have been identified and in other parts of the world, dozens of others have been isolated (Cavanagh, 2008; De Wit *et al*, 2010). In some areas in Asia, Africa, Australia and Latin America, regional variants have developed (Jones *et al,* 2009).

The way of transmission between continents are often unknown and the role of wild migratory birds is not yet established (Jones *et al,* 2009; Jones, 2010).

Infectious bronchitis in Brazil

The major Brazilian poultry regions are located in the Southern, Southeastern, Northeastern and Central western parts of Brazil. The avian population density within these regions is very high and the flocks and farms are located in a close distance (Villarreal *et al,* 2010). Infection with IBV is a huge problem in all categories of poultry, i.e. broilers, breeders and layers (Brandão *et al,* 2009) and despite intensive use of vaccines, outbreaks with clinical disease are common (Villarreal *et al,* 2007a). The share of flocks being positive for IBV has been shown to be as high as 50-100 % (Brandão *et al,* 2009).

An important factor contributing to the challenge for the poultry industry in Brazil to fight IBV infections is that only one type of live attenuated vaccine is approved by the Brazilian Ministry of Agriculture, Massachusetts – serotype vaccine. This presents a big concern since the genotypes of the IB virus in Brazil are highly divergent and the majority belongs to Non-Massachusetts types (Villarreal *et al,* 2010, Brandão, 2010). A specific Brazilian genotype Variant closer related to the European serotype D274 than to the Massachusetts serotype is present (Villarreal *et al,* 2007a) and the cross-protection between this Variant and Mass serotypes is thought to be low- moderate (Villarreal *et al,* 2010).

The significant variation in genotypes circulating among the Brazilian poultry was confirmed by a study performed by Villarreal et al in 2007-2008 (Villarreal *et al,* 2010). 85 % (17/20) of the strains identified in IBV positive flocks belonged to Non-Massachusetts strains. Three quarters belonged to the Brazilian genotype and within this genotype, three subclusters were outlined. Furthermore, two strains of the genotype 4/91 (also known as 793 B), not before detected in Brazil, were found (Villarreal *et al,* 2010). The authors suggested that this may be an indication of the emergence of this genotype into Latin America (Villarreal *et al,* 2010), especially since a strain similar to this has been detected before in a non specified country in Latin America (Jones *et al,* 2009).

Current research, not yet published, are being performed by the Coronavirus research group in Brazil regarding IBV infection in quails and the possibility of quails serving as a reservoir for IBV (Brandão, 2010). Results obtained so far indicates that this may be the case. This would require even further measurements for the poultry industry in Brazil since farms with both chicken and quails are not uncommon and usually quails are not vaccinated against IBV (Brandão, personal communication, 2011-11-03).

Aim

The aim of this study was to: I) achieve further knowledge of the disease Infectious Bronchitis and the causative virus through a literature study; II) to study the presence of the IB virus in a number of flocks in Bastos, one of the major areas for layers in Brazil; and III) to identity the genotypes of the virus obtained as part of the continuously ongoing screening for strains circulating in the country. As a minor part, IV), the biosecurity measures were observed and blood samples for antibody analysis were collected.

LITERATURE STUDY

Etiology

Infectious bronchitis virus (IBV) belongs to the family *Coronaviridae* in the order *Nidovirales*. The family *Coronaviridae* is divided into two subfamilies: *Coronavirinae* and *Torovirinae* (Cavanagh, 2008; Cavanagh, 2007; IVCT website). Virus within the Coronavirinae can infect a number of animals including humans, birds, cattle, pigs, dogs, cats and rodents (Pattison *et al,* 2008). *Coronavirinae* are divided into the genera Alphacoronavirus, Betacoronavirus and Gammacoronavirus. IBV is now called Avian Coronavirus, a species in the genus *Gammacoronavirus* (IVCT website, nd).

Morphology

The morphology of the IBV virion is round to pleomorphic. The envelope is approximately 120 nm in diameter and carries club-shaped projections called spikes (Cavanagh, 2008). The spikes are relatively large membrane bound glycoproteins with a mean size of 20 nm (Cavanagh, 2007). An electron micrograph photo of IBV is shown in Figure 1.

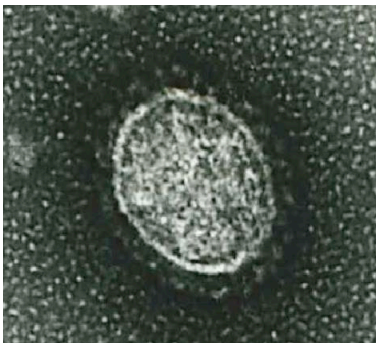

Figure 1. Electron micrograph of Infectious Bronchitis virus particles. (Photo: Siamak Zohari, SVA)

Gene function and organization

The IBV genome consists of a single- stranded positive sense RNA and contains about 27 600 nucleotides representing both structural and non-structural genes (Cavanagh, 2007).

The Spike (S) represents one of three major structural proteins (Cavanagh 2007). In IBV, along with some other corona viruses, the spikes consist of two subunits called S1 and S2 (Cavanagh, 2007). The part of the spike with the appearance of a bulb is mostly represented by the S1-unit while the S2- unit is the part anchored to the virion membrane. The function of the S-protein is attachment of the virus to the receptor at the host cell (S1) and induction of fusion of the membranes of the cell and the virion in order for the viral genome to be released into the cytoplasm (S2) (Cavanagh, 2007). S1 and S2 consist of approximately 500 and 600 amino acids, respectively. The S2 subunit is much more conserved among serotypes than the S1 (Cavanagh, 2008).

The other major structural proteins are the membrane (M) glycoprotein and the internal nucleoprotein (N), the latter being closely wrapped around the genome to form the ribonucleoprotein. A smaller membrane protein (E), not present to the same extent as the others, is associated with the envelope (Cavanagh, 2008). Both the M-protein and the E-protein is needed for the formation of the virus particle. The E-protein consists of less than half of the number of amino acids as the M-protein, 100 and 230 amino acids respectively (Cavanagh, 2007).

Apart from the structural proteins, non-structural proteins needed for RNA replication and transcription, and small non-structural proteins, most of these with unknown function, are encoded in the coronavirus genome (Cavanagh, 2007). IBV carries 15 non-structural proteins for replication and transcription and these are encoded in gene 1, or ORF1. The number and the location of the small non-structural proteins vary between coronaviruses (Cavanagh, 2007). The gene organization of IBV is shown below (Figure 2). The small non-structural proteins genes of IBV are named 3 and 5 and they encode three and two proteins, respectively. 3c is the small membrane E-protein (Cavanagh, 2007).

5' UTR	Gene 1	S	3a	3b	3c (E)	M	5a	5b	N	3'UTR

Figure 2. The genome organization for IBV (modified from Cavanagh, 2007). The sizes of the boxes in the figure do not correlate to the sizes of the genes.

The replication of IBV occurs in the cytoplasm. The transcription process is discontinuous and results in five messenger RNAs. The virions are formed through a budding process in the endoplasmic reticulum and are transported to the surface in smooth vesicles. The mechanism behind the final release of the virions from the cells is still unknown (Cavanagh, 2008).

Classification

IBV exist in numerous serotypes and genotypes and new variants continue to emerge due to mutations as well as recombination (Jones, 2010). The cross protection between different serotypes is poor (Cavanagh, 2008). Both genotype and serotype classification is generally based on the S1-subunit of the S-protein (Cavanagh, 2008). S1 is the major inducer of virus neutralization antibodies and traditionally, cross-neutralization tests have been used for serotype classification

(Cavanagh, 2008; Jones, 2010). Genotypes are defined via Reverse Transcriptase Polymerase Chain Reaction, usually targeting the S-gene, followed by nucleotide sequencing or restriction endonuclease analysis (Cavanagh, 2008).

A change of only 2-3 % of the nucleotide sequence in the S1-gene, representing some 10-15 amino acids, may alter the serotype of the strain (Cavanagh, 1997). Some regions of the S1-gene are of greater importance than others when it comes to epitopes inducing neutralizing antibodies. The location of these areas is mainly in the first and the third quarters of the S1-gene (De Wit, 2000).

Mean nucleotide difference of the S1-unit is approximately 20-25 % between serotypes. Usually the correlation between genotype and serotype is good. However, there are strains with greater nucleotide difference that still have been grouped in the same serotype using virus neutralization tests. Exceptions to this are also available where, although similar in nucleotide sequence of the S1-unit, virus neutralization tests defined the strains in different serotypes (Cavanagh, 2008).

Strain classification in IBV Diagnosis

Strain classification during IBV diagnosis is very important (Cavanagh, 2008). Classification can be based on biological function of the virus using functional tests or on the viral genome using non-functional test (De Wit, 2000).

Functional tests group viral strains in immunotypes or protectotypes (strains that induce protection against each other), in serotypes (strains that induce serotype-specific neutralization antibodies) or epitope-types (strains that contain specific epitopes) (De Wit, 2000). Functional tests are useful for the selection of vaccine programs since they provide information about the antigenicity of the strain (De Wit, 2000).

Non-functional test groups the strains into genotypes (De Wit, 2000). Methods used are genome sequencing, detection of part of the genome that is specific for a genotype via RT-PCR or determination of specific enzyme cleavage sites. When sequencing is used, part of the S-gene or the N-gene is most commonly analyzed. In genotype-specific RT-PCR genotype specific primers are used in combination with a universal primer resulting in amplicons of different sizes (De Wit, 2000). Cleavage sites are identified via restriction enzymes that cut the PCR product at specific cleavage sites, resulting into fragments that produce different electrophoresis patterns. These patterns can then be compared to patterns of known serotypes (OIE Terrestrial Manual, 2008; De Wit, 2000).

