Infectious Bronchitis Virus and Infectious Bursal Disease Virus; a Study Performed at the Universidad Nacional of Costa Rica

by

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Abstract
Infectious Bronchitis and Infectious Bursal Disease, also called Gumboro Disease, are two important poultry diseases, causing serious illness in chickens and major production losses. In Costa Rica, as in many other developing countries, poultry production is an important industry and their products are important parts of every day food supply.
During ten weeks the author was working at the laboratory of virology at Universidad Nacional in Costa Rica. Three viral isolates of Infectious Bronchitis Virus, IBV, were characterized by Restriction Fragment Length Polymorphism (RFLP), one isolate was found to be a Massachusetts strain and the two others a new Costa Rican variant the IBV-CR-53 strain. Sera from 182 wild birds, sampled between 2000 and 2002, were investigated for presence of antibodies to IBV using a blocking ELISA. Two pigeons of the species *Zenaida asiatica* were positive and two pigeons of the species *Columba fasciata* were suspected positive. Two commercial ELISA kits were compared and the results were similar.
Seven samples from macerated bursas and cell cultures from suspected cases of Infectious Bursal Disease, IBD, were characterized using a antigen-capture ELISA and were shown to be classical strains of IBD virus. In virus neutralization test using chicken sera sampled at three, ten and 18 days of age it was shown that the neutralizing antibody titers were significantly lower against a wild type strain than against a vaccine strain and that the half-life of antibodies was 2.5 days rendering the chickens with little protection at 18 days of age.
A Costa Rican chicken production plant was visited in order to view typical premises. The pens had open net walls and thus some problems with biosecurity. Blood samples sent to the laboratory for routine antibody control showed that in spite of vaccine strategies, protection against IBV was low at 44 days of age.

Sammanfattning
Infektiös Bronkit och Infektiös Bursit, eller Gumborosjuka, är två mycket betydelsefulla fjäderfäsjukdomar. De orsakar allvarliga sjukdomsutbrott och ekonomiska förluster. I Costa Rica är fjäderfäproduktion en viktig industri som inte bara ger arbetstillfällen och uppehälle utan även mat till befolkningen.
Under tio veckor hade författaren möjlighet att arbeta på virologilaboratoriet vid Universidad Nacional i Costa Rica för att studera dessa virus närmare. Tre isolat av IBV karaktäriserades genom RFLP-teknik. En befanns vara en Massachusetts stam och de andra två en ny Costa Ricansk variant med benämningen IBV-CR53. Dessutom jämfördes två kommerciella ELISA-kit; en blocking och en indirekt ELISA. De båda kiten befanns vara jämförbara på flocknivå. Serum från 182 vilda fåglar som provtagits år 2000 och framåt testades för prevalens av antikroppar mot IBV. Två duvor av arten *Zenaida asiatica* visade sig vara positiva och två *Columba fasciata* misstänkt positiva.
Sju prover från misstänkta Gumborofall undersöktes med en DAS-ELISA och alla var klassiska stammar. Genom en virusneutralisations test jämfördes serum från kycklingar provtagna vid tre, tio och 18 dagars ålder för att se hur det maternella antikroppsskyddet minskade. Den neutraliserande effekten jämfördes mellan en vaccinstam och en virusstam som isolerats vid ett sjukdomsutbrott och visade sig vara signifikant lägre mot det senare viruset. Halveringstiden för antikropparna beräknades till 2.5 dagar och titrarna var mycket låga vid 18 dagars ålder.
En Costa Ricansk fjäderfäanläggning besöktes för att studera typiska förhållanden. Byggnaderna som hönsen hölls i var nättäckta vilket förklarar en del av problemen som finns med att upprätthålla adekvat smittskydd. Blodprov som inskickats för antikroppsanalys visade dessutom att kycklingarna vid 44 dagars ålder hade mycket låga halter antikroppar mot IBV.

Resumen
La Bronquitis Infecciosa y la enfermedad de Gumboro son dos de las enfermedades mas importantes en pollos y gallinas alrededor del mundo. Ambas causan brotes serios de enfermedad que conllevan a grandes pérdidas económicas. En Costa Rica la industria avícola es muy importante porque genera un gran oferta de trabajo y es fuente de proteína de origen animal para la población.
Durante diez semanas la autora tuvo la oportunidad de estudiar estos virus en el laboratorio de Virología de la Escuela de Medicina Veterinaria en Universidad Nacional de Costa Rica. Tres aislamientos del Virus de la Bronquitis Infecciosa (IBV) fueron caracterizados con las técnicas de RT-PCR y RFLP. Uno de ellos resultó ser una cepa del serotipo Massachussetts y las otros cepas variantes nuevas denominadas VBIA-CR-53. Además, se comparó dos kits comerciales de ELISA para el diagnóstico de anticuerpos contra IBV: un ELISA competitivo y un ELISA indirecto. A nivel de grupos, aplicados a parvadas comerciales, ambos kits resultan equiparables.
Conjuntamente se realizó otro estudio serológico, pero esta vez en aves silvestres. Sueros de 182 aves silvestres, colectados desde el año 2000, fueron analizados con el kit de ELISA competitivo para detectar anticuerpos contra el IBV. Dos palomas, especie *Zenaida asciatica* resultaron positivas y dos palomas, especie *Columba fasciata* resultaron sospechosas.
En lo referente ala enfermedad de Gumboro, siete muestras de tejido bursal y dos aislamientos virales costarricenses obtenidos en cultivo celular (Línea BGM-70) fueron caracterizados con un ELISA de captura antígeno (Synbiotics), resultando todos cepas clásicas. Adicionalmente, con el propósito de evaluar la duración de la inmunidad materna, se analizó mediante la técnica de neutralización viral y empleando dos antígenos diferentes (Cepa vacunal Bursine-2 y una cepa variante aislada de tejido bursal de pollos de Costa Rica) sueros provenientes de tres grupos de pollos, sangrados a los días 3, 10 y 18 de edad. Se encontró que los títulos contra la cepa de campo resultaron estadísticamente inferiores a los obtenidos contra la cepa vacunal.
Finalmente, se realizó una visita a una planta de pollos en Costa Rica, en la cual se constató algunos problemas con la bioseguridad en los edificios tipicos que tienen paredes de tela metálica.

Contents

Abbreviations

AEC	Amino ethylcarbazole
CAV	Chicken Anemia Virus
CPE	Cytopathogen effect
D/kD	Dalton/ kilodalton
DNA	Deoxy-ribonucleic acid
E. coli	Escherichia coli
ELISA	Enzyme linked immunosorbent assay
FAE	Follicle associated epithelium
IBD/IBDV	Infectious bursal disease/infectious bursal disease virus
IB/IBV	Infectious bronchitis/infectious bronchitis virus
ND/NDV	Newcastle Disease/ Newcastle Disease Virus
OIE	Office International de Epizooties
PBS	Phosphate buffered saline solution
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RT	Reverse transcriptase
$TCID_{50}$	Tissue culture infective doses in 50% of the cultures
VNT	Virus neutralization test
VP	Viral protein
VvIBD	Very virulent infectious bursal disease

Background

Introduction and purpose

The purpose of this study was to assist the laboratory of virology at Universidad Nacional with their project on Infectious Bronchitis Virus, IBV, and Infectious Bursal Disease Virus, IBDV, and to do a literature study of the pathogenesis and basic virology of these viral diseases. IBV and IBDV have a major impact on the avicultural production in Costa Rica as in other parts of the world.

Even though ten weeks in Costa Rica is a short time to be able to draw any major conclusions it was possible to screen some of the wild bird population and to characterize some samples of both IBV and IBDV. Although the plan was to characterize all isolates of IBV and IBDV, this was not possible due to problems with the PCR enzymes. Financially Astra Zeneca and the August Carlsson foundation of the Veterinary Faculty of the Swedish University of agricultural studies, SLU, supported this project. SVANOVA biotech AB also contributed with training in ELISA and ELISA kits.

Costa Rica is a small country, about two-thirds the size of Scotland, situated between Nicaragua and Panama. It is a country famous for its biodiversity and has, for example, over 850 different species of wild birds. Today 11% of the land is protected national parks. The country is inhabited by almost 4 million Costa Ricans and is visited by an increasing amount of tourists (Rachowiecki, 2002).

