THE ISOLATION, IDENTIFICATION AND CHARACTERISATION OF THE CAUSATIVE AGENT INVOLVED IN THE "PARALYSIS SYNDROME" AND THE IMPLEMENTATION OF SUITABLE CONTROL MEASURES

by

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DECLARATION

These studies represent original work by the author and have not been submitted in any other form to another institute. Work of other authors has been duly acknowledged in the text.

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ABSTRACT

"Paralysis syndrome" was first described by Dr M.J Versveld in 1993. The disease appeared to be confined to the Natal area with a few sporadic outbreaks in Gauteng, but none in the Cape region , nor in non-Rainbow flocks. The syndrome tended to re-appear on affected farms but not necessarily in the same houses. There was no correlation between affected flocks and their parent flock or hatchery identity (Maharaj, pers.comms.1994).

The disease was characterised by sudden onset, low morbidity and high mortality. Affected birds displayed sternal recumbency, ataxia and with severely affected birds displaying a fine head tremor. Feed samples from affected houses did not contain toxic levels of any significant toxins and the disease was not reproduced in specific pathogen free (SPF) birds that were fed feed and water removed from houses of affected broiler flocks. The severely affected chickens developed hypoglycaemia. The post mortem and histopathology analysis were inconclusive.

The early mortality caused by the disease resulted in significant financial loss, especially in Rainbow Farms (Pty) Ltd which has an integrated poultry structure. It was therefore imperative to isolate, identify and characterise the causative agent in an attempt to implement suitable control measures.

No pathogenic bacteria or fungi were isolated from brain and kidney samples of affected birds. However, an entero-like virus was isolated from the brain of at least five cases and in one instance from the kidneys of affected birds.The virus exhibited a hexagonal shape which indicates an icosahedral symmetry and was

between 28 and 32 nanometers in diameter. The virus grew optimally in yolk sac but not as well in allantoic sac of embryonated SPF eggs. The isolate was resistant to chloroform and was stable when exposed to heat for 1 hour at 56°C. There was no visible cytopathic effect in chicken embryo fibroblast and chicken embryo kidney cells.

There are striking clinical similarities between "paralysis syndrome" and "spiking mortality syndrome", with the fundamental difference being that the former is believed to be caused by an Entero-like virus and the later by an Arenavirus or Adenovirus.

The differentiation and characterisation of an Enterovirus by Computer-Assisted Viral Protein Fingerprinting was done by Holland, et.al, 1998. Four characteristic structural genes were identified as (viral protein) VP1, VP2, VP3 and VP4. The molecular weights were 30 kDa, 27 kDa, 24 kDa and 7 kDa. These molecular weights did not correspond with that of isolate H02283/94. Further analysis involving Western Blotting and reverse transcription - polymerase chain reaction (PCR) is planned on isolate H02283/94.

A killed oil emulsion (KOE) vaccine was prepared and administered intramuscularly to parent flocks at 18 weeks and this provided intermediate protection to the progeny.

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CHAPTER 1

1.0 INTRODUCTION

1.1 The Poultry Industry in South Africa

South Africa is a third world country which has an extensive poultry industry responsible for approximately one percent of the world's poultry production and is already the largest poultry meat producer in Africa, producing approximately 394 000 metric tons per annum, which comprises 22 percent of Africa's total production (FAO, 1992). The South African poultry industry has constantly increased at an estimated rate of 10 000 metric tons per annum from 1989 to 1991. To maintain the continued growth of the poultry industry it is essential that disease control is achieved.

The South African poultry industry has an integrated structure which differs from the European and American poultry industries. In South Africa, a few major companies own the entire poultry structure (ie. farming and abattoir) whereas in Europe and America separate individual companies own different levels. A typical poultry structure in South Africa consists of the following: great grand parent flock (which is imported), hatchery, grand parent flock, parent flock (consisting of rearing, breeding and laying flocks), hatcheries, broiler flocks and abattoir. It is therefore imperative for these major companies in South Africa to have strict disease control at each level to ensure profitability and continued flow of this cycle.

Disease control involves both direct and indirect testing procedures. The indirect

testing procedures involves routine serological evaluations (Enzyme Linked Immunosorbent Assay (ELISA) tests, Rapid serum plate agglutination (RSPA) tests, Haemagglutination (HA) tests, Haemagglutination inhibition (HI) tests and Serum Neutralisation (SN) tests of various flocks at different ages for elevated serum antibody levels for detection of common poultry diseases. Any significant increase in serum antibody levels may indicate a field challenge of a particular disease which will initiate appropriate action.

The direct testing procedure involves isolating and identifying causative agents from various samples following clinical examination of suspect flocks. A rapid and accurate diagnosis is therefore necessary to initiate treatment which will ideally cure the disease and prevent further spread of the pathogen.

"Paralysis syndrome " is a name given to describe a condition first seen in young broiler chicks at Ezakheni in the KwaZulu Natal midlands in 1992 and later seen on farms in the Hammarsdale area since mid 1993 (Versveld, pers.comms.1993). The syndrome tends to re-appear on affected farms but not necessarily in the same house. There seems to be little correlation between affected flocks and their parent or hatchery identity (Maharaj, pers.comms. 1994). The syndrome was confined to young broiler flocks in the Natal region, with few sporadic outbreaks in Gauteng but no outbreaks in the Cape region, nor in any non-Rainbow flocks in Natal.

The disease symptoms are characterised by ataxia, mild tremor and general

lethargy in affected chicks. Dead chicks are often found in lateral recumbency with survivors being undersized and unthrifty. The signs persist for up to 5 days, with the mortality reaching three percent per day in severely affected flocks. Post mortem examination of affected chicks reveals nothing other than frequent litter eating, with no other macroscopic lesions (A.J. Morley, pers.comms. 1994).

No pathogenic bacteria have been isolated from affected chicks. Virus isolation attempts from brain of affected chicks had proved successful on at least five occasions, with one positive isolate from the kidney. The isolates appear to be similar when inoculated via the yolk sac causing stunted, congested and oedematous embryos. The virus grows poorly in allantoic sac and produces no macroscopic cytopathic effect in chicken embryo fibroblast (CEF) and chicken embryo kidney (CEK) cells.

With an estimated mortality of 3% per day during an outbreak, Rainbow Farms (Hammarsdale) will loose approximately R1 964 250 per cycle. The Hammarsdale region completes 6.5 cycles per annum and this will result in an annual loss of approximately R12 767 625. The objectives of this study was therefore to isolate, identify and characterise the causative agent involved in the "Paralysis Syndrome" and to implement suitable control measures in an attempt to significantly reduce early broiler mortality in the Hammarsdale area.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Symptoms and Pathology of " Paralysis Syndrome "

"Paralysis Syndrome" is a newly recognised disease that is characterised by low morbidity but high mortality in broiler chicks between the ages of 5 and 18 days. The disease is characterised by sudden onset of approximately two percent mortality per affected house. Affected chickens display sternal recumbency, ataxia with both legs sometimes grossly out-stretched, severe depression and in severe cases a fine head tremor is visible (Maharaj, pers.comms. 1994). These symptoms generally last for approximately three days with the severely affected chickens dying (Versveld, pers.comms.1993).

The incidence of paralysis syndrome is not widespread on a multiple house farm, with few houses showing signs of the disease in an outbreak. Feed samples from affected houses did not contain toxic levels of aflatoxins such as citrinin, kojic acid, ochratoxin, patulin, penicillic acid, sterigmatocystin, some trichothecenes and zearalenone (Dutton, pers. comms. 1994). The disease was not reproduced in specific pathogen free chickens that were fed feed and water removed from houses of affected broiler flocks. Feed change in affected flocks did not have any impact on flock improvement (Maharaj, pers.comms. 1995).