Non-functional tests are useful in epidemiological studies. Although these methods do not provide information neither about the biological function nor the antigenicity of the virus (De Wit, 2000), genotyping is increasingly replacing traditional serotyping with VN and HI for identification of field strains (OIE Terrestrial Manual, 2008). Nucleotide sequencing of important fragments of the S1-gene are the most common method used (OIE Terrestrial Manual, 2008).

Pathogenicity

IBV is an epitheliotropic virus that replicates mainly in the epithelium in the respiratory tract causing characteristic lesions in the trachea, the primary site of replication for all IBV pathotypes. IBV can also replicate in epithelium in other tissues such as kidney, intestinal tract and gonads of both males and females (Cavanagh, 2008). Furthermore, IBV can replicate in lymphoid organs such as the Harderian gland in the eye socket and the bursa of Fabricius (Dhinakar & Jones, 1997).

In chicken of very young age, the development of air-sacculitis due to secondary bacterial infection or the development of kidney disease will increase the severity of the disease (Cavanagh, 2008). Different strains differ in virulence and hence in clinical features. Primary nephropathogenic strains exist, some of them causing mortality of up to 25 % (Cavanagh, 2008). However, many strains can cause nephritis to some extent (Cavanagh, 2007).

Replication in the enteric tract result in excretion of the virus in the faeces but clinical disease is considered rare but not impossible (Villarreal, 2007a; Cavanagh, 2007). IBV may also be associated with lesions in the testis and infertility in roosters (Villarreal, 2007b). Furthermore, there are strains of IBV that induces myopathy in the pectoral muscles in broilers (Gough *et al,* 1992) and others that cause lesions in the proventriculis (Cavanagh, 2008).

The determinants of pathogenicity of IBV are poorly known (Cavanagh, 2007). In other coronaviruses the S-protein has been demonstrated to affect the tissue tropism pattern (Kuo *et al,* 2000). *In vitro* studies for IBV have shown that the S-protein affect the host cell range (Casais *et al,* 2003). The function of the small non-structural proteins have been discussed both when it comes to possible involvement in pathogenicity and in immune response. However, further research is required in this area (Cavanagh, 2007).

Transmission and incubation period

The virus is spread within a flock through breathing air, direct contact and contaminated faeces (Engström *et al,* 2010). All animals in a naive herd will be quickly infected if the virus is introduced. The period of incubation is 18-36 h. (Cavanagh, 2008; Engström *et al,* 2010). Spreading between flocks can occur through contaminated objects, equipment and people (Engström *et al,* 2010). The frequency of air-borne spread between flocks is unknown (Cavanagh, 2008).

Extended and intermittent shedding of virus occurs both due to persistent infection and re-excretion. Persistence of IBV infection may be seen in non-respiratory tissue such as the kidney and intermittent shedding of virus can occur via nasal secretions and faeces (Cavanagh, 2008; Dhinakar & Jones, 2007). Re-excretion has been seen in hens at time of start of lay, if they had been infected as younger chickens. The re-excretion occurred although they had recovered from respiratory signs and they had had a period of time when viral samples were negative (Jones *et al,* 1987). Vaccination can also contribute to intermittent excretion since vaccine virus may persist in various internal organs for months (Cavanagh, 2008).

Hosts

IBV was long considered to be limited to infect chicken (*Gallus gallus*) but results from recent research indicate that the host range is much wider (Cavanagh, 2008). The emergence of the Severe Acute Respiratory Syndrome, SARS, in 2003 largely increased the interest of Corona Virus research which resulted in the identification of new coronaviruses both in humans and animals (Muradrasoli *et al,* 2010). Avian coronaviruses, some with great identity with IBV, have for example been isolated in pheasants, peafowl, teal, partridge and guinea fowl although they did not necessarily cause disease (Cavanagh, 2008). However, as mentioned previously, current research in Brazil indicates that quails may carry and develop disease from IBV. Symptoms with impact on egg production and quality, similar to those seen in layers, have been observed (Brandão, 2010). Furthermore, turkey and pheasant coronaviruses have shown to be genetically related to IBV although they are considered to be distinct species from IBV (Cavanagh, 2008; Muradrasoli *et al,* 2010). During a study of wild birds in the Bering area, diverse coronaviruses were found in 18 different avian species. The authors suggested that avian coronaviruses may be a genetic reservoir for future emerging pathogenic coronaviruses and the need for more studies within this area in order to better understand the ecology and the virus -host-interaction was illuminated (Muradrasoli *et al,* 2010).

Clinical signs

The most severe symptoms are seen in young chickens and include several respiratory symptoms such as coughing, sneezing, gasping, tracheal rales and nasal discharge (Cavanagh, 2008). Reduced general condition as well as depression and huddling are also part of the clinical features. Reduction in food consumption and in weight gain is common. The mortality in chicken less than 6 weeks of age can reach 25 %, the major cause being secondary bacterial infections. Infection in newly-hatched chickens, one day of age, can result in permanent damage to the oviduct (Cavanagh, 2008).

In chickens older than 6 weeks and in adult birds the disease can pass unnoticed or with mild respiratory signs. Mortality is unusual (Cavanagh, 2008) unless complications with secondary bacterial infections occur (Engström *et al,* 2010). However, since the pathogenicity between strains of IBV varies, some strains have shown to induce severe disease also in older chickens (Gough *et al,* 1992).

Reduced egg production and impaired quality and sustainability of the eggs are seen in IBV infected layers. Reduced hatchability, soft-shell, misshapen and rough-shelled eggs are some of the features. Respiratory signs may or may not be present. The egg production in herds that has undergone infection may be permanently reduced (Cavanagh, 2008). Both the time of lay (Eck & Van, 1983) and the virus strain (Cook *et al,* 1986b) has shown to be factors that influence on the severity of the production drop.

Nephropathogenic strains can induce symptoms of uremia in broilers, usually after a phase of respiratory signs and a recovering period from these signs (Cavanagh, 2008).

Mortality and morbidity

The morbidity within a flock is high and often reaches 100 % (Cavanagh, 2008). Mortality varies due to several factors such as virulence of the infecting serotype, age, status of immunity, and stresses like cold or secondary bacterial infections. In nephropathogenic strains sex, breed and nutrition are further factors that will influence on the severity of the kidney disease (Cavanagh, 2008)

Gross lesions and histopathology

IBV causes an exudative inflammation primarily in the upper respiratory tract i.e. trachea, nasal passages and sinuses. The tracheal mucosa is edematous with loss of cilia and a decrease in ciliar activity. Impact on air sacs and lungs may or may not be present (Cavanagh, 2008; Engström *et al* 2010).

Degeneration and loss of cilia is seen in the epithelial and glandular cells of the oviduct in adult hen (Cavanagh, 2008; Villarreal, 2007b). Lesions in the reproductive tract of one-day-old chicks are mostly located in the middle third of the oviduct (Cavanagh, 2008).

Lesions in the kidneys caused by nephropathogenic strains include swollen and pale kidneys with distended tubules and ureters (Cavanagh, 2008).

Infection in newly-hatched chickens, one day of age, can result in permanent damage to the oviduct. Re-excretion of virus has also been shown experimentally at time of lay, i.e. around 19 weeks of age, possibly as a result of the stress induced by the start of this process (Jones *et al,* 1987; Cavanagh 2007; Cavanagh, 2008).

Immunity

Both antibody and cell mediated immune responses seem to be involved in the protection against IBV (Cavanagh, 2008; Pei *et al,* 2001). Several studies have been performed in order to evaluate the role of the B-cells (Dhinakar & Jones, 1997). A number of adverse effects were seen followed B-cell-depletion such as more severe symptoms, prolonged disease duration and more severe lesion in tissues such as the kidney (Dhinakar & Jones, 1997). However, further mechanisms are also involved as shown in other studies where chickens resisted IBV challenge, despite lack of B-cells (Cavanagh, 2008). A variety of evidence for cell-mediated immune responses has been reported for example activity of natural killer cells and cytotoxic lymphocytes, and infiltration of cytotoxic T-cells (CTL) in respiratory and kidney tissues (Cavanagh, 2008). A good correlation has been shown between the peak in CTL present 10 days post-infection and the clearance of virus from lungs and kidneys (Cavanagh, 2008).

The S1-subunit plays an important role of the induction of VN and HI antibodies and the induction of protective immunity (Ignjatovic, 1994; Cavanagh, 2008). The latter was shown in a study where a recombinant chimera of the non-pathogenic IBV Beaudette strain and the S-protein gene from a pathogenic strain M41 was

created. The recombinant chimera called Beau-R-M41-(S) induced a considerable higher level of protection towards challenge with a M41 strain that did the original Beaudette although the recombinant virus was non-pathogenic itself (Hodgson *et al,* 2004).

Local antibody is considered very important (Dhinakar & Jones, 1997) and is believed to be involved in the protection of the respiratory tract (Cavanagh, 2007). The Harderian gland may also contribute to local immunity (Cavanagh, 2008) since it stores plasma cells and is the source of immunoglobulins in the lachrymal fluid (Dhinakar & Jones, 1997). Furthermore, local antibodies have been detected in the oviducts of hen (Dhinakar & Jones, 1997).