Poultry is an important part of the Costa Rican diet, which can be reflected in the vast amount of food chains that exclusively serve chicken. In Costa Rica there is not only the “Kentucky Fried Chicken”, but also the big fast food chain “Rosti Pollo” and numerous small chains like “Tico pollo” and “Pollo a la leña”.

Poultry production - world wide and in Costa Rica

The production and consumption of eggs and poultry is increasing worldwide; over the last quarter of a century the consumption of eggs has doubled and chicken meat has tripled (Jordan & Pattison, 1996). In 1999, 54.9 million tons of poultry meat was produced in the world, compared to 39.4 million in 1993. The consumption is highest in North America where they consumed 37.8 kg/person/year of meat and 12 kg/person/year of eggs in 1996. In Europe 13.9 and 10.8 kg of meat and eggs respectively were consumed per person that year. In Central America twice this amount (27.3kg) of meat was consumed and half the amount of eggs (5.0 kg). Generally, there is a higher consumption of eggs in developed countries (Jordan & Pattison, 1996).

In developing countries, especially in Asia and Latin America where it is common for people to keep some birds, poultry still is an important source of bringing cheap and simple food to the families. In contrast to cattle and swine,

poultry is not a large investment and does not require much attention and space, but gives regular protein and vitamin in the form of eggs to the family diet. The birds can also be slaughtered at regular intervals. In large scale producing units, poultry production is a highly effective way of changing cheap grains into eggs or meat for the market.

In Costa Rica some large production units have developed but household breeding is still common. In many remote parts of the country, it is common to keep some poultry for the household, often consisting of both chickens and ducks or turkeys. Even in the cities it is still possible to see hens on the streets and hear roosters crow in the mornings. According to the Avicultural Union of Costa Rica, there are approximately 8 000 people working directly and a total of 70 000 indirectly in the poultry production. It is estimated that 12 000 families are dependent on aviculture for their sustenance (Echeverría, 2003).

Poultry diseases

With increasing industrialization and intensification of rearing systems the disease pattern in fowl are changing. There are increasing problems with nutritional and genetic diseases due to higher demands on growing and food conversion capacities. Outbreaks of infectious diseases are often due to the increased stress the animals are subject to and the high infectious pressure of thousands of animals in a confined space. In developing countries the infectious diseases still have a predominant role. According to Sainsbury (1992), tropical countries have more problems with infectious diseases due to the climatic circumstances. It is harder to keep food and water free from bacteria and other pathogens, even for human consumption. Also it is difficult to keep buildings free from insects and other animals, especially considering the increased demands on ventilation.

Two of the most important diseases in poultry are Infectious Bronchitis, IB, and Infectious Bursal Disease, IBD, also called Gumboro Disease (Murphy et al., 1999). The latter disease has not only an importance in causing illness, death and reduced growth in the chickens, but also in inducing a severe immunosuppression that renders the animals susceptible for other pathogens and unable to produce protective antibodies after vaccination (Jordan &Pattison, 1996; De Wit, 2001).

The total cost of IBDV infections is difficult to calculate since it depends on multiple factors such as the breed and the age of the chicken, the strain of virus, previous protection from vaccination, natural infection or maternal antibodies, the level of immunosuppression caused and secondary infections. The costs not only include the losses of dead and diseased animals, but also the costs of extra labour, veterinarians, medications and disinfection strategies. There is no doubt, however, that the disease causes serious economical losses. For example it is estimated that the money saved after having introduced vaccination in 1988-89 in the USA was 400 million US dollars in 1990 and 580 million in 1998 (De Wit, 2001). In the United Kingdom the cost of Gumboro in 1994 was estimated to be 15 million GBP (Jordan & Pattison, 1996).

Although Infectious Bronchitis is a long known disease it is still not possible to control it (Pei, Briles & Collison, 2003). In spite of the use of vaccines, the virus still breaks through protection and causes outbreaks (Niesters, 1987; Rosenberger, 1998).

Infectious Bronchitis was recently actualised in a worldwide perspective as it was suggested a possible origin of Severe Acute Respiratory Syndrome, SARS, in humans, although later examinations of the genome showed this to be highly unlikely. However coronaviruses have a high capability to mutate (Niesters, 1987) and are important disease agents in many species worldwide.

Differences in anatomy and physiology between birds and mammals

Birds differ from mammals on both their anatomy and their physiology and it is important to understand these differences. Infectious Bronchitis Virus (IBV) has its main implications on the respiratory system and Infectious Bursal Disease Virus (IBDV) infects an organ that does not even exist in mammals and the latter disease has severe effects on the immunesystem of the bird. A brief description of these features will be given here in order to increase understanding when reading about the diseases.

Bursa Fabricii and other lymphatic organs

One very noticeable difference between mammals and birds is the presence of the Bursa Fabricii in the avians. The Bursa Fabricii gave name to the B-lymphocytes since it was shown that they differentiated in that organ, comparable with the thymus the organ in which T-lymphocytes differentiate. In mammals it is suggested that this organ would be replaced in function by either the bone marrow or the gut associated lymphatic tissues (Gerschwin, Krakowa & Olsen, 1995). The bursa is a diverticulum of the dorsal cloacae and is connected to this through a short duct. The bursa reaches its maximum size when the chicken is between five and eleven weeks old. At that time the organ is about the size of a cherry, located mostly within the pelvic cavity and contains approximately 100 000 follicles. Successively the bursa begins to involute, starting with the follicular cortex, and the bone marrow adapts the function of the bursa. By one year of age the bursa has reduced to the size of a pea (Heider & Monreal, 1992).

The bursa contains longitudinal primary and secondary folds (Heider & Monreal, 1992). The surface of the folds is covered with epithelium. About 90% of the epithelium is columnar and the rest is a polygonal epithelium with pinocytic capacity (Ridell, 1996). This modified epithelium is found immediately above the follicles and is referred to as Follicle Associated Epithelium, FAE (Farner, King & Parkes, 1983). It has an important function in bringing antigens to the bursal follicles from the lumen of the bursa. The bursa also uses continuous movements to

suck material in from the cloacae and thus increases the amount of antigens (Heider & Monreal, 1992).

Normally other cells of the immunosystem also are encountered in the bursa. It is constantly populated by a number of macrophages, dendritic cells and T-lymphocytes. Close to the duct there is an especially large accumulation of T-cells (Ridell, 1996).

A tripeptid has been identified that is responsible for the B-cell differentiation in both mammals and birds. This is referred to as bursin or bursopoietin and is supposed to be secreted from cells close to the FAE. This protein is similar to the thymosin, which induce differentiation in the thymus). The importance of the bursa in chicken has been shown by surgical or testosterone-induced bursectomy. Birds, bursectomized before day 17 of incubation, were incapable of producing immunoglobulin postnatally. B-lymphocytes bind more testosterone than T-cells and the bursa is thus more sensitive to increased plasma levels (Farner, King & Parkes, 1983).

Birds also have a thymus with prominent cortex and marrow and as in mammals Hassall bodies can be seen. It reaches its maximal size about three or four months after hatching. The spleen does not have much blood storage capacity but is more important in phagocytizing old erythrocytes, which in birds contain a nucleus, and cleaning away circulating antigen-antibody complexes. A certain lymphocytopoeises also takes place. Phylogenetically lymph nodes started developing in birds although there are none in certain species as for example chickens. Birds also have fewer and thinner lymphatic vessels than mammals and as embryos they have lymphatic hearts pumping the lymph. In some species such as geese and ducks these hearts persists even after birth (Heider & Monreal, 1992).

Chickens have lymphoid accumulations in the walls of both blood and lymphatic vessels, called mural lymphatic nodules. These are developed at four weeks after hatching. Birds have the same lymphoid aggregation along the Ileum, Peyers patches, as mammals, approximately seven in the chicken. In the walls of the entrances to the paired ceacums are aggregations known as caecal tonsils. Similar structures can be found by the bifurcation of the trachea. Lymphocytic accumulations in the ventromedial orbita form the gland of Hardersch (Heider & Monreal, 1992).

Respiratory system

The nasal cavity is located within the upper part of the beak and there is one paranasal cavity, the infraorbital sinus. The choanes are wide and allows communication with the oral cavity. The larynx lacks epiglottis and thyroid cartilages as well as vocal cords. The tracheal rings are closed dorsally and ossificate with advancing age. In the caudal part of the trachea, which is dilated, is the syrinx, the vocalization organ of birds (Krahmer & Schröder, 1976).