Affected chickens develop hypoglycaemia with blood glucose levels ranging from 3.5 to 5.0 millimoles per litre in moderately affected birds and between 0.5 to 2.5

millimoles per litre in severely affected birds. Normal chicken blood glucose levels range from 9 to 15 millimoles per litre. The reduced glucose level is a probable cause for the nervous symptoms displayed by affected chickens. Subsequent treatment with sugar displayed steady improvement of the affected flock. Intravenous and intra peritoneal administration of sugar showed more rapid recovery rate when compared to oral administration. Oral administration had to be administered for at least 2 days in order to induce steady recovery of the affected flock (Maharaj,pers.comms.1994) .

Post mortem analysis of severely affected and dead chickens displayed gross dehydration of internal organs, slightly enlarged kidneys, presence of shavings in the gizzard which is possibly due to the inaccessibility of the chickens to feed and water. No other abnormalities were noted and the post mortem analysis was therefore inconclusive (Morley, pers. comms. 1994).

Pooled organ samples (brain, heart, proventriculus, small and large intestine, pancreas, lung, spleen, bursae of fabricus and spinal cord) of affected chickens were examined for histopathology. No evidence of neuropathy was evident which eliminates the possibility of avian encephalomyelitis (Travers, pers. comms. 1995). No abnormalities were seen in the heart, kidney, spleen, cerebellum and liver. The pancreas showed possible cell degeneration and degeneration of vacuoles and some debris possibly due to single cell necrosis. The proventriculus displayed focus of lymphocytes in granular pockets. The central nervous system displayed no gliosis, no cuffing, no changed Purkinje cells or molecular large cerebellum.

Numerous histological reports proved inconclusive which eliminated avian encephalomyelitis but suggested mild to gross lymphocytic infiltration of the pancreas, consequently suggesting viral activity with no definitive commitment to the causative agent.

2.2 Global Distribution of 'Spiking Mortality' & 'Paralysis Syndrome'

'Paralysis syndrome' is a novel disease that was first seen in young broiler flocks at Ezakheni, KwaZulu-Natal, in February 1993 (Versveld, pers.comms. 1993). In the United States of America a clinically similar disease called 'spiking mortality syndrome' (SMS) was first described by Brown, et al., 1991.

Spiking mortality is a newly recognised low-morbidity, high mortality clinical syndrome seen in previously normal 7 to 14 day old broiler chickens in the U.S.A (Brown, pers. observations, 1991). The early incidence of this disease displayed no conclusive evidence of a causative agent, but more recent studies have positively identified an Arena-like virus (isolated in flocks from Georgia, U.S.A.) which is believed to be the causative agent of this syndrome (Davis, et al., 1994). A fowl Adenovirus was also isolated from a field challenge on the Delmarva peninsula U.S.A. (Mendelson et al., 1995).

To date, experimental and morphological data from affected flocks in Georgia, USA indicates that the putative agent behaves like an Arena virus. The virus reacts with antiserum to known Arenavirus by immunohistochemistry and morphologically appears strikingly similar to Arenavirus by transmission emission microscopy using

negative staining with phosphotungstic acid (Davis, et al., 1995).

The disease (SMS) is characterised by a sudden onset of greater than one percent daily mortality for 3 to 5 days. Affected chickens develop opisthotonos, recumbency, depression, hypoglycaemia and die within 2 to 6 hours, often in sternal recumbency with the legs and neck extended (Brown et al., 1991).

The fundamental difference between spiking mortality syndrome and the paralysis syndrome is that the former is caused by an Arena-like virus or an Adenovirus and the latter is believed to be caused by an Entero-like virus (Maharaj, pers. comms, 1995).

2.2.1 The Arena virus

Arena viruses have a membraneous envelope and are negative sense, single-stranded RNA virus with round, oval or pleomorphic shapes and a varied size range between 50 to 300 nanometres (nm). The buoyant density of this virus in sucrose is 1.17 to 1.18 g/cm^3. The morphology of different Arenaviruses have been studied by several investigators who have reported that Arenaviruses replicate in the cytoplasm of infected cells and release virions by budding from the cell membrane. The virion has glycoprotein spikes (5 to 10 nm) and a sandy appearance with granular ribosomal-like inclusions seen within virion in thin sections of the virus infected cells (Bishop, et al., 1990).

To ascertain the similarities of the Arena-like virus particles to a known Arenavirus

(Pichinde), a comparative morphologic study was done (Table 1). The morphologic characteristic of the Arenavirus (Pichinde) was compared by negative staining to the Arena-like virus particles found primarily in faecal specimens of affected broilers. Previously healthy broiler flocks in Pennsylvania and Georgia were reported to be absent of these particles (Castro et al., 1994). The transmission emission microscopy findings indicated that the particles from affected broiler flocks were similar to the Pichinde virus in the size, spike or projection on the surface and their pleomorphic appearance.

Table 1: Comparison of the characteristics of the Arena-like virus and a known Pichinde Arenavirus

Virus	Pichinde	Arena-like (Field-birds)	Experimental birds
Particles measured	7	24	25
Size (nm)	50 to 200	79 to 250	133 to 292
Mean (nm)	183	126	194
Morphologic spike	9 to 12	10 to 13	10 to 12
Appearance	Pleomorphic, oval, round, sandy external		

Particles were measured by A.E. Castro from print of negative (Castro, et al., 1995).

The present findings suggest that this agent (whatever its eventual classification) appears closely associated with the SMS and hypoglycaemia seen in broilers. The lack of recognition in prior studies of this described syndrome could lie in the fact that it has never been seen in poultry previously, its pleomorphic appearance and also its inability to be detected by routine viral isolation methods.

2.2.2 The Adenovirus

Another recent field outbreak of spiking mortality syndrome was seen in broiler flocks in the Delmarva Peninsula, U. S. A. in 1990. A fowl adenovirus belonging to the strain of fowl adenovirus FAV 12 was isolated by cross-neutralisations and by restriction enzyme tests and these results were confirmed by nucleic acid hybridization tests (Bauer and Monreal, 1986).

Fowl adenovirus (FAV) is ubiquitous in poultry flocks throughout the world and its pathogenic effects is usually low. Certain strains of fowl adenoviruses were isolated during epidemics in Germany, Pakistan, New Zealand, Australia and the U.S.A. in recent years (Brown et al., 1991).

Its numerous striking similarities with regards to the disease pattern in the broiler flocks in Georgia and that in the Delmarva Peninsula. In the latter the disease was observed in 11 to 18-day-old broiler chicks and characterised by a sudden onset of greater than 1 % daily mortality lasting 3 to 4 days. Severely affected chicks are recumbent and depressed and may die within a few hours after first showing clinical symptoms. Flock recovery is usually rapid with the most obvious gross lesions being enlarged and mottled livers, thymus atrophy, enlarged hearts and hydropericarditis. Histological findings are hepatocellular necrosis with inclusion bodies and lymphocyte necrosis in the thymus or bursa of fabricus.

Most fowl adenovirus infections are subclinical and multiple factors may influence clinical outbreaks and disease severity. Therefore there is a certain degree of uncertainty with regards to pathogenicity of conventional clinical outbreaks. It should also be considered that if the first infection of parent flocks with highly virulent FAV strains occurs during the egg laying period, FAV may be vertically transmitted to progeny not sufficiently protected by maternal antibodies. Early contact of parent flocks with a wide range of FAV strains would be an important prophylactic measure, ensuring adequate titres of specific maternal antibodies in broilers.