Post infection, some immunity is developed towards the specific virus strain that caused the infection but the immunity to other strains varies (Cavanagh, 2008). The virus titer recovered after a challenge is much lower if the birds had been vaccinated with a homologous strain prior to the infection (Cook *et al,* 1986a). Although humoral response to IBV is good, there is no clear correlation between the antibody titer in sera and the resistance to infection (Dhinakar & Jones, 1997).

Maternal antibodies

Maternal derived antibodies (MDA) decrease the efficiency of the immune response in chicken vaccinated at one day of age if the vaccine is the same that had been given to the mother. However, MDA may provide protection against IBV challenge in one day old chickens (Cavanagh, 2008).

Control of immunity

Evaluation of protective immunity after immunization or challenge can be addressed in different ways (Cavanagh, 2008). Failure of isolation of IBV from trachea a few days post-challenge has been used a single evidence for respiratory protection. In order to evaluate the range of protection, a score resulting from the assessment of a number of criteria such as absence of tracheal lesions, presence of tracheal ciliar activity, failure of IBV recover in kidneys and ovaries and absence of clinical signs of IB, can be used (Cavanagh, 2008). Evidence of IB protection in strains where respiratory signs are not the major issue, is based on the ability of the immune system to suppress the lesions and clinical signs produced by the certain type of virus strain i.e. protection against mortality of kidney disease in nephropathogenic strains and protection against egg production drop in layers (Klieve & Cumming, 1988; Box *et al,* 1988).

ELISA targeting the immunoglobulin M (IgM) may be used as an indicator of the efficacy of IBV vaccination at around 10 days post vaccination since IgM are short-lived and usually only detectable from three- five days until two weeks post-infection /vaccination (De Wit,2009b).

Diagnosis

In addition to clinical history and lesions, diagnosis of IB is based on different methods for detection of the virus (i.e. antigen, virus isolation or viral RNA) or

detection of an antibody response (i.e. seroconversion or an, at minimum four fold rise, in IBV antibody titers) (Cavanagh, 2008; De Wit, 2000). As mentioned in the chapter of strain classification, identification of serotype or genotype should be included in diagnosis of IBV due to the great number of existing types (Cavanagh, 2008).

Regarding ongoing or recent disease outbreaks, the exclusion of other possible causes should be made since IBV can be persistent and because long-term recovery has been reported (Chong & Apostolov, 1982; Alexander and Gough 1977).

Methods for viral detection

Collection of samples

Trachea is the sample site of choice for birds with symptoms of respiratory disease, especially when sampling is performed within the first week of infection. If more than one week has passed, cloacal swabs or caecal swabs are preferred since the virus spread to non-respiratory organs after the initial replication in the respiratory tract (Cavanagh, 2008; De Wit 2000). Lungs, kidneys and oviduct may also be sampled, depending on the history and the clinical features of disease (Cavanagh, 2008).

Virus isolation and detection of IBV antigen

Virus isolation is commonly performed via inoculation of the sample in embryonated Specific Pathogen Free (SPF) eggs or chicken tracheal organ cultures (TOC). Even if typical IBV induced changes can be seen such as embryo dwarfing and curling (figure 3), feather dystrophy and mortality, and ciliosthasis in TOC, fluid must be collected and submitted to a further method for confirmation of the diagnosis after 2- 3 days of inoculation (Cavanagh, 2008). Methods used include serological analysis like VN, HI and ELISA, and immunohistochemistry, nucleatic acid analysis and electron microscopy (Cavanagh, 2008).

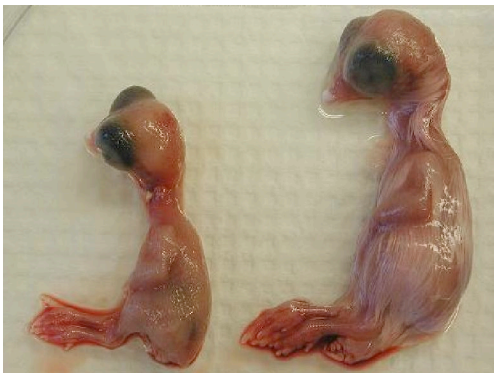

Figure 3. Embryo dwarfing caused by IBV. (Photo: Siamak Zohari, SVA)

In addition to virus isolation, direct detection of IBV antigen can be done from tissue material using antibody-based methods such as immunofluorescence (IFA), Immunoperoxidase assay (IPA) and Agar-gel Precipitation test (AGPT) (De Wit, 2000). All methods for antigen detection use either antisera containing antibodies

against different parts of the virus or monoclonal antibodies (Mabs) against one or more epitopes (De Wit, 2000). IFA and IPA are more commonly used than AGPT and antigen ELISA because of the general image of low sensitivity of the AGPT and the relatively high amount of virus needed for the antigen-ELISA (De Wit, 2000).

Detection of IBV genome

The use of Polymerase Chain Reaction (PCR) for IBV genome detection is increasing (De Wit, 2000). In identification of field strains, this method has replaced the traditionally used HI and VN tests because of its ability to identify a wide range of different genotypes (Cavanagh, 2008). As mentioned above, initial multiplication of the virus may be performed *in vitro* using embryonated SPF eggs or TOC in order to increase the viral titer and hence, the sensitivity of the analysis (De Wit, 2000). Before submitting the samples to RT-PCR, genomic RNA is extracted from the samples and cDNA is created using the enzyme reverse transcriptase. A Nested PCR step adds more sensitivity to the test since a second amplification step is performed using the first round PCR product as template (Cavanagh, 2008; De Wit 2000).

Factors that may influence the outcome of the RT-PCR is the possible presence of non-specific inhibitors of the PCR enzyme in the samples (Kwon *et al*., 1993) and the amount of cations such as Mg2+ and Mn2+ (Jackwood *et al*., 1997). Nonspecific inhibitors can be reduced by RNA purification prior to the RT-PCR (Kwon *et al*., 1993).

After the RT-PCR, the PCR product is further analyzed using nucleotide sequencing, restriction enzyme length polymorphism (RFLP) or S1 genotype specific RT-PCR (Cavanagh, 2008). In RFLP different genotypes can be identified due to unique electrophoresis banding patterns as a result of the digestion of the SI RT-PCR product by restriction enzymes (Cavanagh, 2008). In S1 genotype specific RT-PCR a combination of a universal primer and genotype specific primers are used in order to identify different genotypes (Keeler *et al*., 1998). Both RFLP and S1 genotype specific RT-PCR is limited to identification of already known genotypes (Cavanagh, 2008). Nucleotide sequencing, however, has the advantage to be able to identify new, previously unknown, genotypes. Sequencing of the S1 gene is the most useful method for IBV differentiation and also the most widely used. Further advantage with sequencing includes the possibility of phylogenetic studies where the relatedness of the strains is outlined (Cavanagh, 2008)

Methods for antibody detection

For serology analysis, paired samples should be obtained at onset of disease and two to four weeks after. Methods available are AGPT, ELISA, Hemagluttinin inhibition (HI) test and Virus Neutralization test (VNT) (De Wit, 2000). VN, HI and ELISA are usually used for routine diagnosis (De Wit, 2000) where ELISA and HI-test are considered to be most useful (OIE Terrestrial Manual, 2008).

ELISA is a group-specific test and detection of antibodies can be made one week post infection (De Wit, 2000). Several commercial kits for ELISA are available (Cavanagh, 2008). Although HI and VN test are considered to be type specific, cross reactions exist, especially in HI test (Cavanagh, 2008). In young, unvaccinated birds, VNT is golden standard for determination of serotype (De Wit, 2000). However, this is seldom the case in the field where most chicken have been vaccinated against, and possibly infected with, IBV resulting in a sera containing a broad antibody response (Cavanagh, 2008). AGPT is not recommended for antibody detection because of the relatively low sensitivity and because of the short lives of the precipitating antibodies (Cavanagh, 2008; De Wit *et al.* 1997).

Several factors affect the detection of antibodies. The humoral response may be decreased or delayed due to factors such as young age, presence of maternal derived antibodies (MDA) and recent vaccination or infection with homologous serotypes. In older birds that have received several vaccinations, or been previously infected, cross-reactions between antibodies of different serotypes results in the use of serology being limited to monitor prevalence (De Wit, 2000).

Differential diagnosis

For the clinical acute respiratory disease differential diagnosis include infectious laryngotracheitis (genus *Iltovirus* within family *Herpesviridae*) (Guy & Garcia, 2008), low pathogenic avian influenza, infectious coryza (*Avibacterium paragallinarum*) (Blackall, 2008) and lentogenic pneumotropic strains of Newcastle disease (genus *Avulavirus* within family *Paramyxoviridae*) (Alexander & Senne, 2008) (Cavanagh, 2008). The production and quality drop of eggs may resemble the egg drop syndrome caused by adenovirus (Cavanagh, 2008).

Treatment

Treatment for IBV consists of supporting procedures and minimization of stresses such as cold stress and overcrowded animal facilities (Cavanagh, 2008). Antibiotics may be used if complications such as air sacculitis due to secondary bacterial infections are present. In cases of nephritis, electrolyte replacers may be supplied in the drinking water (Cavanagh, 2008).