The lungs are comparably small and located at the dorsal aspect of the thorax with the main bronchi entering ventrally. Since the bird only has one body cavity there is no pleura and no diaphragm. In spite of their small size the lungs double their capacity by using the air sacs. These are mucus structures permitting the air to pass twice through the lungs. There are a total of nine sacs; one cervical pair, one abdominal pair, two thoracic pairs (cranial and caudal), and one single interclavicular. They are situated between the organs and the body wall and also invaginate into some of the skeletal bones (Krahmer & Schröder, 1976).

Infectious Bronchitis Virus (IBV)

History

The first case of IB was diagnosed in 1931 in the USA. At that time it was a disease affecting chickens but in the 1940s it was already causing significant losses within the laying industry and in the 1960s the first cases of nephropathic syndrome was observed. The virus was isolated in 1936 and in 1956 the first report of multiple serotypes was published. The first commercial vaccine appeared in the 1950s (Charlton, 1996). There has been intense research concerning the disease and its prophylaxis since the discovery, but it is still one of the most important poultry diseases in the world with a virus constantly changing to new serotypes and strains requiring the development of new empirical vaccines.

At least since the 1970s the disease has been prevalent in Sweden (Engström et al., 2003) with major outbreaks in the 1990s with vaccination commencing in 1997 (Farsang et al., 2002). Today it is to be reported to the Swedish authorities upon diagnosis.

The virion

The Infectious Bronchitis Virus belongs to the family *Nidoviridae*, genus *coronavirus* (Murphy et al., 1999). IBV is considered the prototype of its genus (Mc Ferran & Mc Nulty, 1993). Coronaviruses are today divided in three groups. Infectious Bronchitis is the only member of group III (Murphy et al., 1999). This genus contains multiple viruses causing disease in almost every species. Examples of important diseases caused by coronaviruses are Feline Infectious Peritonitis (FIP), in cats, Severe Acute Respiratory Syndrome (SARS), in humans, Porcine Transmissible Gastroenteritis (TGE) in porcines and Winter-dysenteries in Cattle. Coronaviruses often are part of respiratory and gastrointestinal diseases of especially young animals and children. The viruses have an affinity for respiratory or gastrointestinal epithelium and a tendency to cause rather mild symptoms in adults and more severe symptoms in young individuals (Murphy et al., 1999).

Coronaviruses are enveloped viruses containing a single stranded positive RNA genome. By Electron microscopy lollipop-shaped projections sticking out from the surface like a crown are shown, hence the name coronavirus. Coronaviruses are more or less spherical and have a diameter between 60 and 220 nm. The genome consists of only one strand and is the largest of all groups of RNA-viruses. The

virus replicates in the cytoplasm and the RNA works directly as a messenger RNA for the RNA-polymerase, after which a negative sensed RNA is formed. The RNA-polymerase then transcribes both the whole positive sensed RNA and a nested set of subgenomic RNAs (Murphy et al., 1999).

IBV has a genome of 27,6 kbp and encodes for the structural proteins: The nucleocapsid protein, N, with a weight of 45 kD, the membrane or matrix protein, M, with a core weighing 23 kD and having aspergine-linked oligosaccarids embedded in and projecting from the lipid bilayer of the membrane. Another smaller transmembrane protein, E, is important since it assists the M-protein with virus assembly (Murphy et al., 1999). The spikeprotein, S, is a peplomere consisting of a multiple of two or three copies of the subunits S1 and S2, which are glycoproteins. S2 is of a very conserved structure found in coronaviruses of different species. It is bound to the membrane, whereas the S1 is non-covalently bound to S2. The spikeprotein binds the cell receptors and causes fusion and is responsible for inducing neutralizing antibodies. The more variable S1 part is most important for this induction (Niesters, 1987). It has also been demonstrated (Casais, 2003) that the S protein has an importance for cell tropism of the coronavirus. In some coronaviruses the spikeprotein has hemagglutinating capacities but the IBV lacks these (Charlton, 1996). The virus is assembled and buds into the endoplasmatic reticulum and subsequently is released through exocytosis. After exocytosis many virions remain attached to the cellular membrane (Murphy et al., 1999).

Coronaviruses with genetic resemblance to the IBV have been found in other species. In pheasants, coronaviruses were the infectious agents most commonly found (Welchman, 2002) and genetically coronaviruses in pheasants are very close to IBV (Lin, Loa & Wo, 2002). The differences between the coronaviruses of the respective species are equal to the differences observed when serotypes within IBV are compared (Cavanagh, 2002). IBV has been isolated from competition pigeons and is thought to be able to cause disease (McFerran & McNulty, 1993; Murphy et al., 1999).

Clinical and pathological features

IBV is the avian virus spreading most rapidly by air within a flock with usually 100% morbidity (Engström et al., 2003). The incubation period can be as short as 24 to 72 hours (Rosenberger, 1998; Engström et al., 2003) and the disease usually lasts less than two weeks if no secondary infection occurs. Mortality is below 30% if the animals are not secondarily infected. Young animals are more seriously affected (Jordan & Pattison, 1996; Murphy et al., 1999). Trachea is the primary target of the virus, but some strains have not only predilection for respiratory epithelium, but also for epithelium in the kidneys, intestines and oviducts (Niesters, 1987; Fenner et al., 1993). After a primary replication in the respiratory epithelium, viremia occurs and the virus is spread (McFerran & McNulty, 1993)

The symptoms can be divided into respiratory, reproductory or subclinical. When the disease is mainly respiratory the chickens show depression, have reduced

weight gain, a swollen face, rales, cough, dyspnea, gasping, sneezing and a watery nasal and ocular discharge (Murphy et al., 1999; McFerran & McNulty 1993). After five weeks of age, the disease is not so serious and often passes suclinical if secondary infection does not occur (McFerran & McNulty 1993). Secondary infections often give rise to purulent airsacculitis with thickened and opaque air sac membranes (Jordan & Pattison, 1996; Charlton, 1996). At necropsy there is mucosal thickening and catarrhal exudate in the respiratory tract. There might be pneumonia, sinusitis, conjunctivitis (Murphy et al., 1999) and pulmonary stasis and oedema (Fenner et al., 1993).

There are two distinct syndromes of reproductive disease (Jordan & Pattison, 1996). If the bird is infected during the first days of life the development of the oviduct may be incomplete. The malformations are most often localized in the middle third of the oviduct and can also be caused by severe reactions to live vaccines. Some of these birds will be “blind” or “internal” layers; at the time when they should start laying they will behave correctly but the eggs will be deposited in the body cavity because of the deformed oviduct. If a mature bird gets infected during laying, the oviduct will be damaged and the production and quality of the eggs impaired. It takes about 4-6 weeks for the bird to recover but when it starts producing eggs again they are often malformed and the bird will not resume its peak production. Misshaped eggs can be small, lack shell or have a bad quality shell or albumen (Jordan &Pattison, 1996). Secondary complications to the reproductory forms are often in the form of egg peritonitis. It is common to see yolk in the abdomen at autopsy of infected birds but this can often be observed in laying birds that are stressed or have other diseases, and it is not a specific finding (Charlton, 1996). Histologically, degeneration of the epithelium and infiltration of lymphocytes can be seen in the oviduct according to Rosenberger (1998) but Engström et al., (2003) reports no pathological changes in the oviduct after infection. In cases of developmental deformities hypoplasia and cystic lesions might be seen in the oviduct (Charlton, 1996). If the oviduct is infected, virus can be present in eggs (McFerran & McNulty, 1993) but the disease is not transmitted vertically (Engström et al., 2003).

In some cases birds can be persistently infected and the virus can stay latent for several months. Usually virus shedding resumes at the time of laying or at some other stressful event (Jordan & Pattison, 1996). IB can also result in a nephropathic syndrome in which the kidneys are swollen with accumulations of urates and distended tubuli (Rosenberger, 1998; Jordan & Pattison, 1996). If virusinfection persists in spite of high antibody titers, immunocomplexes could cause nephritis (Fenner et al., 1993).