Extensive studies are currently underway in the U. S. A. to detect and eliminate other viruses or microorganisms which may also be associated with the spiking mortality syndrome. To date only two of the field or experimental preparations which causes disease, including a preparation of the purified agent, contained identifiable causative agents.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Isolation and propagation of the Causative agent

3.1.1 Sample type

Broiler chickens displaying recumbency, ataxia with both legs outstretched, severe depression and in severe cases a fine head tremor were selected for sampling. The chickens were humanely euthenised with CO_2 gas. Brain and kidneys were aseptically sampled from these chickens. The external occipital surface of the head was sterilised using technical grade methanol and with a sterile post mortem scissors the skin at the occipital quarter of the skull was exposed. The brain was exposed by cutting the roof of the cranium with a sterile number 22 scalpel blade.

The kidneys were exposed by making a small incision at the base of the xiphoid process. The skin was gently removed upwards towards the head, thus exposing the breast muscles and rib cage. The rib cage was then aseptically cut at both sides (ensuring no contact was made with any internal organs), thus exposing the abdominal cavity. The intestines were aseptically moved aside thus exposing the kidneys. Isolate number H02283/94 (sample from brain) was used for all the confirmatory tests. The above isolate was chosen for two reasons, firstly this was the first outbreak from which live birds were sent to the laboratory for examination and secondly this was the only isolate that had suspicious viral activity in both the brain and kidney of affected birds.

3.1.2 Bacteriology

The brain and the kidneys were sampled by stabbing a sterile bacterial loop through these respective organs. The loop was then aseptically streaked onto one MacConkey agar (Oxoid CM 7) and three blood agar (Oxoid CM 854) plates. These plates were streaked out for single colonies. The MacConkey agar was incubated aerobically at 37°C for 48 hours. The blood agars were incubated aerobically with five percent CO_2 and anaerobically (Oxoid Gas Generating Kit BR 38) at 37°C for at least 48 hours, after which they were examined for growth.

3.1.3 Mycology

The brain and kidneys were sampled aseptically with a sterile bacterial loop and plated onto Sabourauds Dextrose (Oxoid CM 41) and Rose Bengal Chloramphenicol (Oxoid CM 549) agars. Pieces of the brain and kidneys were also aseptically removed with sterile forceps and placed on Sabourauds Dextrose and Rose Bengal Chloramphenicol agars. The plates were incubated at 32°C for up to 21 days. The plates were examined each day for fungal growth.

3.1.4 Virology

Samples for virus isolations (brain tissue and kidneys) were aseptically collected into sterile universal bottles with sterile forceps into 9 ml nutrient broth containing antibiotics (Appendix, 1) . The organs were homogenised into solution with a sterile pestle and mortar. Each sample was shaken vigorously for 30 seconds and then centrifuged at approximately 2000 x g for 10 minutes in a Heraeus Hettich Universal II benchtop centrifuge to pellet the macroscopic cellular debris. The

supernatant was then filtered with a 0.45 micron individually packed Sterivex filter into sterile universal bottles.

3.1.4.1 Yolk sac inoculation

Six-day-old Valo embryonated SPF eggs (Lohmann, Germany) were candled and the centre axis of the airspace of five fertile eggs were marked. These eggs were placed on the egg flats with the air space facing upwards. The external top surface of each egg shell was disinfected above the air cell with methanol. A hole was punctured on the centre axis at the top of each egg with a sterile steel needle. Using a 2 ml sterile syringe fitted with a 21 gauge needle, each egg was aseptically inoculated with 0.2 ml of the original inoculum by inserting the needle vertically to its full length. To verify the needle position in the yolk sac, the syringe plunger was gently withdrawn and aspiration of the yolk was observed for. The inoculation holes were sealed with Paraffin wax and the eggs incubated at 37°C for 24 hours. The eggs were candled after 24 hours and all dead embryos discarded. The remainder of the eggs were incubated at 37°C for a maximum of 10 days. The eggs were candled twice daily and the dead embryos were immediately removed and chilled at 4°C for 2 hours. The first yolk sac passage material from the dead and live embryos were harvested as described earlier(for the allantoic sac route) and reinoculated into the yolk sac. This was repeated until a consistent pattern of embryo pathology or embryo mortality was achieved. Continued passaging of the sample was done in an attempt to attenuate the virus.

3.1.4.2 Allantoic sac inoculation

Five SPF eggs incubated at 37°C for 11 days in a Buckeye egg setter incubator, were candled by holding them to a bright light and the air cell of the embryonated fertile eggs was marked away from the developing embryo with a pencil. Care was taken to ensure that the air cell was in the normal position (central anterior position). Infertile and dead embryonated eggs were discarded. The eggs were then placed on an egg flat, air cell up and the area directly above the air cell was disinfected with chemically pure methanol (CH_3OH: 32.04). Using a steel needle a hole was punctured into the shell of each candled embryonated egg, approximately 2 mm above the base of the air cell. Approximately 0.2 ml of the filtered sample and unfiltered (treated with equal volumes of antibiotic solution, Appendix, 2) was inoculated using a 2 ml syringe fitted with a 26 gauge needle, into the allantoic sac of the five 11-day-old embryonated eggs. The inoculation holes were sealed with chemically pure molten Paraffin wax (Unilab) and the eggs incubated at 37°C.

The eggs were candled after 24 hours. Embryos that died within 24 hours of inoculation were discarded as traumatics caused by the inoculation procedure or bacterial contamination. The eggs were candled twice daily for up to 7 days. On the seventh day the eggs were chilled at 4°C for 2 hours. The allantoic fluid was aseptically harvested into sterile 20 ml universal bottles with a sterile disposable plastic Pasteur pipette. The resulting allantoic fluid was then lightly centrifuged at 1000 x g for 10 minutes in a Heraeus Hettich Universal II benchtop centrifuge which allowed for the pelleting of any macroscopic particles. The supernatant was

transferred to another sterile universal bottle. This procedure was repeated for three passages.

3.1.4.3 Chorio-allantoic membrane inoculation (CAM)

Five 9 to 11-day-old embryonated SPF eggs were candled and a mark was made midway along the axis (ensuring the area was free from blood vessels) and another mark was made at the centre axis of the air space. The egg was then placed horizontally on an egg flat with the first mark facing up. The entire surface of the egg was then disinfected using absolute methanol. The egg shell was then carefully penetrated midway through the long axis ensuring that the egg shell membrane was not perforated, a second hole was made at the centre of the existing airspace (penetrating the egg shell membrane). A rubber bulb was then used to draw out air from the existing airspace. The resulting negative pressure resulted in an artificial airspace being created midway along the long axis. Using a 2 ml syringe fitted with a 26 gauge needle, 0.2 ml of the 5th yolk sac passage material of isolate H02283/94 that caused death or pathology in yolk sac inoculated embryos, was inoculated into the chorio-allantoic membrane (CAM) at the artificial airspace. Both of the holes were sealed with molten paraffin wax and the embryos incubated at 37°C for 24 hours after which the eggs were candled and dead embryos discarded as traumatics. The remaining live embryos were re-incubated for 7 days. The eggs were candled twice daily and dead embryos chilled for at least 2 hours before harvesting the allantoic fluid and the CAM. Live embryos displaying pathology after 7 days were also chilled and allantoic fluid and CAM harvested.

The chorio-allantoic membrane and allantoic fluid were pooled and the contents homogenised in a Waring blender. The resultant homogenate was centrifuged in a Heraeus Hettich Universal II benchtop centrifuge at 1000 x g for 10 minutes. The supernatant was treated with equal volumes of antibiotics for 1 hour and then re-inoculated onto the CAM's of five SPF eggs. This procedure was repeated for a further five passages.