Biosecurity

Biosecurity includes the management procedures executed in order to prevent the entrance of any infective agents (i.e. virus, bacteria, protozoa, and parasites), insects, rodents and wild birds to the farm which could jeopardize the health status of the flock (Bermudez & Stewart-Brown, 2008). The term includes a wide variety of factors, among them design of animal facilities, location of the farm related to other farms, location of the buildings within the farm to each other, routines for procedures, staff, visitors, transporters etcetera. Other factors such as good quality of feed and water and optimal environment for the birds are also included. Proper cleaning and disinfection between batches and an “all-in-all-out”

routine are desirable when it comes to biosecurity but may not always be possible for example in layer farms where flocks of different ages are present (Bermudez & Stewart-Brown, 2008).

Because of the nature of IBV with rapid transmission, high morbidity and major production loss resulting in financial loss, management measures are often not enough to control disease. Immunization is an important tool and both live and inactivated virus vaccines are being used (Cavanagh, 2008). However, the number of different strains present and the tendency of the virus of constant emerge makes the control hard (Cavanagh, 2008).

Vaccination

Vaccines of Massachusetts serotype such as H120 and M41 are commonly used all over the world (OIE Terrestrial Manual, 2008). In some countries, like Brazil, these represent the only serotypes allowed to be used as live vaccines although other serotypes are present in the field (Villarreal *et al,* 2010; Brandão *et al,* 2009).

Live vaccines are produced from field strains via passage in embryonated egg leading to attenuation (Cavanagh, 2007). Live vaccines are more efficient in inducing a local immunity in the respiratory tract (OIE Terrestrial Manual, 2008) and are considered to provide a greater width of protection (Cook *et al,* 1999). The disadvantage with live vaccines is the risk of regain of pathogenicity and back-passage within the flock. In order to decrease this risk, simultaneous vaccination of all birds is recommended, especially in farms where birds of different age are present (OIE Terrestrial Manual, 2008).

Vaccination routines

Vaccination of broilers with live vaccines is usually performed in the hatchery at one day of age. In areas with high frequencies of IBV re-vaccination may be used, sometimes with another serotype than that of the first vaccination (Cavanagh, 2007). Administration routes of live vaccines include aerosols, application in drinking water and intraocular eye drops (OIE Terrestrial Manual, 2008).

Mass vaccination administration is commonly used but the accuracy may be compromised when it comes to application technique and dosage. Further factors that may affect the level of protection yielded from vaccination include immunosuppression and time between vaccinations and challenge (De Wit, 2009a).

Layers commonly receive a series of live and inactivated vaccines, starting at the age of two or three weeks (Cavanagh, 2007). More than one serotype may be given in order to increase the width of the protection (Cook *et al,* 1999). Before start of lay, killed vaccines are given (Cavanagh, 2007). Inactivated vaccines must be primed with a live vaccine in order to be efficient (OIE Terrestrial Manual, 2008). Administration routes for inactivated vaccines include intramuscular or subcutaneous injections (OIE Terrestrial Manual, 2008).

The use of two heterologous vaccines has shown to significantly increase the protection against variants although the mechanism behind this is still poorly understood (Jones, 2010; Cook *et al,* 1999; Ganapathy *et al,* 2009). In addition, layers that received an inactivated IBV M41 vaccine after initial vaccination with two live vaccines got a better protection both against ciliosthasis and drop in egg production (De Wit, 2009a).

In order to meet the challenge of the antigenic diversity of IBV, molecular technology may be an increasingly important tool in vaccine development in the future. One example of a technique within this area is recombination of different IBVs regarding the S-gene called "spike swapping" (Jones 2010; Britton, 2009). Another field of vaccine research is that of *in-ovo* vaccination (Jones 2010) where already some promising results has been shown (Khalesi, 2009).

METHOD AND MATERIALS

Collection of samples

Farms and flocks

Collection of samples from layer hen was made during one week in September 2011 in Bastos, a small town in São Paulo State in the southwest part of Brazil. Bastos represents one of the main regions for layers in Brazil and the area houses 15 million chickens and 4 million quails.

Organ and blood samples were collected from four farms that had reported problems with respiratory disease. Three of the farms (here referred to as A, B and D) had previously participated in a study regarding quails as reservoirs for IBV. Blood samples were also collected from a fifth farm (referred to as E) where no organs samples were taken. The farms were chosen on the basis of willingness to participate and because of the already-established contact due to participation in previous studies. The total number of birds at the participating farms is presented in table 1. At three of the farms, these numbers were unknown.

Table 1. Number of birds present at the participating farms distributed on chicken and quails

Farm	Total number of birds	Chicken	Quails
A	136 000	86 000	50 000
B	1 800 000	1 300 000	500 000
C	U	U	0
D	U	U	U
E	U	U	0

U = unknown

A total of 66 layers from 13 flocks (3-4 flocks per farm) were included in the study. The flocks were of different age and represented both layers in production and immature hen. Both birds with and without respiratory symptoms were chosen for collection. The birds were selected by local veterinarians and there was little possibility of influencing the selection of individuals. A thorough clinical

examination was not performed due to time constraints. Registration of presence or absence of clinical symptoms was based on history given by the accompanying person representing the farm.

Organ samples

Organs samples were collected postmortem as pools of 5-6 birds. The birds were euthanized via dislocation of the neck. The different pools consisted of trachea, lungs, kidneys, reproductive tract (i.e. ovaries and oviduct) and enteric content, predominantly from the caecum. At collection, gross pathological lesions were registered but no thorough necropsy was performed due to time constraints. The organ samples were kept cold during transport to the laboratory at the University of São Paulo and were then stored in -20 º C.

Blood samples

Blood samples were collected from 5-20 birds per flock resulting in a total number of 182 samples. Five milliliters of blood per bird was collected from the vein v. cutanea ulnaris on the wing. Blood sampling was performed in the same flocks, but not from the same individuals, from which the organ samples were taken. From the fifth farm (E), where no organ samples were retrieved, five flocks were sampled. The birds as well as the number of birds were selected by local veterinarians and there was little possibility of influencing this selection. The blood was kept in outdoor temperature at around 30º for approximately 3 hours and was then kept in room temperature for another 20 hours in order for the sera to separate. After 24 hours the sera was transferred to new tubes and were kept refrigerated until, and during, transport to the laboratory at the University of São Paulo. At the laboratory the samples were stored in -20 º C. 23 samples was discarded due to hemolysis or insufficient amount of sera required for the analysis, resulting in a total number of 159 useable samples.

Additional information

In addition to sample collection, information regarding age and breed were retrieved. Presence or absence of clinical symptoms was registered. Biosecurity was studied through own observations and registration of the routines for visitors at the farms. Vaccination routines for quails against IBV were registered.

Laboratory methods

Background on laboratory methods used

Polymerase chain reaction

Polymerase chain reaction, PCR, is a method for amplification of a specific fragment of DNA using a series of temperature changes initiating the different steps in the procedure. Several components are required for the reaction: termostabile DNA-polymerase to catalyze the synthesis of DNA, a pair of synthetic oligonucleotides to prime DNA synthesis, equimolar amounts of the

deoxynucleoside triphosphates (dNTPs) dATP, dTTP, dCTP and dGTP, buffer solution to maintain pH, divalent cations such as magnesium (Mg^{2+}) or manganese (Mn) ions, and a monovalent cation, such as potassium chloride (KCL). For routine PCR Taq-polymerase is the most commonly used termostabile DNA polymerase. The buffer solution normally constitutes of Tris-Cloride. The presence of free divalent cations is crucial for the activity of the enzyme. Since both the dNTPs and the primers bind cations, the amount of the latter must be greater than the molar concentration of the phosphate groups of the dNTPs and the primers. Hence, the optimal concentration of Mg^{2+} differs between reactions and optimization is made empirically. The design of the oligonucleotides is very important since they have a prominent influence on the efficiency and the specificity of the amplification reaction (Sambrook and Russel, 2001).

The main processes involved in the PCR reaction is denaturation of the double stranded DNA template by heat, annealing of the primers to the target sequence of the template and the extension of the primers by termostabile DNA polymerase. The steps are repeated in a number of cycles in order for the target sequence to be amplified in a satisfactory amount that largely exceeds that of the unwanted nonspecific amplification products, which should be barely detectable or, preferentially, not detectable at all (Sambrook and Russel, 2001).

In a Nested PCR, the product of the first round PCR is used as template and the oligonucleotide primers are replaced in order to reduce the size of the amplicon. Nested PCR increase the sensitivity of the PCR (Cavanagh 2008) and decrease the risk of non-specific amplification of DNA (Brandão, Personal Communication 2011-10-31).

The result of the PCR is made visible through an agarose gel electrophoresis. The PCR product fragments run through the gel with a migration correlated to their size and a band pattern will be created. The bands are then compared with a DNA ladder, containing DNA fragments of known size, and a positive control.

Sequencing

The PCR product can be further analyzed through a number of methods including DNA sequencing. DNA sequencing allows positive IBV samples to be distinguished into genotypes. The Sanger method of DNA sequencing is widely used and is based on Dideoxy-mediated Chain Termination (Sambrook and Russel, 2001). The dideoxynucleoside triphosphates (ddNTPs) differ from deoxynucleotides in that they lack a 3´-hydroxyl residue and instead carry a 3´hydrogen residue. This does not affect the ability of the ddNTPs to be incorporated into the DNA chain by DNA polymerases, but an extension of the strand past the ddNTPs is not possible since the 3´hydroxyl residue is needed for the formation of a 5´→ 3´phosphodiester bond. Hence, the ddNTPs will work as a terminator (Sambrook and Russel, 2001). Since the possibility that the DNA polymerase will add a dNTPs to the DNA strand is equal to that of the addition of a ddNTP, all possible fragments will be created if a sufficient number of cycles is performed.