IBV has been a suggested agent in the etiology of the Swollen Head Syndrome, a disease with low morbidity but high mortality affecting mostly female broilers. The birds show a mild tracheitis, peritonitis centered on the ovaries, opisthotonus, incoordination, dilated pupils and subcutaneous edema around their head. Other pathogens involved might be *Escherichia coli*, paramyxoviruses, pneumoviruses and combinations of these. High levels of ammoniac gases and dust might also be

important (Jordan & Pattison, 1996). Infectious Bronchitis is often associated with colibacillosis, and subsequent increased mortality. It has been shown that both vaccine and virulent strains cause an increased susceptibility for *E.coli* infections (Matthijs et al., 2003).

Different strains of IBV cause different syndromes. Using RFLP these strains have been characterized and are often named from the location where they were isolated. "Massachusetts" is probably the original prototype and is pathogenic for both respiratory and reproductory tracts but not for the kidneys, the same with "Arkansas", whereas "Holland" causes disease in all three systems. "Connecticut" causes only mild respiratory disease and "T" causes a syndrome with highly mortal nephritis, mostly seen in Australia. Some strains like "Beaudette" are laboratory mutants (McFerran & McNulty, 1993). New variants are continuously being identified.

Prophylaxis

The coronaviruses have a large RNA genome, which constantly undergoes mutations giving new serotypes. This is the main problem for the development of protective vaccines (Niesters, 1987; Rosenberger, 1998).

There are two types of vaccines at present; live attenuated and inactivated, “dead”, vaccines. The live vaccines are usually administered in the forms of sprays or aerosols. Aerosols have particles less than 5 µm in diameter and can penetrate down to the smaller structures in the lungs. This requires sterile water for administration but there is a risk that ubiquitous pathogens like *E.coli* also enter. This, in combination with the inflammation produced by the live vaccine in the bronchioles, can cause severe bronchitis with the formation of obstructive caseous mucus plugs that can cause dyspnea and death of the chickens (Ridell, 1996; Jordan & Pattison 1996). Sprays with a particle size of more than 10 µm are considered a safer alternative. Both sprays and aerosols have the disadvantage that it is hard to know if all birds received their dose. The administration of the vaccine as ocular or nasal droplets is a more secure way for ensuring that every chicken will receive their dose but it is a highly time consuming procedure. These locally applied vaccines give a good protection of the upper respiratory tract, mainly mediated through IgA antibodies, even though the number of circulating antibodies may be low (Niesters, 1987). Vaccination by drinking water is time efficient but is sensitive to water pollution and it requires a rapid consumption of water (Jordan & Pattison 1996).

For an inactivated vaccine to be effective it has to be proceeded by a vaccination with live vaccines at least eight weeks before the injection of the inactivated vaccine (Jordan & Pattison, 1996). This is given to breeders in order to have a sufficient maternal protection and is usually administered some weeks before onset of lay. The chickens are hatched with the same level of circulating IgG as their mother (Murphy et al., 1999). Injectable vaccines are often produced as a combination between two or more viruses. Polyvalent vaccines containing IBV and

New Castle Disease viruses, NDV, have been shown to be less effective, since the IBV component interferes with the replication of NDV (Gelb, 2003).

The importance of maternal protection against IBV has been shown in a study by De Herdt et al. (2001), who found that economic losses of IB often are associated with flocks that had low maternal protection at the time of hatching. Studies of the antibody pattern show that it is common with field infections breaking through the vaccinial protection (De Herdt et al., 2001).

There is always a risk with attenuated live vaccines since they can regain their virulence and the risk is even greater with viruses, like coronaviruses, that have high frequencies of mutations. In Sweden it was shown that a vaccine strain has been involved in multiple outbreaks at the time of vaccination (Farsang et al., 2002).

The new advances in biotechnology have made it possible to construct IBV vaccines consisting of DNA and this vaccine showed to be protective if either injected in ovo followed by live vaccination at 14 days of age or if injected intramuscularly at 1 and 14 days of age (Kapcynski et al., 2003).

The complexity of the immune response against IBV is the importance of both humoral and cellular immunity (Niesters, 1987). It has been shown that memory T-cells from chickens infected several weeks earlier could be given to other chickens before infection and yield protection (Pei, Briles & Collisson, 2003) and it is possible that new techniques like DNA-vaccines stimulate these defense mechanisms more than the conventional ones. Other important means of protection against viruses are hygiene and disinfection. IBV is enveloped and thus sensitive to many disinfectants and drought.

Differential diagnosis

Respiratory diseases are a common problem in the poultry production. Two serious diseases that need differentiation from IBV are the Avian Influenza (an orthomyxovirus) and Newcastle Disease (an avulovirus). These viruses cause high mortality and morbidity and symptoms are usually more severe than IB. Newcastle outbreaks can also be distinguished by the presence of signs from the Central Nervous System (Rosenberger, 1998).

Infectious laryngotracheitis is a disease causing similar symptoms to IB but the former is caused by a herpesvirus, which spreads more slowly. Avian Pneumovirus not only causes respiratory symptoms in chickens but also rhinotracheitis in turkeys. It has been associated with development of Swollen Head Syndrome. (Jones, 2003)

Some primary bacterial diseases like *Mycoplasma* and *Ornithobacterium rhinotracheale* are possible differentials. Other bacteria are capable of infecting the respiratory tract once it has been damaged primarily by a virus and can complicate the diagnosis (Engström et al., 2003).

Infectious Bursal Disease Virus (IBDV)

History

The disease was discovered in 1962 in Gumboro, Delaware, USA, but had been present since 1957. Chickens between two and three weeks of age were affected and showed signs of disease and a transient immunosuppression. The first vaccine was made from bursas of diseased animals and was given to chickens in the drinking water at two weeks of age. In the 1980s the disease changed character. If the birds were infected within the first week of life the bursa would atrophy and a subclinical immunosuppression develop, leading to increasing diseases of the skin, gastrointestinal and respiratory systems (Giambrone, 2001).

Later a new strain appeared, causing a more rapid bursal atrophy, immunosuppression, diarrhoea and weight loss. Even vaccinated birds were affected and the natural immunity declined faster than after infection with the old type of virus. The mortality in Leghorns reached up to 90% and the morbidity almost 100%. In the beginning of the new millennium more variants were discovered causing different syndromes like proventriculitis (Giambrone, 2001).

Three different syndromes are referred to as “classical”, “very virulent” (vvIBD), and “variant” infectious bursal disease respectively (Giambrone, 2001). According to de Rosa (2003), the OIE states that over 95% of member states have reported IBD and 80% have had cases of the very virulent strains. In some important poultry producing countries like the USA, Australia and New Zealand, no cases of vvIBD have been reported (de Rosa, personal communication). However Murphy et al. (1999) reports that the vvIBD strain started developing in the USA. The frequency of variant diseases is unknown since they are difficult to detect (de Rosa, 2003). IBDV was diagnosed in Sweden 1978 and in 2000 the first cases of vvIBD were reported (Engström et al., 2003). Today confirmed cases of vvIBD have to be reported to the Swedish authority.

The virion

IBDV is a bisegmented RNA virus. It belongs to the family *Birnaviridae*, genus *avibirnavirus*. In this family other members cause disease in fish and insects. Infectious Pancreatic Necrosis of fishes is the most well known disease and is considered the prototype of the whole family. The virus lacks an envelope and is thus resistant to many disinfectants and heat treatment. The capsid consists of 32 capsomeres (McFerran& McNulty, 1993) and there are no projections, glycoproteins or lipids on the surface (Murphy et al., 1999). The icosahedral virus is about 60 nm in diameter (Murphy et al., 1999). The virus persists many months in the environment and has been shown to persist for months in insects and worms. The virus can thus be spread mechanically over long distances and a farm will have

to be considered contagious for a long time after the outbreak of the disease (McFerran & McNulty, 1993).