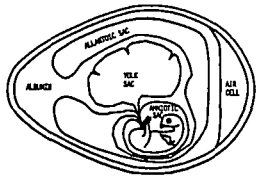

Fig.1. Structure of the embryonating egg (Hawkes, R.A. 1979)

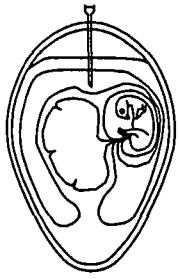

Fig.2. Allantoic route of inoculation Method A (Hawkes, R.A. 1979)

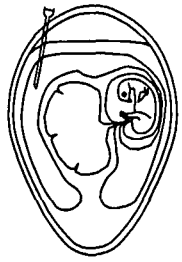

Fig.3. Allantoic route of inoculation Method B (Hawkes, R.A. 1979)

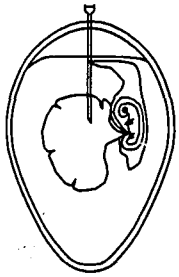

Fig.4. Yolk sac route of inoculation (Hawkes, R.A. 1979)

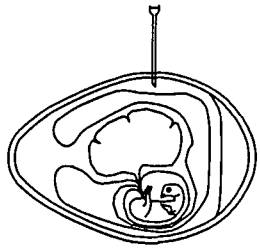

Fig.5. Chorioallantoic membrane (CAM) route of inoculation (Hawkes, R.A. 1979)

Illustration of the different routes of egg inoculation

3.1.4.4 Transmission Studies

Once a constant embryo death or pathology pattern was established in SPF eggs an attempt was made to reproduce the disease symptoms in chickens.

The fifth yolk sac passage was used for the transmission studies. Allantoic fluid from the fifth yolk sac passage was diluted 10^{-1} in nutrient broth with antibiotics (Appendix 1) and was used as the challenge material.

Eight groups, each consisting of 10 1-day-old chicks were used. There were four SPF chick groups and 4 Hubbard commercial broiler chick groups. The first SPF group was challenged intracerebrally, using a 1 ml syringe fitted with a 26 gauge needle with 50 ul of the challenge material. The second SPF group was the negative control group, challenged similarly with 50 ul of sterile distilled water. The third SPF group was challenged orally, using a sterile Pasteur pipette with 0.5 ml of challenge material. The forth SPF group was the negative control challenged similarly with 0.5 ml of sterile distilled water. Each group was separately reared in sterile isolation units with controlled feed, water and air flow. The commercial Hubbard groups were treated similarly to the four SPF groups. The chickens were monitored twice daily, with the dead chicks removed and re-isolation of the causative agent attempted from brain and kidney material. Post mortems were also done on the dead chickens. The chicks were kept for a maximum of 21 days, after which the survivors were humanely euthenised with CO_2 gas. Post mortem analysis was performed on each bird and reisolation of the causative agent was attempted.

3.1.5 Cell culture methods

3.1.5.1 Growth in Chicken Embryo Fibroblast Cells (CEF)

All cell culture procedures were carried out under a laminar flow system (Labotec Bio-Flow). Eleven-day old embryonated SPF eggs were used for the chicken embryo fibroblast cell production. Five 11-day-old embryonated eggs were candled for viability. The outside surface of the egg shells were sterilised with chemically pure methanol (CH_3OH: 32,04). The egg was held with gloved hands and the excess methanol was allowed to drip off. Sterile forceps were used to cut around the shell just below the air cell and flip the shell open. The embryo was removed by placing the forceps under the neck of the embryo and lifting gently to enable the embryo to break away from the yolk sac without rupturing the yolk. Embryos soiled with yolk were discarded as yolk is inhibitive to the growth of the cells. The embryo was placed in a sterile petri dish and its head and limbs were aseptically cut off. The remaining body of the embryo was then homogenised by passing it through a 2 ml syringe into a sterile beaker (250 ml). The homogenate was then washed three times to remove excess red blood cells.

Approximately 50 ml of a 0.25 percent solution of trypsin (Highveld Biologicals) was added to the homogenate and the embryonic tissue was disrupted into single cells by magnetic stirring at 37°C for 20 minutes. The trypsinated cells were then aseptically transferred through a sterile muslin cloth into a sterile 250 ml Erhlenmeyer flask (containing 5 ml foetal calf serum), thus removing gross macroscopic debris. The trypsin activity was inactivated by the serum. The trypsinised tissue was then transferred aseptically into sterile Greiner tissue culture

tubes (10 ml) and centrifuged at 1250 x g in a Heraeus Hettich Universal centrifuge for 30 minutes.

The pellet was resuspended in 5 ml of Eagles minimum essential medium (Highveld Biologicals, EMEM) with ten percent foetal calf serum. The pellet was broken up by gently flicking the Greiner tube. The cells were then quantified in a haemocytometer and diluted accordingly with EMEM containing ten percent foetal calf serum to achieve a minimum concentration of 2×10^5 per ml. The cell suspension was then dispensed in 5 ml aliquots into 50 ml tissue culture flasks (Greiner Labortechnik) and incubated at 37°C for 24 hours in order to obtain a confluent monolayer of cells which was viewed under a Leitz Diavert inverted microscope.

The fifth yolk sac passaged material was inoculated onto the confluent monolayers. The monolayer was first washed three times with Hanks balance salt solution (HBSS) and then 1 ml of inoculum (allantoic fluid) was aseptically introduced into the tissue culture flask. The inoculum was then incubated at 37°C for 1 hour, thus allowing sufficient time for viral adsorption. The inoculum was then discarded aseptically and the monolayer washed a further three times with HBSS. After the final wash, 5 ml Eagles minimum essential medium without foetal calf serum was added to the flask and incubated at 37°C for a maximum of 14 days. The monolayer was viewed daily with a Leitz Diavert microscope for cytopathic effect (CPE). A control flask was also aseptically inoculated with 1 ml of normal allantoic fluid and treated similarly to the test flask.

After 14 days the cells were viewed for CPE. The tissue culture flask was then frozen for 1 hour at -70°C and then allowed to thaw. This was done to release any viral like particles from the cell. The flask contents were then aseptically transferred into sterile universal bottles and centrifuged lightly at 1500 x g in a Hereaus Hettich Universal centrifuge for 10 minutes. One ml of the resulting supernatant was then inoculated onto a new monolayer. This procedure was repeated for a minimum of five passages.

3.1.5.2 Growth in Chicken Embryo Kidney Cells (CEK)

This cell line was prepared using five 19 to 20-day-old SPF embryos or 5 day-old SPF chicks. The embryo was aseptically removed from the egg. The kidneys were then aseptically removed from the embryo and placed in a sterile petri dish containing HBSS. The day-old chicks were euthenised by cervical dislocation and dipped in a disinfectant. The skin around the abdominal area was aseptically peeled back to expose the abdominal cavity. The kidneys were then aseptically removed and placed into a sterile petri dish containing HBSS. The kidney cells were then washed three times with HBSS in a sterile 100 ml Schott bottle. Sufficient washing of the kidney cells was accomplished by vigorously shaking the bottle allowing the chunks to settle and discarding the supernatant fluid. Washing was not done with CEF.

Trypsinisation was achieved by adding 50 ml of 0.25 percent trypsin solution (vigorously shaken) and allowed to react for 5 minutes at 37°C. The supernatant was harvested by passing through a sterile muslin cloth into a sterile 250 ml

Erhlenmeyer flask consisting of 5 ml of foetal calf serum . These cells were kept at 4°C. The kidney cells were retained in the bottle for further trypsinisation until the kidney tissue was exhausted. Trypsin activity was stopped by adding 5 ml of sterile foetal calf serum. These cells were then poured into sterile tissue culture tubes and centrifuged at 1250 x g for 30 minutes. The resultant pellet was then aseptically resuspended in 50 ml of EMEM containing 10% foetal calf serum. These cells were then quantified as for CEF. The cells were diluted to obtain a suspension of 0.5 to 0.6 x 10^6 cells per ml. Five ml of this suspension was added to Greiner cell culture flasks and incubated at 37°C for 48 hours.