The components needed for the DNA sequencing process are DNA template, a DNA polymerase like *Taq* polymerase, primer, buffer solution and equimolar

amounts of the four deoxynucleotides (dNTPs) and the four dideoxynucleoside triphosphates (ddNTPs). The latter is labeled with a fluorescence marker that is color-specific for each base. The DNA sequence is then analyzed in a sequencing machine. The sequencing machine used in this study was an ABI 3500 containing capillaries with polymers inside where laser beams register the color which each represents one base.

Genotype Specific Multiplex PCR

Genotype specific Multiplex PCR is a method for identification of already described genotypes. The PCR include the use of several primers specific for given genotypes, in combination with a universal primer. Annealing of the different primers will yield amplicons of different size and the fragment sizes can then be used to recognize the genotypes using an agarose gel electrophoresis (Keeler *et al,* 1998).

Execution at the lab

Extraction of RNA

The organ samples were cut in small pieces and mixed with DEPC-treated water in a 50/50 suspension.

The organ suspensions were frozen, thawed and homogenized via vortexing three times in order to break the cellular membranes. Liquid nitrogen was used to accelerate the freezing process. The organ suspensions were then clarified through centrifugation in 4 º C, 5000 g (RCF) for 10 minutes. 250 µl of the supernatants were collected and added to new tubes containing 750 µl Trizol (containing Guanidine, Phenol pH 4 and pH indicator) for further breakage of cellular membranes and denaturation of proteins but preservation of the integrity of the RNA. 250 µl of DEPC-treated water and 250 µl of a virus reference strain were used as negative and positive controls, respectively. The virus reference strains included the Massachusetts vaccine strain MA5 and Ceva Big Vacina and the Massachusetts field strain Cobb 3/6p

Following homogenization via vortexing and 5 minutes in room temperature, 200 µl of Chloroform was added to the tubes in order to separate the solution into an aqueous phase and an organic phase. Another session of vortexing was performed and after 10 min in room temperature the samples were centrifuged in 4 ºC, 12 000 g (RCF) for 15 minutes. 500 µl of the supernatant was added to new tubes containing 500 µl of Iso-propanol in order to agglutinate RNA by removing the water molecules between the RNA-molecules. After careful mixing, centrifugation at same temperature, velocity and time as mentioned above was performed.

The liquid phase was discarded in a container with one part water and one part chloride. The tubes with the remaining pellet were left to air-dry upside down before 950 µl of 75 % Ethanol was added to clean of remnant of Iso-propanol. The procedure with centrifugation (4 º C, 10 000 g, and 10 min), discarding the liquid phase and air-drying (57º C, 10 min) was repeated. Finally, 20 µl of DEPC-treated water was added and the samples were mixed via a short episode of

vortexing followed by a quick spin and was then heated in 57 º C 5 minutes. The RNA was then kept in a box with ice until storage at -80º C.

Reverse Transcription

3, 5 µl of the RNA yielded from the extraction was denaturized at 94 º C for 5 minutes, using a termocycler. A reverse transcription master mix was prepared containing random primer, dNTPs, di-thio-threitol (DTT), 5x Strand buffer, DEPC-treated water and reverse transcriptase (RT) enzyme. The RT enzyme had been prepared from a murine retrovirus named Maloney Murine Leukemic Virus. 6, 5 µl of the mix was added to the RNA and the reverse transcription was performed in the termocycler using the program RT 37.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) 3'UTR

A Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and a Nested PCR was performed to screen for IBV. The target sequence of the first round PCR was a fragment of 266 bp located in the 3' untranslated region (UTR). The primers used were UTR 41+ and UTR 11-. In the second, nested step the primer UTR 11- was replaced with UTR 31 – in order to generate an amplicon of 179bp. The process was carried out as described by Cavanagh *et al.* (2002).

A PCR master mix was prepared by the addition of the primers UTR 41 and UTR 11 and DEPC-treated water to a pre-mix containing Taq-polymerase, MgCl, buffer and dNTPs. 2.5 µl of cDNA was added to 22.5 µl of mix. The program used in the termocycler was 3 UTR. The specific cycles for 3 UTR, and the general function of each step, are presented in Table 2. Step 2-4 were repeated 24 times.

Table 2. The cycles for, and the general function of each step, of RT-PCR 3'UTR

Step number and title	Temperature (ºC)	Time (min)	Function
1) Denaturation	94	4	Denaturation of antibodies bound to the Taq- polymerase i.e. enabling function of the enzyme
2) Melting	94	1	Separation of DNA strains
3) Annealing	48	1,5	Activation and binding of reverse and forward primer to the DNA strain
4) Extension/elongation	72	2	Activation of polymerase. Extension of strands
5) Final extension	72	10	Final extension
6) Hold	4	forever	Hold

Nested RT-PCR 3'UTR

The product amplicon obtained in the first round of PCR was submitted to a second nested round of PCR. A master mix was prepared according to the procedure described above and the same program was used in the termocycler. The primers used were UTR 41+ and UTR 31-.

Electrophoresis

An agarose gel of a concentration of 1.5 % was prepared from agarose powder and 0.5 x TBE buffer solution. A DNA ladder was deposited in the first and the last well followed by the deposition of the nested PCR products in the remaining wells. The electrophoresis process, where the negative charged DNA runs towards the positive pool, was performed at 10 V per centimeter of gel for approximately 90 minutes. Before the DNA was detected by ultraviolet transillumination using the software Gene snap, the gel was stained with ethidium bromide. The size of the fragments representing the bands seen on the gel, were estimated via comparison with the DNA ladder where each step represented 100 basepares.

RT-PCR and Nested RT-PCR Spike gene

In order to be able to determine genotype of the IBVs detected by the 3' UTR RT-PCR, the positive samples were submitted to partial amplification of the spike gene, Spike gene RT-PCR. Genotype identification is made through analysis of the DNA sequence of the Spike gene RT-PCR product (also called amplicon). The target of the Spike gene RT-PCR was a 390 bp segment between the nucleotides 705 and 1094 of the S1 coding region, corresponding to the amino acids 234-364 of the S1 protein. The primes used for the first round PCR was SX 1+ and SX2- and for the second, nested step SX3+ and SX4-. The primers used are considered universal for most of the known strains of IBV but the region between them varies between individual genotypes and genotype identification is therefore possible (Worthington *et al.* 2008). The process was carried out as described by Worthington *et al* (2008). The program used in the termocycler was named S-JONES.

DNA purification and sequencing preparations

The nested PCR product was amplified in order to achieve a total volume of 45 µl. The amplified products were submitted to an agarose gel electrophoresis at 100 V for four hours. The bands were cut out of the gel with a scalpel, using ultraviolet light for improved visibility, and were put in DNAse-free microcentrifuge tubes.

The DNA was purified using Illustra GFX PCR DNA and Gel Band purification Kit (GE Healthcare) according to the manufacturer's instructions. The main steps included were 1) addition of a capture buffer to the sample consisting of the amplified Nested PCR product in order for the proteins to denaturate and the agarose gel to be dissolved; 2) separation of the DNA by filtration through a membrane to which the DNA was attached 3) removement of salt and other

contaminants by a wash and dry step; and 4) addition of an elution buffer in order to release the DNA from the membrane.

Sequencing

The purified Nested PCR product was submitted to the sequencing reaction which was executed using Big Dye terminator sequencing kit. Two identical mixes were prepared with sequencing buffer and Big Dye, containing dNTPs, ddNTPs, DNA polymerase and buffer. A forward primer was added to one of the mixes and a reverse primer to the other. The mixes were distributed to two sets of tubes. The purified DNA was then added as template to both sets of tubes. The tubes were submitted to the sequencing machine ABI 3500.

Genotype Specific multiplex RT-PCR

In addition to genotype identification through DNA sequencing, another method for genotyping, Genotype Specific multiplex RT-PCR, was performed on the samples that were positive for 3'UTR Nested PCR. Primers that were used were the universal reverse primer IBVNA and the forward primers 793S, D274S and H120S, specific for the genotypes 4/91 (also called 793B), D274 and Massachusetts, respectively. The target fragments were 153 bp for 4/91-793 (nucleotide 958-1111), 217 bp for D274 (nucleotide 895-1111) and 295 bp for Massachusetts (nucleotide 817-1111). The genotypes included in the PCR were chosen based on availability of already designed oligonucleotide primers. The process was carried out as described by Cápua *et al* (Cápua *et al.* 1999). The program used in the termocycler was IBV TYPES.

ELISA

The 159 sera samples were analyzed using commercial Blocking Enzyme Linked Immunosorbent Assay (blocking ELISA) kits from Svanova. In addition, 62 of the samples were sent to the lab AVIPA (Avicultura Int. E Patol. Animal) in Campinas, São Paulo, for analysis using IDEXX kits. The samples were added to the Blocking ELISA microtiter plate which was pre-coated with antigen. Antibodies from the samples, if present, will then bind the antigen and block their binding sites. Monoclonal antibodies conjugated with Horseradish peroxidase (HRP) directed to the antigen were then added to the wells and if no antibodies were present in the samples, the binding sites on the antigen-coat will be available for the monoclonal antibodies. Upon addition of a substrate, a change of color will indicate binding of the monoclonal Horseradish peroxidase antibodies. Hence, no change in color indicates absence of antibodies in the samples and a negative result. The results were confirmed using a microplate photometer at the optical density (OD) of 450 nm.