Virus replication occurs in the cytoplasm and does not affect the cells own synthesis of RNA and proteins (Murphy et al., 1999). The two double stranded DNA-segments, A and B, code for different viral proteins (VP). In segment A (3 261 nucleotides) the gene VP2 encodes for the protein of the outer capsid with antigenic properties, VP3 encodes the inner layer protein and VP4 and VP5 both non-structural proteins. Except from VP5, all the other proteins are cleaved from one precursor protein (Brandt et al., 2001). Segment B (2827 nucleotides) contains only one gene, VP1, which codes for the virus polymerase of 97kD (Eterradossi, 2001). Genetically there is a clear difference between the very virulent strain and other strains regarding the gene VP1 (Islam, Zierenberg & Muller2001). Probably a mutation of one amino acid in a hypervariable locus in the VP2, caused a complete change of epitope and gave rise to some of the new variants arising from the USA (Giambrone, 2001). Antigenic determinants are in the VP2 protein (Fenner et al., 1993).

The non-structural protein VP5 has been shown to be important since it causes lysis of the host cells and release of the virus. Expression of VP5 in a cell is cytotoxic: The protein accumulates in the plasma membrane and causes cell deformation prior to cell lysis (Lombardo et al., 2000). In a study by Brandt et al. (2001) it was shown that markers for virulence and phenotype are found in the VP2 gene and that the VP1 gene has some influence on the cell tropism. VP4 is the protein responsible for cleaving the large precursor protein (Brandt et al., 2001).

There are two serotypes of the virus, which are both capable of infecting chicken as well as some other species of domestic poultry like turkey and ducks. However, only serotype 1 is known to cause disease and only in chickens. Serotype 1 has a rather limited range of host cells and preferably replicates in B-lymphocytes whereas serotype 2 is less specific (Engström et al., 2003). These two serotypes show minimal crossimmunity (Murphy et al., 1999). The presence of virus or antibodies in wild birds has not yet been studied (Eterradossi, 2001). Using molecular techniques, serotype 1 has been divided into six distinct groups. There are however some strains that do not belong to any of these groups (de Rosa, personal communication).

Clinical and pathological features

IBD is a disease only affecting chickens, and mainly those around 3-4 weeks old when the bursa is well developed. In younger birds the disease usually has a subclinic course. Diseased chickens are pale, depressed, dehydrated, anorectic, shivering, have an insecure, atactic pace and ruffled feathers (Murphy et al., 1999). They often develop transient diarrhoea that can be white with red or green staining. Often there is self-inflicted vent picking (Charlton, 1996).

If the chickens survive they recover rapidly within three, four days without noticeable damages, but even in mild or subclinical cases immunosuppression

occurs (Murphy et al., 1999). When the infection occurs earlier in life the immunosuppression probably will be permanent. In other cases, it usually is transient. Some birds may also show growth retardation after recovery (Sainsbury, 1992). No disease can occur after 15-16 weeks of age when the bursa is regressed (Engström et al., 2003) and chickens over six weeks of age seldom are sick but seroconvert. This age dependent disease is caused by the virus exclusive infection of only B-lymphocytes during differentiation (Murphy et al., 1999). The virus spreads mainly by faeces and the incubation period is 2-3 days (McFerran & McNulty, 1993).

The morbidity can be almost 100% and mortality 90% in Leghorns affected with vvIBD. Susceptibility of the birds varies with breed and the Leghorns are the most sensitive (Giambrone, 2001; Jordan &Pattison, 1996). In outbreaks with classical strains mortality is usually 20-30% (Murphy et al., 1999).

Within three to four days post infection there is an inflammatory hypertrophy of the bursa and apoptosis of B-lymphocytes. In disease caused by some North American subtypes this development is more rapid but the atrophy is not inflammatory (Eterradossi, 2001). The less virulent strains can also give histological changes in other lymphoid organs such as the thymus, spleen, bone marrow and ceacal tonsils. The extent of the thymic lesions varies with different strains of virus (Inoue, Fukuda & Miyano 1994) and Sharma et al. (1993) have found evidence that lesions can occur in the thymus without virus replication.

The incubation period is usually two to three days. Following oral infection the virus will replicate in lymphoid cells of the gastrointestinal organs before spreading further to the liver through the porta system, where replication occurs in Kupffer cells and subsequently to the Bursa Fabricii through the blood (McFerran & McNulty, 1993). After replication in the bursa a second viremia occurs, spreading the virus to kidneys, spleen, thymus and other lymphoid organs (Murphy et al., 1999). The primary target of the virus is B-lymphocytes, and especially those expressing IgM. The immunodeficiency caused by the virus depends on viral strains, age of the chicken and co-infection with other pathogens (De Wit, 2001). Antibody production against other antigens than IBDV is repressed but high titers against IBDV are produced from B-cells that are already mature at the time of the infection (Murphy et al., 1999). Very virulent strains are capable of inducing a pancytopenia (Ridell, 1996).

Within two days of infection there can be a complete depletion of cells in the follicular cortex and after a week the follicular structure may not be visible at all (Heider & Monreal, 1992). Histologically coagulation necroses can be seen with cystic structures and connective tissue reparation. At four to six days post infection the bursa is swollen, hemorrhagic and might be covered by a gelatinous exudate and virus can be isolated from different lymphoid tissues (Rosenberger, 1998). The bursa will reach five times its normal size during acute infection before atrophy begins (Murphy et al., 1999). Ten days post infection the bursa will be one eighth of its original size and after two months a repopulation of the bursa might occur

(Ridell, 1996). At necropsy an atrophied, grey bursa is seen with no B-lymphocytes in the bursal follicles or in other lymphoid tissues (Murphy et al., 1999).

Recently it has been shown that T-cells also are affected by IBDV infection. This might be explained by a macrophage-mediated inhibition of the T-cells ability to respond to mitogenic stimulation or an activation of T-suppressor cells (Sharma, 2003). The immunosuppression caused by IBDV can be significantly increased by co-infections with IBV. This is especially associated with an increase of respiratory *E.coli* infections that causes mortality and economic losses (Naqi et al., 2001).

At necropsy, enlarged kidneys with urate deposits can be observed, possibly caused by physical obstruction of the ureters by the bursa, by secondary dehydration or by circulating immunocomplexes. Petechiae can be seen in the musculature, especially of the thigh, but the etiology of this is unknown. Sometimes petechiae are seen in the proventriculus and the liver is swollen with infarcts (Jordan & Pattison, 1996; Rosenberger, 1998; Murphy et al., 1999).

Variant viruses may cause a syndrome known as infectious proventriculitis. At necropsy enlarged and fragile thymus and proventriculus are found, sometimes the proventriculus even burst upon manipulation. Histologically the glands are enlarged and the organ is edematous (Giambrone, 2001).

Prophylaxis

Since IBDV lacks envelope it is resistant to many types of disinfectants (Murphy et al., 1999). Thus it is important to use mechanical cleaning, correct disinfectants and ensure a period in which the stables are kept empty before new groups of chickens are introduced.

There are many different vaccines on the market. The live attenuated vaccines can be composed of mild, intermediate, intermediate plus or hot strains (De Rosa, 2003). These are classified due to the virus' ability to cause symptoms in the chicken. The more virulent the strain of vaccine the more rapid a high level of protective antibodies will be developed, but at the same time immunosuppression and symptoms of disease may occur (Jiménez, 2003). Live vaccines also have capacity to spread and mutate. The ideal vaccine would elicit a high level of protection without causing the disease and without being able to spread. Today such a vaccine does not exist.

For breeders both live and inactivated vaccines can be used in order to provide maternal antibodies. The best strategy is to use first a live vaccine that will prime the immune system to respond better when the inactivated vaccine is used. Inactivated vaccines are usually supplied in oil suspension and are administered subcutaneously or intramuscularly. For broilers only live vaccines are used since they rapidly need a strong protection. There are many different ways to administer the live vaccines, such as oral in the drinking water, spray, and nasal or ocular droplets (See the IBV section for further details on vaccination methods).

De Rosa (2003) recommends the use of different vaccine strains depending on the challenge of virus the chickens are subject to. She recommends a strategy using three vaccinations. With high infectious pressure the first vaccination can be made with an intermediate strain and the others with hot strains. When no disease pressure is present only intermediate strains should be used. If the breeder animals are vaccinated and the chickens have maternal antibodies the day of the first vaccination has to be postpone, if not, they should be vaccinated the first day of life.

Munoz (2003) recommends continuous monitoring of the vaccination responses and the levels of maternal antibodies to determine the best day for the first vaccination. Usually the variations in antibody responses after vaccination are higher with live vaccines than with inactivated ones. The half-life of maternal antibodies is approximately 3 days (Murphy et al., 1999) and maternal protection often lasts 2-4 weeks after hatching (Engström et al., 2003; Charlton, 1996).