Confluency of the monolayers were achieved after 48 hours, at which time they were inoculated as described for CEF with the fifth yolk sac passage of the virus. A minimum of five passages in CEK monolayers were performed.

3.1.5.3 Growth in Vero cell monolayer

Vero cells from King Edward viii Hospital were used. Upon receipt of the Vero cells, the monolayer was washed three times with 1 molar phosphate buffered saline (1 M PBS pH 7.2, Appendix, 3). The monolayer was then trypsinised by the addition of 5 ml of a 1:1 mixture of 0.25 percent trypsin and 0.1 mm Ethylenediaminetetraacetic acid (EDTA) (Highveld Biologicals). The solution was in contact with the monolayer for 60 seconds and then aseptically poured off leaving approximately 0.5 ml on the monolayer. The flask with the monolayer was then allowed to stand at room temperature for approximately 150 seconds during which time the cells gradually detached from the flask forming single cells. The detached single cells

were resuspended in approximately 30 ml EMEM containing 10 percent foetal calf serum and an aliquot of 5 ml were dispensed into each of six new 50 cm^3 tissue culture flasks (Greiner Labortechnik).

A confluent monolayer, viewed with a Leitz Diavert inverted microscope, developed within 24 to 48 hours after incubation at 37°C. The confluent monolayers were maintained with EMEM containing no serum. The cell lines were propagated by trypsinisation of uninoculated monolayers after every 3 to 4 days. Confluent monolayers were inoculated with the allantoic fluid from the fifth yolk sac passage. A control was maintained for each passage by inoculating a monolayer with healthy allantoic fluid. Five passages, each lasting 14 days were performed with the sample and control material.

3.1.5.4 Titration of the virus

The activity of biological suspensions, such as a virus, can be quantitatively measured by procedures that require preparing serial dilutions of the suspensions and determining the highest dilution (end point) at which a particular biological activity can be detected.

This means that embryo infective dose or EID_{50} (where 50 percent of a given population is affected or killed) was used to titrate the virus. The criteria used to determine infectivity was embryo mortality and general embryo pathology associated with the Entero-like virus.

Allantoic fluid from the fifth yolk sac passage was serially diluted in nutrient broth consisting of antibiotics from 10^{-1} to 10^{-7}. The virus was titrated via the allantoic

sac and yolk sac routes. Five SPF eggs were inoculated with 0.2 ml of each dilution (starting from the highest dilution, 10^{-7}) and thereafter aseptically transferred into 5 SPF eggs (per dilution) via their respective routes. These eggs were then sealed with wax and incubated at 37°C for up to seven days for the allantoic sac inoculation and up to 10 days for the yolk sac inoculation. The eggs were candled every day and the mortality recorded. The surviving embryos were aseptically evaluated for embryo pathology.

The infective dose 50 percent (ID_{50}) of the virus in each route was calculated according to a modification of the Spearman-Karber method (Villegas and Purchase, 1989) depicted below:

$$ID_{50} = 10^{L + L1\,(\text{sum of mortality or pathology} - 0.5)}/x \text{ ml}$$

L = highest dilution with mortality or pathology

L1 = difference in consecutive dilutions

x = volume of inoculum used per egg

3.2 Physical and chemical characterisation of the virus

3.2.1 Introduction

The diagnosis of viral diseases is based on the use of various direct and indirect (serological) methods. The direct method uses the clinical specimen to detect the presence of a virus or to demonstrate specific changes caused by the virus. False positive and negative results may be obtained and this can compromise the identification of the virus. It is therefore essential that the direct method be confirmed by an indirect method (Versteeg, 1985).

Many methods are based on the fact that many viruses cause characteristic changes in the cell of the inoculated or infected host. These cause the production of infectious viral particles in host cells, secretions and excretions. The production of specific antibodies is the general response of the host (Hawkes, 1979).

The present criteria for the classification of animal viruses is based on the physical and chemical characteristics of the virus. After initial isolation and subsequent propagation of the virus, it is essential to determine the type of virus isolated. There are seven families of deoxyribonucleic acid (DNA) containing animal viruses and sixteen families of ribonucleic acid (RNA) containing animal viruses, it is important to determine which of these two groups the virus belongs (Lukert, 1989). Other tests such as determining the presence or absence of an envelope, susceptibility to increased temperature and the ability to agglutinate red blood cells, are important characteristics for classification. Serological tests are also important

in characterising the virus. The most common serological tests are enzyme linked immunosorbent assays (ELISA), serum neutralisation (SN), haemagglutination (HA) and haemagglutination inhibition (HI). The inoculation of purified virus into SPF animals and monitoring the animal serologically for significant elevated antibody levels would provide suggestive evidence as to the identity of the virus (Hawkes, 1979).

Molecular biological techniques are more recent and these advanced techniques can be used to identify viruses. These will be briefly discussed later.

3.2.2 Haemagglutination Test (HA)

The test was done at the end of each passage by aseptically removing 2 ml of clear allantoic fluid with a sterile pasteur pipette and placing approximately three drops on a clean white tile (Villegas, 1987). An equal volume of a 10 percent suspension of chicken red blood cells (collected from the brachial wing vein of SPF chickens into Alsevers solution, Appendix 4, and washed three times with PBS pH 7.0), was added to the allantoic fluid. The plate was gently swirled, manually, for two minutes to mix the allantoic fluid and red blood cells. A positive control consisting of allantoic fluid infected with Newcastle disease virus (NDV) and a negative control consisting of healthy uninoculated SPF allantoic fluid, were used for each set of HA tests. A positive HA test was identified by the agglutination of red blood cells which appears grainy.

3.2.3 Heat stability Test

The fifth yolk sac and allantoic sac passage were used for this test. Approximately 1.5 ml of allantoic fluid, from each route, was placed into two separate 2 ml, thin-walled, glass, screw top vials (San Bio vials) and one vial of each route was placed into a water bath at a temperature of 56°C for one hour. The remaining vials were kept at 4°C until required. After one hour the heat treated vials were immediately transferred to a beaker containing ice. Serial ten fold dilutions of the heat treated and the non-heat treated samples were made in nutrient broth and the 10^{-1} to 10^{-4} dilutions of the heat treated samples and the 10^{-3} to 10^{-6} dilutions of the control samples were aseptically inoculated into 7-day-old SPF eggs for yolk sac inoculation and 9-day-old SPF eggs for the allantoic sac inoculation. Five eggs were used for each dilution. The eggs were incubated at 37°C for seven days or until the mortality of the embryos.

A heat labile and a heat resistant control sample consisting of Infectious Bronchitis H_{120} vaccine virus (IBV) (TAD Pharmazeutisches) and a chicken embryo lethal orphan (CELO) virus (Rainbow Farms Veterinary Laboratory),respectively, were included in the test and treated identically to the test samples. The eggs were opened after seven days and examined for pathology.

3.2.4 Chloroform sensitivity Test

Four millilitres of the fifth yolk sac allantoic fluid passage was separately aliquoted into two sterile bijou bottles each and 0.4 ml of chloroform added to one bijou bottle. The untreated sample was kept at 4°C until required. The chloroform sample

was inverted several times to mix for ten minutes with the bottle placed in a container of ice between mixes. The treated sample was centrifuged at 1500 x g for thirty minutes in a Universal II centrifuge. The upper layer was aseptically transferred with a pasteur pipette into a sterile bijou bottle. The bottle was left opened in a laminar flow hood for thirty minutes to allow traces of chloroform to evaporate. Serial dilutions of 10^{-1} and 10^{-2} dilutions of the treated and untreated samples were made in nutrient broth and separately inoculated into the yolk sac (5 per dilution) of SPF eggs. A chloroform-resistant and a sensitive control sample, consisting of CELO virus and IBV H^{120} (TAD) virus, respectively, were used as controls and treated identically to the test sample.