RESULTS

Laboratory results

More than half of the samples were positive for IBV according to the 3'UTR Nested PCR. The positive samples were submitted to the Spike-gene RT-PCR but unfortunately, no results were obtained on this PCR. Because of lack of results on the Spike-gene RT-PCR, genotyping using DNA sequencing was not possible. Although it would not result in identification of genotypes, the positive samples from 3'UTR RT-PCR were submitted to the DNA sequencing reaction but no results were obtained in this process either. An additional method for identification of genotypes was used, Genotype-specific Multiplex RT-PCR, which resulted in the identification of three 4/91 genotypes among the positive samples. The rest of the positive samples were identified as not belonging to neither Massachusetts, D274 nor 4/91 genotypes. Almost all of the sera were positive for anti-IBV antibodies on the blocking ELISA analysis, both according to the Svanova kit and the IDEXX kit. The PCR results are summarized in table 3 and 4.The results are presented in more details below the tables.

Table 3. Overview of the positive and negative samples, the classification according to the Genotype Specific RT-PCR and information regarding farm, flock and breed from where the samples were derived

Flock	Farm	Breed	Trachea	Lungs	Kidneys	Reproductive tract	Enteric content
35	A	Hisex	+	-	4/91	-	+
36	A	Hisex	+	-	+	-	4/91
37	A	Hisex	-	-	-	-	+
38	B	Isa Brown	+	+	-	-	+
39	B	Decalb brown	+	-	-	-	+
40	B	Bovan	-	-	-	-	+
42	C	U	+	+	+	+	+
43	C	U	+	+	4/91	-	+
44	C	U	+	+	-	-	+
45	C	U	+	+	-	-	+
46	D	Hisex	-	+	-	-	-
47	D	Isa Brown	+	-	+	+	+
48	D	Isa Brown	-	+	+	-	-
Total			9	7	6	2	11

+ = IBV positive according to 3' UTR RT-PCR; Negative for 4/91, Mass and D274 genotypes according to Genotype specific Multiplex RT-PCR

4/ 91 = IBV positive according to 3' UTR RT-PCR; Positive for 4/91 according to Genotype specific Multiplex RT-PCR

U = unknown

Table 4. Overview of the absolute number and the proportion of positive samples, and the proportion of positive samples belonging to Non- 4/91, Non-Mass and Non-D274 "Variant" genotypes and 4/91 genotype, per organ pools

Organ Pool	Positive samples per organ pool	Proportion of positive samples per organ pool (%)	"Variant" %	4/91 %
Trachea	9 /13	69.2	100% (9/9)	-
Lungs	7 /13	53.8	100% (7/7)	-
Kidney	6 /13	46.2	66.7% (4/6)	33.3% (2/6)
Reproductive tract	2/ 13	15.4	100% (2/2)	-
Enteric content	11/ 13	84.6	90. 9 % (10/11)	0.09 % (1/11)
Total	35 / 65	54.4	91.4 % (32/35)	8.6 % (3 /35)

3' UTR Nested RT-PCR

All the 13 flocks sampled had a least one organ pool positive for IBV on the 3' UTR RT-PCR. The total frequency of IBV detection was 54.4 % (35/65). The percentage of positive samples per organ pool were 69.2 % (9/13), 53.8 % (7/13), 46.2 % (6/13) and 15.4 % (2/13) of trachea, lungs, kidneys and reproductive tract, respectively. For the pools of enteric content, only 2 out of 13 pools were negative for IBV, resulting in a percentage of positive samples of 84.6 %. The results of the 3'UTR RT-PCR for the enteric content pools is presented in figure 4.

Spike-gene Nested RT- PCR

The samples that were positive for 3'UTR were further analyzed via Nested RT-PCR targeting the Spike-gene; however, no results were obtained on this PCR. For the analysis of the organ pools of trachea, lungs, kidneys and reproductive tract even the positive control (vaccine"Ma5") failed to produce a band. However, the ladders were functional indicating that the reason for failure must have been prior to the electrophoresis step. For the analysis of the enteric content, one of the two positive controls (field strain Cobb 3/6) produced a band but none of the samples.

Sequencing, Genome analysis and Phylogenetic analysis

Due to failure of the Spike-gene RT-PCR, the positive samples for 3'UTR was instead submitted to the DNA sequencing process. However, the sequencing process was not successful and no sequences were generated. No genomic analysis or phylogenetic analyses were therefore possible.

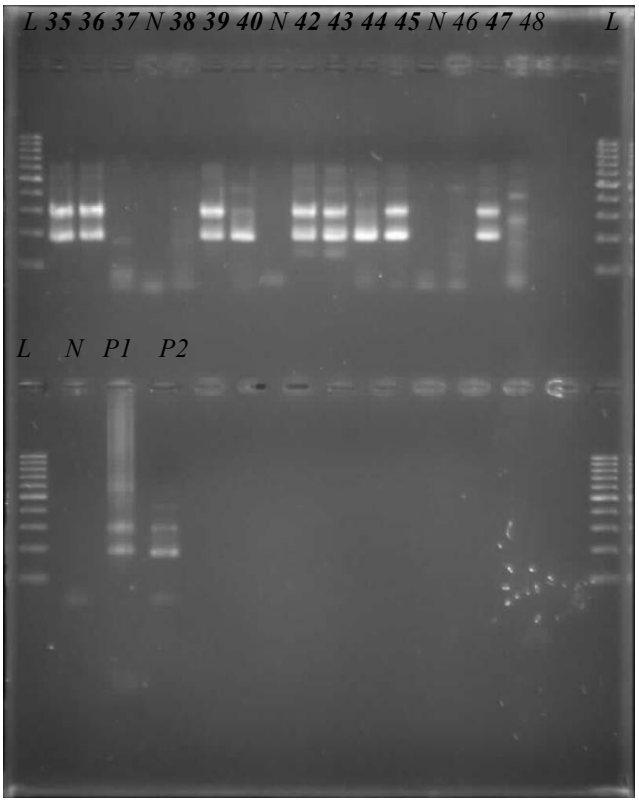

Figure 4. Electrophoresis pattern for 3' UTR Nested PCR of the 13 pools of enteric content. *11 out of 13 samples were positive generating a fragment of approximately179 basepares. 2 of the positive samples (37 and 38) were weak positive and did not generate bands when amplified before sequencing. Negative controls (DEPC-treated water) did not produce any bands. Bold figures indicate positive samples.*

L = Ladder

N = negative control

P = Positive control (P1 = Field strain "Cobb 3/6 p"; P2 = Vaccine "Ceva Big Vacina")

Genotype Specific Multiplex RT-PCR

The samples that were positive for the 3'UTR RT-PCR was submitted to the Genotype Specific Multiplex RT-PCR. Three of the samples, two kidney pool samples and one enteric content pool sample, was identified as genotype 4/91 (153 bp) because of the generation of fragments of the size of approximately 150 basepares (bp). No samples produced bands of the size of 217 bp (D274) or 295 bp (Mass H120). The samples classified as 4/91 represented 8.6 % (3/35) of the total positive samples. The remaining samples, 91.4 % (32/35) were classified as Non- 4/91, Non-Mass and Non-D274 genotypes, therefore called "Variants". The results of the Genotype Specific RT-PCR for the enteric content pools are presented in figure 5.

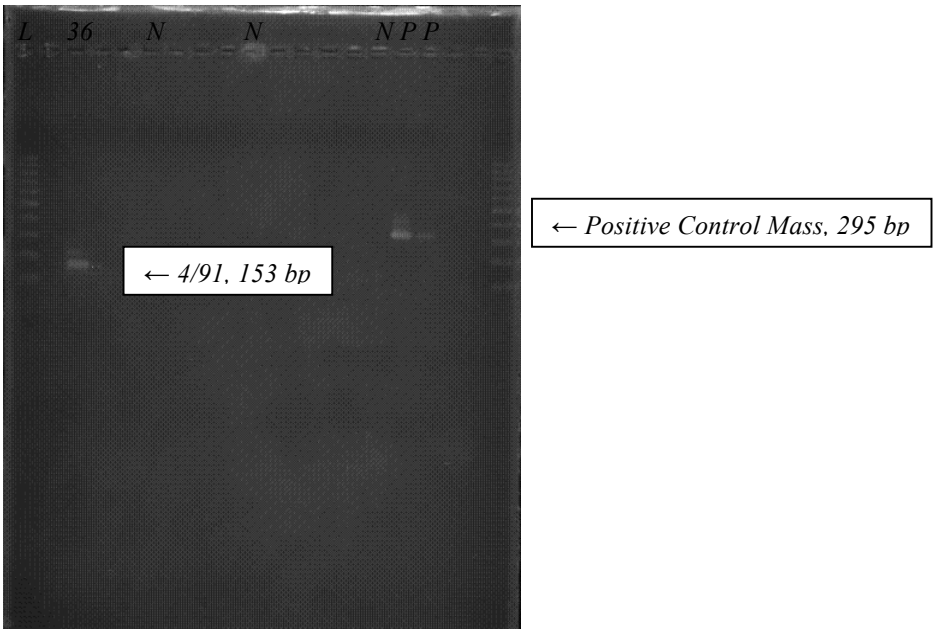

Figure 5. Electrophoresis pattern for Genotype Specific RT-PCR targeting the serotypes/genotypes 4/91 (153 bp), D274 (217 bp) and H120 Mass (295 bp) of the 13 pools of enteric content. *One sample (36) produced a band corresponding to a fragment size of approximately 150 basepares indicating the genotype 4/91. The remaining samples did not produce any bands and were therefore classified as "Variants". The two positive controls produced bands corresponding to fragments sizes right below 300 basepares, as expected for Mass genotypes (295 bp).*

L = Ladder

N = negative control

P = Positive control

Serum antibody analysis with blocking ELISA

The vast majority of the sera samples were positive for anti-IBV antibodies (155/ 159 = 97.5 % Svanova; 60/62 = 96.8 % IDEXX). Only two of the 159 samples (one from flock 35 and one from flock 42) were negative according to the results from the Svanova kit. Two samples were classified as doubtful (both from flock 42). The negative sample from flock 35 was also negative according to IDEXX. One of the doubtful samples was positive on IDEXX; the other one was not analyzed. One sample that was positive for Svanova was negative on IDEXX (from flock 36). An overview of the ELISA results is presented in table 5.