Differential diagnosis

The clinical symptoms shown by acute sick chickens are common with many diseases and it is difficult to differentiate based only on clinical symptoms (Engström et al., 2003). Other viruses, capable of causing immunosuppression, are the Chicken Anemia Virus (CAV, a circovirus), Mareks' Disease Virus (a herpesvirus) and Avian Reovirus (Sharma, 2003). A co-infection with IBDV and CAV can cause subcutaneous haemorrhages and decreased haematopoiesis (Rosenberger, 1998).

Non-infectious causes to bursal atrophy are different mycotoxins (Rosenberger, 1998; Jordan & Pattison 1996). Aflatoxins cause a degeneration of both thymus and the bursa and trichothecenes generally depress lymphocytopoesis. High doses of zearalenon also cause decreased bursal weight. As in other species corticosteroids cause apoptosis and decreased production of lymphocytes and animals in poor nutritional states or stressed from other reasons have smaller bursas (Ridell, 1996; Farner, King & Parkes, 1983).

Materials and Methods

A visit was made to some farms of the medium sized Costa Rican Company, "Ricura". Due to recent outbreaks of Newcastle Disease in other parts of Central America biosecurity measures have been increased. The entrances to the farms visited were controlled and all workers and visitors had to pass through showers and change clothes before entering the chicken houses. Moreover, in front of every entrance there were possibilities to disinfect hands and boots. The chicken houses had walls of mesh net and the bottom decimetres are of concrete (adult birds are high enough to see over). For a schematic picture of a typical chicken house see figure 1.

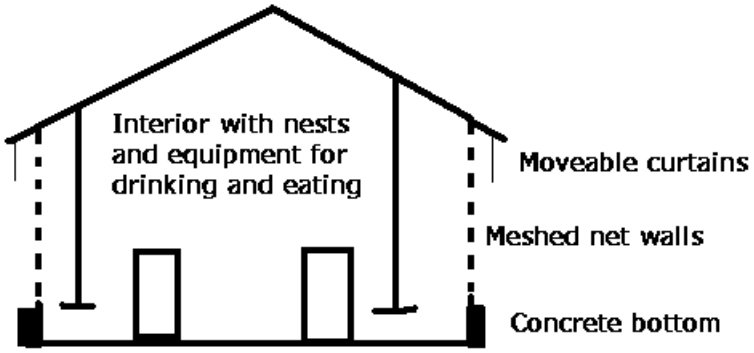

Fig 1. Schematic draw of a Costa Rican chicken house

Vaccination against IBV and IBDV is routinely performed within the "Ricura" company. The breeders and layers are vaccinated three times with a live vaccine and before the onset of lay, also one inactivated vaccine. The chickens are vaccinated against IB at day 1 and against Gumboro disease at day 10. Blood samples were regularly sent to the laboratory for routine screening of antibody titers. During August to October 2003 640 samples were analysed with an ELISA kit (IDEXX). The different groups of birds were not sampled at the same day of age but were grouped according to the day when most birds were sampled as followed: 1 day old (n=40 for IBDV and n=20 for IBV), around day 44 (including birds sampled day 42-45 of age, n=112 for IBDV and n=83 for IBV), around day 168 (day 126-189, n=27 for IBDV and n=112 for IBV), 224 (n=28 for IBDV and n=28 for IBV), 280 (day 180-187, n=25 for IBDV and n=46 for IBV) and 336 days (n=43 for IBDV and n=56 for IBV).

Enzyme-Linked Immunosorbent Assay (ELISA)

The principle of ELISA is that antibodies are attached to their specific antigen by linking an enzyme to an antibody following the addition of the substrate. There are many different ELISAs. The most common ELISA is the indirect ELISA. These have antigen attached to the polymer base. After that, a serum sample is added and if there are specific antibodies they will bind to the antigen. After this a second antibody against the first antibody, (produced by for example in a goat against chicken antibodies) is added and this is linked to an enzyme. If there is a positive sample, the antibody will attach and react with the substrate. Thus the positive samples will develop colour (Dinter, 1989; Stryer, 1995). In DOT-ELISA samples of antigen are filtrated through nitrocellulose paper to which the antigen attaches. The presence of antigens can be detected addition of conjugated antibodies.

In the competitive or blocking ELISA, the enzyme-linked antibody is directed against the antigen and there is a competition between the enzyme-linked antibody and the antisera. When the sera are positive no conjugate will attach and vice versa. This test is sensitive for antibodies that binds to the antigen and is not dependent on recognizing antibodies from a particular species. Other ELISAs are so called Double-antibody sandwiches, DAS, or antigen capture ELISA, which can be both indirect and direct. Here antibodies against a specific epitope were attached to the solid phase and the sample containing the antigen is added. Subsequently antibodies against another epitope on the same antigen were added. Either this antibody is labelled with an enzyme, direct DAS ELISA, or a second antibody with enzyme is added that can react with the first antibody (Dinter, 1989).

Comparison between two different IBV commercial kits

The ELISA used at the laboratory of virology in Costa Rican for the detection of antibodies against IBV is the indirect ELISA produced by IDEXX and provided with a computer program. Positive samples are grouped according to their titers. This kit was compared with a blocking ELISA kit from SVANOVA biotech AB without a computer program. The results have to be calculated manually or by software like Excel and are expressed as negative, positive or at the borderline.

264 random serum samples from birds sent to the laboratory for screening were used for this comparison. There was no individual labelling of the samples so only the number of positive and negative samples were calculated and compared.

Presence of antibodies against IBV in wild birds

As a part of an internationally financed project during the years 2000 and 2001 pigeons in Costa Rican countryside were examined for the presence of antibodies against various diseases and parasites. Using the competitive ELISA kit from SVANOVA, 91 sera from pigeons *Zenaida asiatica,* a lowland migratory pigeon, (n=40) and *Columba fasciata,* a highland stationary pigeon (n=48), were tested for the presence of antibodies against IBV. Three other pigeons of unidentified species

were also tested. Using another blocking ELISA from SVANOVA biotech, 52 samples were also screened for detection of antibodies against Avian Pneumovirus. Moreover, 91 sera from 31 different species of wild birds captured in nets in the south pacific region of Costa Rica between October and December 2002 were analysed by the laboratory of histopathology of the Universidad Nacional. (For details of the species of these birds, see the appendix).

Isolation and detection of IBV by DOT-ELISA

Five tracheas from birds with respiratory symptoms were macerated using laboratory sand, suspended in PBS, centrifuged and frozen and thawed twice. Five other samples from previous inoculations stored at –70º C were also tested. For virus isolation, embryonated 9 days old eggs were used. The suspension (0,2 ml) was inoculated into the allantoic cavity fluid. To avoid damage of the foetus and the yolk sac the angle of injection was as vertical as possible. Before inoculation, the eggs were disinfected with chlorine and alcohol and a small hole were made in the shell with a sharp scissor. The eggs were incubated at 37º C for five days after which they were opened. If the embryos were dead the eggs were discarded. From non-affected eggs the allantoic fluid was collected avoiding contamination with the yolk sac, which might contain maternal antibodies to IBV and give false results.

After sampling the allantoic fluid, the embryos were examined. If more than one embryo survived only allantoic fluid from one egg was chosen for DOT-ELISA. 100 µl of allantoic fluid was applied to a nitrocellulosa sheet and fixed with a mixture of PBS and casein (1%). Monoclonal rat antibodies against IBV were added and incubated over night at room temperature. After washing with PBS, conjugated anti-rat antibodies were added and incubated one hour at 37º C. Finally the substrate containing a mixture of ten ml sodium-acetate, 300 µl of 3% hydrogen peroxide and 600µl AEC was added and incubated at 37º C until the positive controls developed a colour. The positive allantoic fluids were used for RNA extraction following a protocol with Trizol.