3.2.5 Determination of nucleic acid

The type of nucleic acid was determined indirectly using specific metabolic inhibitors that interfere with virus replication. This method was especially valuable as the unknown virus did not have to replicate to a sufficient high titre to be concentrated, purified and consequently characterising the nucleic acid.

The thymidine analog 5-iodo-2'-deoxyuridine (IUDR) was used to react with the virus to determine whether the virus contains DNA (Lukert, 1989 and Villegas 1987).

The fifth yolk sac passage of isolate H02283/94 was diluted from 10^{-3} to 10^{-6} in nutrient broth with antibiotics. To each dilution 50 ug/ml of IUDR was added. Each dilution was then inoculated into five 7-day-old SPF eggs and incubated at 37°C for up to seven days. Untreated samples (without IUDR) of each dilution was also

inoculated into five 7-day-old SPF eggs. A DNA positive virus (CELO reference strain) and a RNA positive virus control (Clone 30 strain of NDV Intervet) was also treated with IUDR. The Clone 30 was diluted from 10^{-6} to 10^{-9} and the controls were treated in the same way as the test virus.

The titre of each virus, in the presence and absence of IUDR was calculated according to the modified method of Spearman Karber (Villegas and Purchase, 1989).The virus was diagnosed as containing DNA if the titre is 1 $\log_{10}$ lower with the analog than without.

3.2.6 Serum Neutralisation Test

Five 6-week-old SPF white leghorn birds were orally inoculated with 0.5 ml allantoic fluid (fifth yolk sac passage) of the H02283/94 isolate. These birds were pre-bled via the brachial vein. These pre-bleed samples were tested by Mr D. N. Bhoora (Rainbow Farms Laboratory) for antibodies to NDV, IBV, Reo virus, Infectious Bursal Disease Virus (IBDV), Turkey rhinotracheitis virus(TRTV), Mycoplasma gallisepticum (MG), M synoviae (MS), Avian encephalomyelitis virus (AE) and Bacillary white diarrhoea (BWD). The SPF birds were reared in a filtered air positive pressure house (FAPP) with controlled feed and water. No laboratory staff were allowed in the FAPP house for the duration of the trial. Two weeks later these birds were given a further oral inoculation of 0.5 ml allantoic fluid of the H02283/94 isolate. These birds were then bled weekly terminating at six weeks post initial inoculation.

The serum was centrifuged at 2000 x g and the supernatant filtered through a 0.22 micron Sterivex-GS filter. The serum was then heat treated for one hour at 56°C to inactivate complement that could cause false positive results. The sera was tested by Mr Bhoora, using haemagglutination inhibition for antibodies to NDV, by ELISA for antibodies to IBV, Reo virus, IBDV (Delta Bioproducts), TRTV (Pathasure), AE (IDEXX) and by the rapid serum plate agglutination (RSPA) test for antibodies to MG, MS and for antibodies to Salmonella pullorum and Salmonella gallinarum (BWD).

The alpha neutralisation procedure was used (Beard, 1989). This procedure involved the use of serial dilutions of virus mixed with a standard constant dilution of serum. The entero-like mono-specific serum was diluted 1:10 in nutrient broth containing antibiotics. The fifth yolk sac passage of the entero-like virus was serially diluted from 10^{-1} to 10^{-7} in nutrient broth with antibiotics and 1 ml of the 10^{-3} to 10^{-7} added to a sterile bijou bottle containing 1 ml of 10^{-1} mono specific entero-like antisera. These bottles were shaken and allowed to stand at room temperature for one hour. A virus control consisting of 1 ml virus and 1 ml of nutrient broth with antibiotics (10^{-3} to 10^{-7}) was also added. Each dilution (0.2 ml) was inoculated via the yolk sac into five 7-day-old SPF eggs and incubated at 37°C for seven days. The eggs were opened and the mortality and pathology recorded. The titre of the virus and their neutralisation indices were then calculated. The indices were calculated by subtracting the titre of the serum-virus complex from the titre of the virus. Cross neutralisation was also carried out with known viruses and H02283/94 antisera. No monospecific antisera to a known enterolike virus

was avaliable to test against the H02283/94 virus.

3.2.7 Haematoxylin and eosin staining of monolayers

The fifth Vero cell passage was serially diluted from 10^{-3} to 10^{-7} and separately inoculated onto preformed Vero cell monolayers. The lowest dilution was diluted 10^{-1} and re-inoculated onto Vero cell monolayers five days post inoculation. This was repeated with the third Vero cell passage being used for the test. The inoculated and uninoculated control monolayers were subsequently stained by the haematoxylin and eosin (H and E) staining methods. Monolayers were grown on sterile coverslips placed in disposable 60 mm tissue culture dishes (Greiner Labortechnik).

The coverslips with the inoculated monolayers were stained three days post inoculation. The H and E staining method used was similar to that of Villegas (1987). The coverslips were washed with PBS (pH 7.2) and the cells fixed with Bouins fixative for one hour. The coverslips were rinsed in 100 percent ethanol and transferred to 70 percent ethanol for five minutes. This was washed under slow running water for five minutes and stained with Harris' haematoxylin containing four percent glacial acetic acid for 8 minutes. After haematoxylin staining, the coverslips were briefly washed under tap water and differentiated with two dips in acid alcohol (0.5 percent in 70 percent alcohol). To stop differentiation they were immediately washed under running tap water for 20 minutes.

The coverslips were counter-stained with a water soluble eosin-phloxine solution

(1:1) for thirty seconds and the cells on the coverslip were dehydrated by dipping once each in a graded alcohol series (70, 96 and 100 percent). The coverslips were placed in 100 percent alcohol for a further thirty seconds and left in xylene until ready to fix onto a slide with DePex mounting medium (BDH Chemicals). After H and E staining the monolayers were viewed with the Zeiss Axioskop light microscope for inclusion bodies and other cytopathic effects.

3.2.8 Sodium Dodecyl Sulphate (SDS) PAGE of the virus

3.2.8.1 Introduction

Modern immunological and molecular biology techniques have provided diagnosticians with powerful new tools to enhance morphologic and chemical laboratory diagnosis. The application of this technology to pathology represents the transfer of recent basic research tools into the laboratory environment (Fenoglio-Preiser and Willman, 1987).

Polyacrylamide gel electrophoresis (PAGE) in the presence of the anionic detergent sodium dodecyl sulphate (SDS), has proven to be useful for the separation of proteins and the determination of their molecular weights (Sigma Chemical Company, 1988). The main application of this technique is therefore to assist in the identification and classification of pathogens.

SDS-PAGE is normally performed with a discontinuous buffer system where the buffer in the reservoir is of a different pH and ionic strength to that used to cast the gel. A reducing agent, heat and SDS are used to disassociate the proteins in

a sample before loading onto a gel. The polypeptides bind SDS and attain a negative charge. The amount of SDS bound by the polypeptide is proportional to the molecular weight of the polypeptide, resulting in the complex migrating through the gell in accordance with the size of the polypeptide (Sambrook, Fritsch and Maniatis, 1989). The electric current applied between the electrodes of the gel apparatus causes the charged SDS-polypeptide complex to move along with the moving boundary in the gel. The SDS-polypeptide complexes move through the resolving gel and are separated according to size by sieving. The effective range of separation of the SDS-polyacrylamide gels depend on the concentration of polyacrylamide used to cast the gel and on the amount of cross-linking. The sieving properties of the gel are determined by the size of the pores (Sambrook et al., 1989).