Table 5. Overview of results on ELISA analysis

Analyzing kit	Total number of samples analyzed	Number of positive samples (percentage)	Number of negative samples	Number of doubtful samples
Svanova	159	155 (97.5 %)	2	2
IDEXX	62	60 (96. 8%)	2	0

The titers obtained from the IDEXX kit analysis and the classification into titer groups made by the analyzing laboratory is presented in Appendix 1. No further conclusions were drawn from this data due to lack of information regarding age and vaccination date.

Results on Additional information and Biosecurity

Clinical history, clinical symptoms and gross pathological lesions

No thorough clinical examination was performed at time of collection of sample but obvious clinical signs such as nasal discharge, dyspnea and swollen head as well as impaired egg quality were seen in some flocks (Figure 6 and 7). Registration of presence or absence of clinical symptoms were based on history given by the accompanying person representing the farm but the information available varied greatly between the farms. Due to this, in combination with language difficulties and time issues, the total information obtained was considered insufficient and therefore, no further conclusions were drawn from these findings. The data obtained on the ages of the sampled flocks were also considered inadequate and were not presented as results.

Figure 6. Chicken with dyspnea, nasal discharge and sunken eyes. (Photo: Clara Atterby)

Figure 7. Impaired egg quality resulting in a wrinkled shell. (Photo: Clara Atterby)

Gross pathological changes observed during collection of organ samples post mortem included pathological changes in the lungs such as pneumonia, cysts in the oviducts and pathological changes in the liver such as hepatomegali, liverlipidosis and hepatosis.

Biosecurity

Animal facilities

The animal facilities comprised of two rows of cages in two to three levels under a wooden roof with tiles (figure 8 and 9). No walls were present and the ventilation was natural. Two- six birds were present per cage.

Figure 8. No walls were present at the animal facilities. The construction was made of wood. (Photo: Caroline Olofsson)

Figure 9. The animal facilities comprised of two rows of cages in two to three levels. (Photo: Caroline Olofsson)

General conditions

The design of the animal facilities was similar on all of the farms. No walls were present and no protection against wild birds existed. At several occasions, wild birds were seen sitting on the top of the roof and walking around the housed birds. Other animals such as dogs and cats were also present and they were seen both outside and inside the animal houses. A zone free of vegetation or with less vegetation next to the animal facilities was present at some farms and absent at others. The distance between animal houses varied from 6-9 meters (figure 10). In general, routines for staff included one person being responsible for a limited number of animal houses and the work was restricted to these same houses. However, some tasks like collection of eggs and delivery of feed was performed by one person, then visiting all the animal units.

Figure 10. The distance between the animal houses varied between 6-9 meters. (Photo: Caroline Olofsson)

Routines for visitors and Vaccination routines for quails

Farm A. Devices for manual disinfectant of visiting vehicles were available (figure 11). No change of clothes or shoes was required for visitors. Quails were not vaccinated against IBV.

Figure 11. At Farm A devices for manual disinfectant of vehicles were available. (Photo: Caroline Olofsson)

Farm B. Cleaning of tires was mandatory for transport vehicles. Visitors were not allowed to enter the farm if they had been at another farm the same day. No change of clothes or shoes was required. Quails were not vaccinated against IBV.

Farm C. Visiting vehicles had to pass speed bumps where the car and the tires were sprayed with disinfectant. Visitors had to register personal information such as name and phone number in a log. No change of clothes or shoes was required. Quails were not present at the farm.

Farm D. Visiting vehicles were sprayed with disinfectant when entering the farm. Visitors had to shower and change clothes if they had been to another farm the same day. Change of shoes was not mandatory. Quails were not vaccinated against IBV.

Farm E was not included in the investigation on Biosecurity due to lack of information. No person representing the farm was present at time of collection of blood samples and no information was therefore available.

DISCUSSION

IBV is one of the most important diseases in poultry when it comes to financial losses all over the world. Brazil, one of the major producers of poultry products, has significant difficulties regarding the control of the disease. Despite intensive use of vaccines, outbreaks of clinical disease occur (Villarreal *et al.,* 2007a). Constant surveillance in order to identify new strains circulating within poultry populations is crucial (Jones, 2010) and this study was performed as part of this ongoing screening for circulating genotypes in Brazil.

IBV

The frequency of IBV found in the sampled flocks of Bastos was high. The total percentage of positive samples was 54.4 % (35/65). All the flocks had a least one organ pool that was positive, indicating that IBV was present in all the sampled flocks. The vast majority of the fecal samples, 84.6 % (11/13), were positive. However, it is important to keep in mind that isolation of viral genome is solely a proof of presence of the virus and does not conclude whether or not it was actually replicating and causing disease. Nonetheless, with this said, the isolation of IBV in the kidney, a target organ for persistent infection, is more indicative of viral replication than isolation from fecal content (Brandão, Personal communication, 2011-11-03). In this study, almost half of the kidney pools were positive for IBV (6/13; 46.2 %) and it is possible that the isolated strains did represent pathogenic strains.

The initial plan to identify the genotypes of the isolated strains via DNA sequencing part of the S1-gene did not succeed due to lack of results on the Spike-gene RT-PCR. Possible reasons for failure of this RT-PCR include low sensitivity of the primers, a relatively low success rate of the PCR reaction (50%), and low virus titer in the sample (Brandão, Personal communication, 2011-11-03). The protocol used for the S-gene RT-PCR was designed by Worthington *et al* (2008) and has been used successfully in Brazil when identifying both the Brazilian genotype and the 4/91 genotype (Villarreal *et al,* 2010). Therefore, it does not seem likely that the failure was due to "old" primers in the sense that the field strains had emerged to the extent that the primers no longer would fit the strains. There are several possible measures that could have been made in order to increase the likelihood of success of the S-gene RT-PCR. One includes the addition of a bigger amount of sample material to some or all of the steps in the process: more RNA to the cDNA, more cDNA to the PCR reaction or more PCR product to the electrophoresis (Brandão, Personal communication, 2011-11-03). Another include inoculation and multiplication of the virus in embryonated SPF-eggs or TOCs (tracheal organ cultures) prior to RNA extraction in order to increase the viral titer in the initial sample material (De Wit, 2000). Furthermore, a second RT-PCR targeting a different region of the S1- gene could have been attempted.

Because no sequencing analysis could be made on the S-gene, an attempt to sequence the positive samples for 3'UTR was made. Sequencing of this region would not enable determination of genotype, as is the case of S-gene region, but information regarding whether the strain was derived from a vaccine strain or a field strain could have been obtained (Brandão, Personal communication, 2011-10-17). However, as mentioned above in the result section, the sequencing reaction failed. The most probable reason for this is insufficient amount of DNA submitted to the reaction (Brandão, Personal communication, 2011-11-03).

The multiplex Genotype Specific PCR was used as an additional method for genotyping. The genotypes included were chosen due to primer availability and although they did not represent the dominant genotypes in Brazil, Massachusetts is not uncommon and the 4/91-793B genotype has been identified in the country. No primers have been designed for the Brazilian Variant genotype to be used in a multiplex PCR. The reason for this is probably that DNA sequencing is more commonly used for genotyping and hence, no genotype-specific primers are needed.

In the present study, several interesting results were obtained from the Genotype – Specific PCR.

Three of the 34 positive samples were identified as the 4/91-793 B genotype. This genotype was first isolated in Brazil during a screening study in 2007-2008 (Villarreal *et al,* 2010) and the finding was considered an indication of the emergence of this genotype into the country. The isolation of the genotype in this study, although the number of sampled birds was limited, supports the suggestion that the frequency of the 4/91 genotype is truly increasing in Brazil.

A percentage as high as 91.4 % (31/35) of the samples were classified as "Variants" (i.e. they did not belong to any of the archetypical genotypes included in the Genotype-Specific PCR; Mass, 4/91 or D274). Without DNA sequencing, the exact genotype of these samples cannot be determined. However, considering the results of larger studies performed in Brazil the past years, the probability that they belong to the Brazilian Variant genotype is very high. Since this national genotype was first described in 2007 (Villarreal *et al,* 2007), a clear dominance of this genotype have been reported with frequencies as high as 75 % (15/20) (Villarreal *et al,* 2010) and 92 % (65/75) (Sandri *et al,* 2008). With these facts in mind, it is probable that the unspecified genotypes called "Variants" in this study did belong to the Brazilian Genotype.