Characterization of IBV isolates by RT-PCR and RFLP

The principle of PCR is that with two known short sequences of DNA (primers) it is possible to amplify an unlimited number of copies between the two known sequences. In the case of RNA viruses like IBV and IBDV, the RNA has to be transcribed into DNA by the Reverse Transcriptase (RT) (Stryer, 1995). For identifying IBV isolates the primers modified and described by Lee, Hilt and Jackwood (2000) for amplifying the S1 gene were used. By cutting the PCR-products with restriction enzymes different strains can be visualized. Three different endonucleases (Bsty-1, Hae III and Xcm I) were used as restriction enzymes. The PCR products from each of the four samples were mixed with the endonucleases and incubated over night. The samples treated with Bsty-1 were incubated at 60ºC and the others at 37ºC. The following day the samples were loaded on the gel electrophoresis and compared with earlier results.

Serological classification of IBDV

Five individual samples of bursas from disease outbreaks around the country were analysed. The samples, some containing multiple bursas, were macerated in PBS and laboratory sand, frozen and thawed several times. Two previous samples of Gumborovirus from cell cultures were also included.

For classification of the virus a commercial antigen capture DAS ELISA (Synbiotics) was used. The ELISA kit contains strips coated with four monoclonal antibodies (8, R63, B68 and 10). Since classical strains and variants have antigenic differences only the classical strain will bind all four antibodies whereas the variants will show different binding patterns.

Virus neutralization test (VNT) for IBDV

When a virus is mixed with homologous antiserum it will be neutralized and not infectious. This is the principle of a virus neutralization test (VNT) and can be visualized in cell cultures. If the virus usually produces a cytopathic effect (CPE) in cell culture the neutralized virus will not be able to produce it and thereby the effect of the serum can be observed.

For this test the concentration of virus in the sample used for neutralization must be determined by titration. Different dilutions, usually tenfold, of virus is then added to cell cultures and the cytopathic effects are studied. Thereafter the dose is determined whereby half the cultures are infected, the tissue culture infectious dose in 50% of cultures $TCID_{50}$. Two virus isolates were titrated termed IBDV-CR132 and IBDV-CR136.

A predetermined concentration of virus combined with different dilutions of serum (1:200-1:25600) was used for IBDV neutralization test. Since IBDV is difficult to replicate and grow very slowly, cells from monkey kidneys, BGM-cells, were used and incubated with the samples during eight days before a CPE was observed.

This experiment started last year and sera collected from chickens at three, ten and 18 days of age were used. Totally 57 serum samples from three-day old chickens and 54 samples from ten and 18 days old chickens were tested. First against the vaccine strain Bursine-2 and then against a wild type virus called IBDV-CR136 isolated from an outbreak of the disease. The aim of this experiment was to see how the maternal protection declined and if the protection against the vaccine strain and the wild type strain was different.

Results

The results from sera collected from the Ricura Company are shown in figure 2. No negative samples for IBDV were observed. In age category 44 days and 168 days, 24 and six samples respectively were negative for IBV antibodies.

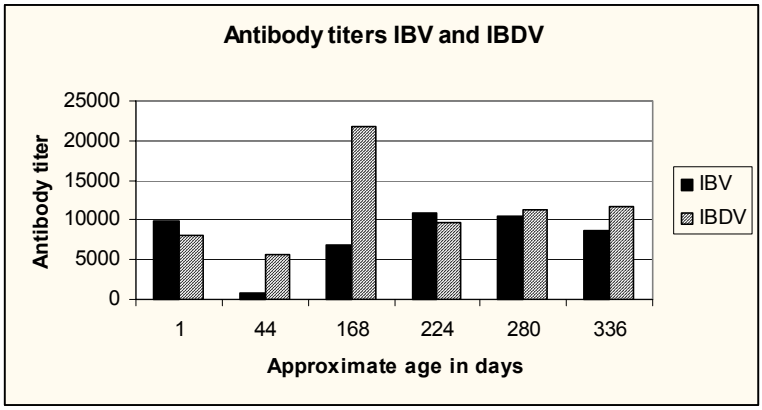

Fig.2. Sera sent in to the laboratory for routine screening of antibodies against IBV and IBDV.

Comparison between two different IBV commercial kits

264 serum samples were tested for the detection of antibodies against IBV with two ELISA kits (Svanova and IDEXX). Using the Svanova kit 253 samples were positive, 9 negative and 2 dubious for presence of antibodies against IBV. Using the IDEXX kit 245 were positive and 19 negative. The borderline cases were never retested but since they were very close to negative they were considered negative. Thus compared to the IDEXX kit the Svanova kit had a specificity of 97%.

Presence of antibodies against IBV in wild birds

Two samples out of 91 (2,2%) were clearly positive for IBV antibodies and two (2,2%) were within the range of borderline cases. The two positive samples were from pigeons of the species *Zenaida asiatica* and the others of the breed *Columba fasciata*. Earlier reports have shown susceptibility of pigeons to IBV (Murphy et al., 1999). No sera from the other 31 species tested were positive or suspected positive. Considering all birds tested, 1,0% were positive and 1,0% suspected positive for presence of IBV antibodies. Since the amount of sera was not

sufficient or there were no possible to repeat the ELISA, the borderline cases could not be rechecked. None of the sera tested for Avian Pneumovirus were positive.

Isolation of IBV

Half of the 30 eggs used had dead foetuses, including one of the controls. The reason for embryonic death might be contamination or physical damage to the embryo at the time of the inoculation. The surviving foetuses were examined. A control foetus measured 8 cm. The other foetuses were between 6,5 and 8 cm in length. The reduced embryo-growth might be a consequence of the inoculation damage or could be dwarfism caused by the viral infection (Engström et al., 2003). The presence of urates in kidneys and mesonephron in embryos inoculated with IBV on initial passages has been described by Villegas (2000). Generally the allantoic membranes of the inoculated eggs contained more urates than the living control eggs and in two of the embryos examined urate deposits in the peritoneum were found. The allantoic fluid from these two eggs proved to be positive for IBV on DOT-ELISA. In a second experiment, the surviving embryos measured between 4.5-6 cm and were not well developed. In this case it was found that the eggs had been kept in room temperature for too long time and survival was therefore reduced.

Detection of IBV by DOT-ELISA and virus characterization by RT-PCR and RFLP

The DOT-ELISA had to be repeated several times because of contamination of the monoclonal antibody. It was not possible to get a clear-cut negative control and it was therefore not possible to see how many samples were positive for IBV antigen. The suspected cases were confirmed by PCR. Due to problems with the PCR reagents this could not be done in this study. However, using older reagents four samples from previous RNA extractions were analysed and were clearly positive for the S1 gene of the IBV genome. The results from RFLP showed identical electrophoresis pattern of two PCR-products and one distinctly different from those two. The RFLP products from one virus isolate could not be visualized. One isolate of IBV had a RFLP pattern resembling the Massachusetts strain and the other two had a pattern not yet classified and referred to the New Variant 53 or VBIA-CR-53.

Serological classification of IBDV

All IBDV samples tested with an antigen capture ELISA reacted with the four monoclonal antibodies and were of the classical type. Jimenez (2003) estimated that approximately 7% of cases of IBD are caused by classical strains and the rest by the variant strain Delaware. No vvBDV strains have been isolated in Costa Rica.

Virus neutralization test for IBDV

Due to bacterial contamination during titration only results using the strain IBDV-CR136 was obtained. Using high concentrations of virus, the Hoff Magnum effect was observed. It is well known that at high concentration of virus, an increasing amount of defect virions are produced and sometimes no CPE can be seen. In lower concentrations though the cytopathic effects were clearly visible after ten days the concentration of virus could be determined. The results from the VNT show that the titers were significantly lower against CR-136 than against the vaccine strain ($P<0.000$, $P<0.021$ and $P<0.003$ for the serum samples from chickens at three, ten and 18 days of age respectively). It was also observed low levels of antibodies in samples from chickens at 18 days of age. The titers of antibodies against the vaccine at three and ten days of age were used to calculate the antibody half-life, which was 2.5 days. The results are summarized in figure 3.