The molecular weight of a given protein can be determined by comparing its electrophoretic mobility (Rm) with known protein markers. A linear relationship is obtained of the logarithms of the molecular weights of the protein markers which are plotted against their respective electrophoretic mobility (Sigma Chemical Company, 1988).

3.2.8.2 Sample preparation for SDS-Page

The fifth yolk-sac allantoic fluid passage of H02283/94 was harvested from valo SPF eggs and centrifuged at approximately 3 000 x g for ten minutes. The supernatant was recentrifuged using a Sorvall S-20 rotor at 10 000 x g for forty-five minutes in a Sorvall RC 28 S centrifuge. The resulting pellet was resuspended

in 0.5 ml of sterile distilled water. Allantoic fluid from healthy 13-day-old embryonated SPF eggs were harvested and treated identically to that of the test sample.

Just prior to SDS-Page, 50 ul of each concentrated sample was added to an equal volume of SDS gel loading buffer (Appendix, 5) and heated to 100°C for three minutes.

3.2.8.3 SDS-PAGE

The method used for SDS-PAGE was according to that of Sambrook,et al., 1989. A 12 percent running gel and a five percent stacking gel were cast according to the formulation in Table 2.

Table 2: The formulation for the preparation of running and stacking SDS polyacrylamide gels

INGREDIENT	RUNNING GEL (ml)	STACKING GEL (ml)
Water	3.3	2.7
30 % Acrylamide mix[@]	4.0	0.67
1.5 M Tris (pH 8.8)	2.5	-
1 M Tris (pH 6.8)	-	0.5
10 % SDS	0.1	0.04
10 % Ammonium persulphate	0.1	0.04
TEMED[*]	0.004	0.004

[@] Solution of 29 percent (w/v) acrylamide and one percent (w/v) N, N'-methylenebisacrylamide in deionised water
[*] N, N, N' - tetramethylethylenediamine

The glass plates for gel casting were assembled and the freshly prepared running gel mixture was introduced between them with the aid of a syringe and a 21 gauge needle to approximately 2 cm from the top of the plate. With the aid of a pasteur pipette, isobutanol was overlaid on the gel to prevent oxygen from diffusing into the gel and inhibiting polymerisation. After polymerisation of the running gel, the overlay was poured off and the top of the gel was washed with distilled water. The

water was drained and the stacking gel mixture was poured on top of the running gel. Immediately thereafter a clean teflon comb with ten "teeth" and of the same thickness as the spacers between the glass plates, was inserted into the stacking gel solution without trapping any air bubbles. After polymerisation the teflon comb was carefully removed and the wells created were washed with distilled water. The gel was then mounted in electrophoresis apparatus and Tris-glycine electrophoresis buffer (Appendix, 6) was added to the top and bottom reservoirs.

Approximately 50 ul of the sample was loaded onto the bottom of the well with aid of a micropipette. The molecular weight marker (Boehringer Mannheim Combithek High Range 14kDa-340kDa) was loaded in lane one. The normal control (normal allantoic fluid) was loaded in lane two. The H02283/94 isolate (allantoic fluid) was loaded in lane three. Two such gels were loaded identically and run together in the same apparatus. The electrophoresis apparatus was connected to a Consort E 702 power pack and run at 180 V for approximately ninety minutes or until the bromophenol blue dye front reached the bottom of the running gel.

One of the identical gels was removed from between the glass plates for Coomassie brilliant blue (CBB) staining. Approximately 0.25g of CBB R-250 was dissolved in 90 ml of a solution of methanol:water (1:1 v/v) and 10 ml of glacial acetic acid.

The solution was filtered through a Whatman No. 1 filter paper and the gel was immersed in this solution with gentle agitation on a micro-orbital shaker for 24 hours at room temperature. The stain was decanted and the gel destained by

soaking in a solution of methanol:water (1:1 v/v) with 10 percent acetic acid on a micro-orbital shaker. The destained solution was regularly changed at 1 hour intervals. After 8 hours of destaining, the gel was photographed with a FCR 10 polaroid camera.

The R_m values of all the protein bands were determined by measuring the distance of the protein band from the top of the running gel and dividing this by the distance of the dye front. A standard graph was drawn by plotting the log of the molecular weights of the protein markers (lane 1) on the y - axis and their R_m value on the x - axis. The molecular weight of the sample (lane 3) was determined from this graph.

3.3 Electron Microscopy

The allantoic fluid from the embryos inoculated via the yolk sac route with the fifth yolk sac passage of isolate H02283/94 was harvested 56 days post-inoculation. The allantoic fluid was harvested just before embryo death, when the embryo displayed stunting and lack of motion upon candling. This was done to ensure that the virus was in the exponential growth phase and that the highest possible virus concentration was achieved. The allantoic fluid was then treated for electron microscopy according to the method of (Doane and Anderson, 1987). The allantoic fluid was centrifuged at approximately 3000 x g for 15 minutes in a Hettich Universal centrifuge to remove red blood cells, bacteria and macroscopic debris. The sample was then centrifuged at 15 000 x g for 45 minutes in a Sigma 2K15 table model centrifuge (Els and Josling, 1998).

The supernatant was carefully decanted and the pellet resuspended in 0.5 ml of sterile deionised water. The supernatant was then gently vortexed to ensure homogenisation of the virus suspension. Using a 1 ml sterile pasteur pipette one drop of each virus suspension was carefully placed on five sterile formvar coated copper grids. Care was taken not to overload the grids, thus preventing running of the droplet. Each grid was then negatively stained for 2 minutes in two percent phosphoptungstic acid (PTA, pH 6.0) and viewed with a Phillips CM 10 transmission electron microscope.

To provide more contrast, one droplet of a three percent glutaraldehyde fixative solution was added before rinsing in water. The three percent glutaraldehyde solution was prepared in PBS (Appendix, 7).

3.4 Vaccine production

The 5th yolk sac passage of the H02283/94 isolate was used as the master seed for vaccine production. The working seed for vaccine production was constituted of an additional passage of the master seed. Seven day-old embryonated SPF chicken eggs were inoculated via the yolk sac with 0.2 ml of a $10^{-3.5}$ dilution of the working seed. The eggs were chilled at 4°C for twenty-four hours when the first sign of embryo mortality was observed in at least 50 percent of the eggs inoculated.

The allantoic fluid was then aseptically harvested. The resulting fluid was then clarified by centrifugation at 4000 x g for ten minutes in a Sorval GSA rotor using

a Sorval RC 28S centrifuge. Two ml of the penicillin/streptomycin solution containing 25 000 units and 25 000 ug per ml respectively (Bio Whittaker) was added to 1 litre of allantoic fluid. This material constituted the antigen for vaccine production and was stored at -70°C until required for use.

Each batch of antigen was tested for sterility and purity. The sterility test involved subbing the live antigen onto blood agar (Oxoid CM 55), MacConkey agar(Oxoid CM 7), Sabarouds dextrose agar (Oxoid CM 41), Freys broth and thioglycolate broth. Each of these media were incubated at 37°C for 48 hours with the Freys broth incubated for up to 7 days. These media were inspected visually for the growth of microorganisms, ie.(turbidity).

The purity tests for the antigen involved reacting equal volumes of a 10^{-1} dilution of live antigen with a 10^{-1} dilution of monospecific antiserum for one hour at room temperature. This was then inoculated via the yolk sac into five seven-day-old embryonated SPF eggs and into CEF monolayers. The SPF eggs were candled for up to 10 days and the CEF monolayers viewed microscopically for up to 5 days for viral activity.

The live antigen was also titrated in 7-day-old SPF eggs via the yolk sac route and a titre of greater than 10^{-6} stored for vaccine production.