Furthermore, there were indications of co-circulation of the "Variant" genotype and the 4/91 genotype within the same flocks. The positive 4/91 strains were isolated from two kidney pools and one enteric content pool. All of the flocks had other pools that were positive for IBV, but these were classified as "Variants". In flock 35 with a 4/91 positive kidney pool, the trachea and enteric content pool were IBV positive and classified as "Variants". In the other flock with a positive kidney pool, flock 42, both the pools of trachea, lungs and enteric content were positive "Variants" and, finally, the flock with the positive 4/91 enteric content pool, flock 36, had positive "Variant" trachea and kidney pools. Co-circulation of different strains has been reported before in Brazil (Sandri *et al,* 2008) and this phenomenon further underscores the diversity of the circulating strains in the country.

Finally, none of the samples were classified as Massachusetts. Since Massachusetts represents the only serotype allowed to be used in live vaccine in Brazil, it can be concluded that none of the isolated strains were derived from vaccine strains.

ELISA

Almost all of the sera samples analyzed were positive for IBV. This is either due to antibody response to vaccination or to antibody response to infection. Unfortunately, the time of vaccination in relation to the time of sampling was unknown and since both intensive immunization and high frequency of disease is present in Bastos, no further conclusions could be drawn from these results. However, a correlation between antibody titer and number of positive organ pools were observed in some flocks. For example, flock 35 and 42 had low antibody titer and several positive organ pools whereas in flock 37 and 40 that had higher titers, viral genome could only be detected in the enteric content pool.

Biosecurity

The results on Biosecurity in Bastos revealed the measurements to be poor: the animal density was very high and the distance between animal facilities within the farms as well as the distance between different farms were short. The construction of the animal houses with no walls present resulted in the possibility for wild birds to come into contact with the housed chicken. At several occasions wild birds were seen sitting on the roof top or walking around next to the facilities; domestic animals such as cats and dogs were also seen inside the animal houses.

As the farms were layer farms, animal of different ages, hence with different immune status, were present, and simultaneous vaccination of all the birds was difficult. No coordination of vaccine time and schedules existed between the farms. Coordinated programs would have been desirable since the distance between them sometimes was short enough for them to be considered the same farm from a biosecurity point of view. The occurrence of close distance farms have been referred to as ”mega farms” by Bermudez and Steward-Brown (2008). The same authors also described the tendency of concentrated poultry areas with high animal density to develop into problem areas when it comes to disease control. The lack of a synchronized strategy for immunization, treatment and prevention of exposure to disease and risk factors was discussed as a deteriorating factor (Bermudez & Steward-Brown 2008).

Three out of four of the visited farms had quails in addition to chickens. None of the farms vaccinated their quails against IBV. The facilities for quails were not placed in a restricted area separated from the chickens as would be desirable, but instead they were most often housed in facilities in the middle of the farm surrounded by houses with layers. Since current research indicates that quails may carry and develop diseases from IBV, the quails at the farms may serve as a reservoir for the virus, further aggravating the difficulties in control of the disease.

Regarding routines for staff and management, Bermudez and Steward-Brown (2008) illuminate the fact that people like owners, managers and supervisors, that frequently visit different poultry farms and farm units, may be the ones that most often jeopardize the health status and the disease control within a farm. On most of the visited farms, staff was responsible for a limited number of animal houses and did not enter the animal houses they were not responsible for. However, only one person was responsible for distribution of feed and collection of eggs, respectively, and these people therefore visited all the farm units, thus representing a possible risk of disease transmission.

A recommended routine for visitors include the presence of a separate area such as a booth, platform or fenced area from where the visitors can inspect the poultry and the procedures at the farms (Bermudez and Steward-Brown, 2008). The routines for visitors at the farms in Bastos mainly comprised of disinfectant of the vehicle. At one of the farms, visitors could not enter if they had been to other farms the same day. At another farm, a mandatory shower and change of clothes were required if other farms had been visited the same day. However, since no change of shoes was required, the net effect of the change of clothes could be questioned.

Limitations of the study

The information retrieved regarding clinical history and clinical symptoms was limited and considered insufficient in order to interconnect with the laboratory results and to draw conclusions. The limitations were due both to language difficulties and time restraint. For example, it would have been interesting to know whether or not the flocks with the positive 4/91 had shown any symptoms and if so, what symptoms they presented. As mentioned above in the strain classification section, methods for isolation of viral genome such as RT-PCR do not provide information regarding active infection, viral replication or pathogenicity of the strain. Furthermore, it would have been valuable to know the date of the last IBV vaccination in relation to time of sample collection; predominantly for the interpretation of the ELISA results. Due to the size of the study the number of farms and flocks had to be limited in order for analysis of retrieved samples to be implementable within the set time frame.

Conclusions about Biosecurity in other parts of the country can not be made since these areas were not visited. However, via personal communication, the situation for layers is considered better in other areas of the country and the biosecurity of broilers, breeders and especially parent farms are apparently much more rigorous (Villarreal, personal communication, 2011- 09-19).

Final conclusions

The poultry industry in Brazil meets a true challenge regarding the control of Infectious Bronchitis with a strikingly high poultry density, a high prevalence of IBV, a large divergence between circulating strains with known poor cross protection, and inadequate possibilities of protection via immunization. The situation is not improved by the properties of the Coronavirus with rapid

transmission, high mutation rate and a tendency for recombination which all result in the constant emerge of new serotypes and genotypes. The title of a lecture held by Brandão at a Workshop on Infectious Bronchitis 2009 summarizes the intricacy of the situation in the country:" Avian Infectious Bronchitis Virus in Brazil -A highly complex virus meets a highly susceptible host population".

Previous studies have concluded that the vaccine failure in Brazil is due to the presence of strains divergent from the Massachusetts with poor cross protection rather than poor cross protection between field Mass strains and vaccine Mass strains (Villarreal *et al*, 2008; Villarreal *et al*, 2010). Furthermore, the development and emerge of variant genotypes in Brazil has been suggested to be a result of the fact that Massachusetts live vaccines are the only ones allowed to be used (Sandri *et al,* 2008). Cavanagh (2008) underlines the importance of correlation between the vaccine schedule and the prevalence of serotypes within a given region in order to provide the birds with adequate protection. This is something that is not met in Brazil under the current circumstances and probably serves as one of the biggest obstacle for IBV control. The reason given by the Brazilian Ministry of Agriculture for only permitting live vaccines of Massachusetts type is the risk of entrance of "new" strains into the country. This despite the fact that researchers have presented several studies that have shown that strains other than Massachusetts already exist in Brazil (Villarreal 2007a; Villarreal 2007b; Sandri *et al* 2008; Villarreal 2010) and despite the fact that vaccination with more than one type of live vaccine has shown to increase the level of protection towards IBV (Cook *et al,* 1999; Ganapathy *et al,* 2009). Altogether, it seems like the poultry industry in Brazil will have a significant disadvantage in the fight towards the Infectious Bronchitis Virus as long as the vaccination restriction is present.

Regarding biosecurity, Bastos face a complex situation. An improvement of the level of biosecurity would require involvement and engagement of all the farms due to the close distance between them. The animal facilities would have to be rebuilt in order to prevent contact with wild birds. Considering the number of birds, and hence the number of animal houses, this would require large investments that may not be feasible for some of the farm owners. Furthermore, routines for visitors and staff would have to be reviewed and would require determination and conviction of the importance of biosecurity.

In conclusion, the results of this study with high IBV frequency, a predominance of non-Massachusetts genotypes and the finding of the 4/91 genotype were consistent with larger studies previously performed in Brazil. Therefore, the present results may be considered valuable in the ongoing screening for circulating IBV genotypes in the country, a screening that hopefully will end up giving Brazil the tools for outlining strategies for optimization of disease control in the future.

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APPENDIX 1.

Antibody titers obtained from IDEXX kit analysis and Classification into titer groups made by the analyzing laboratory

Flock	*Farm*	*Titer*	*Titer Group*
35	A	172	0
35	A	929	1
35	A	1401	2
35	A	2782	3
36	A	200	0
36	A	6439	7
36	A	8423	8
37	A	4964	5
37	A	6961	7
37	A	11703	9
37	A	14480	11
38	B	12330	10
38	B	15579	11
38	B	21581	14
39	B	7791	7
39	B	9698	8
39	B	14995	11
40	B	3510	4
40	B	4532	5
40	B	8204	8
42	C	307	0
42	C	416	1
42	C	438	1
42	C	675	1
42	C	2291	3
43	C	4532	5
43	C	4543	5
43	C	10316	9
43	C	10356	9
43	C	10526	9
44	C	832	1
44	C	1015	2
44	C	1060	2
44	C	2174	3
44	C	2983	3
45	C	10854	9
45	C	12770	10
45	C	15673	11
45	C	17975	12
45	C	21554	14
46	D	1750	2
46	D	6339	7
46	D	7752	7
46	D	9620	8
47	D	8877	8
47	D	9684	8
47	D	15088	11

47	D	18070	13
48	D	3655	4
48	D	4324	5
48	D	14073	11
48	D	18372	13
41	E	7369	7
41	E	9319	8
41	E	12528	10
50	E	539	1
50	E	696	1
50	E	1728	2
51	E	4398	5
51	E	14343	11
52	E	9319	8
52	E	9594	8