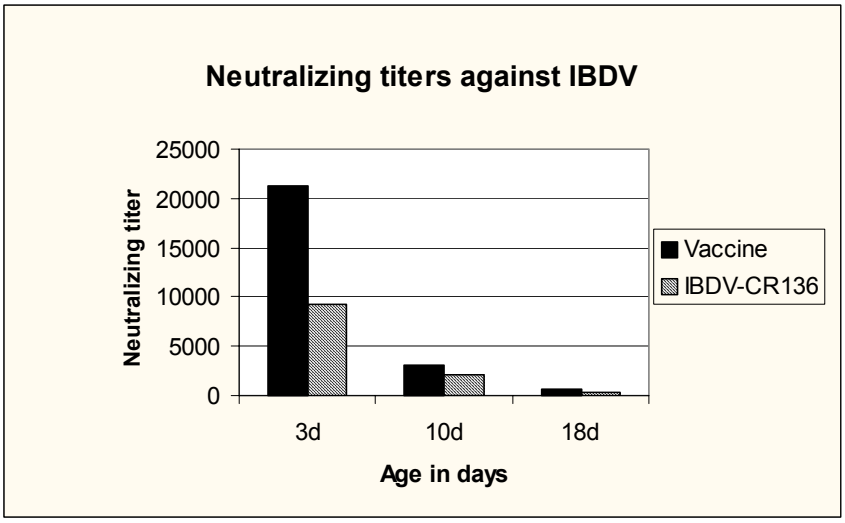

Fig 3. The neutralizing titers were significantly higher against the vaccine virus than against the wild type virus IBDV-CR136 in all age groups. $P<0.000$ in the 3-day-old group, $P<0.021$ in the 10-day group and $P<0.003$ in the 18-day group.

Discussion

The serum from chickens sampled at the Ricura Company had high titers of IBDV antibodies. These does however not necessarily neutralize wild type virus. The results from the VNT showed lower titers against wild type than against the vaccine virus. Vaccination against IB seemed to be less effective considering the high amount of negative birds. It could be worth overlooking the vaccination routines to make sure that all birds are given the correct dose, particularly when spray vaccines are used. Antibody titers were low in the group of birds around 44 days old. Since there is no data on how the titers have changed between day 1 and 44 it is difficult to estimate if there was a sufficient protection. Usually chickens are slaughtered at 42 days of age and it is possible that the immunity is enough to protect them until slaughter. The birds older than 42 days are probably breeders or layers and the protection seems to increase and give a good maternal protection to the one-day offspring.

The differences between the results obtained by the IBV ELISA kits from IDEXX and SVANOVA, are probably due to technical errors at the laboratory, therefore the differences obtained are probable negligible when used on herd level. There was no possibility to run tests in duplicates or to compare with another test like VNT.

The attempts to characterize the IBV isolates with PCR and RFLP were not successful due to problems with the reagents. The same is valid for the DOT-ELISA. It was only possible to perform a successful PCR on four samples, all from earlier isolations. Three out of these samples were further characterized by RFLP. The results showed that the virus belong to types earlier identified in Costa Rica.

Antibodies against IBV were found in sera from pigeons but not in other wild birds. The number of pigeons tested was in majority and therefore no conclusion can be drawn about the possible prevalence of IBV antibodies in wild birds. In pigeons the prevalence of antibody against IBV in *Zenaida asiatica* were 2.2%. In *Columba fasciata* a similar prevalence of suspected cases were noted and should be further investigated. It was not possible due to lack of sera and reagents in this study.

The VNT showed lower titers against the wild type IBDV strain than the vaccine strain. The VNT is more specific than the ELISA and can provide information about the real protection of the chickens. Considering the differences in titers between the two viral strains one understands the importance of knowing which viral strains are present in order to adopt a proper vaccination strategy. The low levels of antibodies in chickens at 18 days of age were expected considering the normal protection of maternal antibodies. It must also be remembered that chickens usually are vaccinated at 10-13 days of age and only stayed unvaccinated for evaluation of maternal protection (Jiménez, 2003). Chickens from unvaccinated mothers should be vaccinated on the first day of life.

In the antigen capture ELISA all samples were positive to the classical strain of IBDV in spite of classical virus being in minority in Costa Rica. However, this material was small and the result may be due to random sampling, or to the same strain causing outbreaks on various farms.

Since IBV can be isolated from different species and causes a variety of syndromes, it could be questioned if the name Infectious Bronchitis Virus is appropriate. Maybe a less specific name, Avian Coronavirus, would be more appropriate. Regarding the nomenclature of IBDV into two serotypes seems rather impractical in view of the antigenic diversities within serotype 1.

Conclusions

In spite of vaccination and increased bio-security, the Costa Rican aviculture still has problems controlling IBV and IBDV. The conclusions made during this study are that Costa Rica has new variants of IBV, which are spread in the country and that antibodies due to vaccination are not equally protective against wild type virus. To fully understand this situation, a continued national screening of tracheas from diseased poultry and a continued characterization of the virus through PCR and RFLP is recommended. Also, in order to study the prevalence of variant and classical IBDV strains it would be of interest to continue the screening of bursas from diseased birds using the antigen capture ELISA. The VNT would be useful to compare other wild type virus strains circulating in the country. The time and resources at disposal were only sufficient to see the complexity of the problem.

It has been shown that pigeons can have antibodies against IBV and although it is unlikely that these pigeons develop symptoms, they should be considered as a potential way for transmission. No antibodies against IBV could be found in other wild birds. This does of course not preclude the possibility of some species being reservoirs, though no indications were found in this study. As mentioned before, the prevalence of Gumboro disease in wild species is uncertain and since there are no commercial blocking ELISAs, and virus neutralization test is time consuming and expensive test, not many studies may be done on this virus. However it should be kept in mind that IBDV is a highly resistant and persistent pathogen that could be mechanically transferred to the birds by vectors, including animals, instruments and humans. Moreover the chicken houses visited would not be possible to clean and disinfect satisfactorily and the virus persist in generations of birds.

Vaccination is an important way for control of the diseases and a continuous surveillance of the strains in national outbreaks is important to choose the appropriate vaccine strains to be used. To minimize the transmission of diseases, a modernization of the poultry industry, including appropriate barriers against wild animals, would be required. This could, however, exacerbate other problems like increased air pollution when natural ventilation is not allowed. In fact Sainsbury, in his book “Poultry health and management” (1992), recommends open poultry houses in hot climates since other types of buildings would require ventilation to

cool down the environment. He also points out the fact that since there is a higher risk of infections in tropical countries it is important to observe some precautions:

- High hygiene standards and keep litter and carcasses disposed as far away as possible
- Control of visitors
- Careful vaccination schemes
- Netted houses to avoid entrance of wild birds
- In stressful situations for the birds, extra protection with antibiotics should be temporarily applied

As far as could be judged by this study these recommendations seem to be generally applied in Costa Rica.

One problem in countries like Costa Rica, where large chicken production plants are kept in proximity to backyard production, is that the companies might have rigorous control and vaccination schemes but backyard poultry will be uncontrolled and probably unvaccinated. These flocks will then serve as continuous sources of virus. Although there is an increasing awareness of diseases and protection within the industry and new effective vaccines are produced, these advances are seldom to aid smaller farmers. Vaccines are often produced for thousands of birds with a small cost for the individual bird, but these are highly uneconomical for the owner of a few birds. This problem is also an increasing concern in countries like Sweden where the hobby poultry population spreads diseases, practically not existing in large production units. Poultry for hobby purposes are not controlled and the birds are often transported between places and in contact with many other birds at exhibitions and markets.

Poultry diseases are an important and interesting part of Veterinary Science and the diseases studied in this work have great impacts worldwide. With increasing industrialization new problems are arising and a continuous surveillance is important. Today most diseases are of global significance since the increase of worldwide transportation of people, animals and goods.

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Appendix

Species	Number
Arremonops conirostris	5
Atlapetes atricapillus	1
Capsiempis flaveola	1
Chlorophanes spiza	1
Elaenia chiriquensis	1
Elaenia frantzii	1
Euphonia imitans	1
Euphonia laniirostris	2
Manacus candei	1
Melanerpes rubricapillus	2
Myiozetetes similis	2
Oryzoborus funerus	3
Pachyramphus polychopterus	1
Pipra mentalis	1
Pitangus sulphuratus	4
Ramphocelus passerini	22
Saltator albicollis	1
Sporophila aurita	16
Sporophila torqueda	1
Stelgidopteryx ruficollis	1
Thraupis episcopus	4
Thraupis palmarum	4
Thryothorus rmodestus	1
Thryothorus rubalbus	1
Todirostrum sylvia	1
Turdus assimilis	2
*Turdus grayi**	5
Tyrannus melancholicus	1
Tyrannus tyrannus	2
Tyrannus verticalis	2

*National bird of Costa Rica