The antigen was then inactivated with 0.3 percent formalin before blending into a vaccine. The inactivation procedure involved allowing the formalin to run down

the neck of the flask containing the antigen. The antigen was then allowed to react with the formalin for 24 hours at room temperature with constant stirring. Antigen inactivation was ensured by repassaging the inactivated antigen into 5 seven-day-old SPF eggs via the yolk sac. The SPF eggs were sealed and incubated at 37°C for up to 10 days. Each day the eggs were candelled to establish viral viral activity.

The blending of the antigen involved combining one part of antigen to four parts of arlacel/oil mixture. All vessels used for blending were sterilised by autoclaving at 121°C for 15 minutes in a Almor autoclave.

The following procedure was used to produce 10 000 doses of killed oil emulsion (KOE) vaccine. Four hundred ml of arlacel A (ICI) and 3600 ml of Marcol 52 (ESSO) was added to a sterile 5 litre Erhlenmeyer flask. This mixture was then transferred to a sterile homogenisation vessel (Silverson L4R mixer) and mixed at full speed (3500 x g) for 2 minutes. The speed was then dropped to 30 percent and the antigen added slowly at a constant rate for 2 minutes. The antigen consisted of 960 ml of allantoic fluid and 40 ml of tween 80. The homogeniser was then turned to full speed and blending was continued for a further 3 minutes. The blended mixture was then transferred to a sterile dispensing vessel which dispensed 500 ml (1000 doses) of vaccine into sterile Sabex pouches, leaving sufficient head space for mixing. The pouches were then sealed using cyclohexamide and a sterile plastic seal. After every five pouches, 50 ml of vaccine was removed from the dispensing container into sterile medical flat bottles for quality control. Each pouch was then labelled with the vaccine type, batch number, date produced, expiry

date, recommended dosage (0.5 ml per bird) and storage instruction which was 4°C.

The quality control of the finished vaccine involved sterility tests, emulsion stability tests, emulsion type, emulsion viscosity, safety tests and degree of vaccine reaction.

The emulsion stability of the vaccine was determined by storing 15 ml aliquots in rubber stopper glass tubes at 37°C for 28 days. Ideally no separation of the aqueous and oil phase should be observed.

The emulsion type of the vaccine was determined by expressing one drop of the vaccine from the tip of a hypodermic needle onto a petri dish containing clear tap water. The drop must retain its physical integrity and must not be diluted by the tap water. This emulsion is one of a high oil content.

The emulsion viscosity of the vaccine was determined by the rate of discharge of 0.4 ml of the vaccine from the orifice of a vertically mounted 1 ml serological pipette. The time for the efflux of 0.4 ml was measured and found to be 3 seconds with the standard international specification being 2.8 to 3.8 seconds. The vaccine was kept for several hours at room temperature and shaken well before this test was carried out.

The chick embryo test was carried out to determine the safety of the vaccine, with

0.2 ml of the inactivated antigen inoculated into 25 seven-day-old embryonated SPF eggs via the yolk sac route. This was done to determine the presence of residual living virus. The eggs were incubated at 37°C for up to 10 days, with candling done daily to determine viral activity. Thereafter the eggs were aseptically opened and the embryos examined for viral activity (stunting, haemorrhaging, oedema, etc). The vaccine passes the test if there is no evidence of pathology.

The degree of vaccine reaction was determined by inoculating 20 healthy 28-day-old SPF chickens intramuscularly into the breast with 0.5 ml of vaccine and observed for 14 days. There should be no abnormal reaction during this period. Thereafter the birds were euthenised (using CO_2) and the degree of reaction determined by incising the breast muscle and observing the breast muscle for necrosis, haemorraging and pus formation. Ideally no necrosis, haemorraging and pus must be visible.

Each bird in a house of 7 000 birds on a laying site was vaccinated with 0.5 ml of KOE vaccine to evaluate the efficacy of the vaccine in the field. Each bird was individually vaccinated in the right breast muscle. These birds were pre bled and bled again 4 weeks post vaccination to evaluate the antibody response of the flock. Due to the absence of a commercially avaliable Enterovirus ELISA kit, the antibody response was evaluated using the serum neutralisation test.

CHAPTER 4

4.0 RESULTS

4.1 Bacteriology

The various attempts of bacterial isolation from affected birds from brain and kidney material, proved to be negative. There was no growth on two blood agar plates and all the MacConkey agar plates. One blood agar plate that was incubated in ten percent CO_2 had scanty growth of Escherichia coli. This growth, however, did not start from the initial streak lines and did not grow on the corresponding MacConkey agar. This gave rise to the suspicion of a possible aerial or plate contaminant.

Five day old SPF chicks were challenged orally, intraocularly and intranasally with 0.2 ml of an overnight nutrient broth culture of the above E. coli isolate. The birds were reared under FAPP house conditions with controlled feed, water and air. These birds did not display any symptoms of the "paralysis syndrome" after 21 days.

4.2 Mycology

There was no growth on any of the Sabourauds Dextrose agar plates and the Rose Bengal chloramphenicol agar plates. Both the brain and kidney samples had no evidence of fungal growth after 21 days post incubation.

4.3 Virology

4.3.1 Yolk Sac Inoculation

There was no embryo pathology or mortality in the first passage and the liver, kidney and other internal organs appeared normal. In the second passage 20 percent of the embryos displayed stunting, slight haemorraging and oedema. In the third passage there were 80 percent affected embryos with 60 percent showing pathology and 20 percent mortality. The embryos were killed 7 days post inoculation and displayed severe congestion and oedema. The embryos displaying pathology were stunted, oedematous and haemorrhagic with mild liver necrosis. By the forth passage,100 percent of the embryos were affected at 5 days post inoculation with 80 percent mortality and 20 percent pathology. In the fifth passage, 100 percent embryo mortality was achieved 5 days post inoculation with the dead embryos displaying oedema and congestion. Liver necrosis was not consistant with approximately 20 percent of the affected embryos displaying mild liver necrosis.

4.3.2 Allantoic Sac Inoculation

The first three allantioc sac passages of the original material displayed no embryo pathology or deaths 7 days post inoculation. The internal organs of these apparently normal embryo displayed no signs of abnormality. Further passaging of the original material via this route was discontinued in order to save cost. However, the fifth yolk sac passage material (egg adapted) was passaged via the allantoic sac route in an attempt to adapt the virus. This material (fifth yolk sac) displayed very inconsistant embryo pathology, with between 20 and 60 percent of the embryos displaying pathology. The affected embryos displayed slight to no stunting, with the affected embryos being pale, slightly haemorragic, oedematous

and slight to gross necrotic liver lesions. Approximately 10 percent embryo mortality was sometimes seen. This, however, was very inconsistent.

4.3.3 Chorio-allantoic membrane (CAM) inoculation

The original material was not inoculated via this route. The fifth yolk sac passage was inoculated via the CAM route. The first CAM passage displayed no embryo deaths or stunting of the embryos. There was slight thickening of the chorio-allantoic membranes in 40 percent of the embryos inoculated 7 days post inoculation, with the embryos displaying slight oedema and very mild liver necrosis. The remaining 60 percent of the embryos were unaffected (live normal). The second passage displayed 100 percent thickened chorio-allantoic membranes, with only 60 percent of the embryos displaying pathology which ranged from oedema, moderate haemorraging and slight to moderate liver necrosis. The third passage displayed 100 percent embryo pathology and thickened chorio-allantoic membranes with no embryo deaths. Embryo deaths were only seen in the forth and fifth passage which ranged from 20 percent to 60 percent of the embryos inoculated. The dead embryos displayed inconsistant pathology which ranged from oedema, haemorraging, pale embryos and slight to gross liver necrosis.

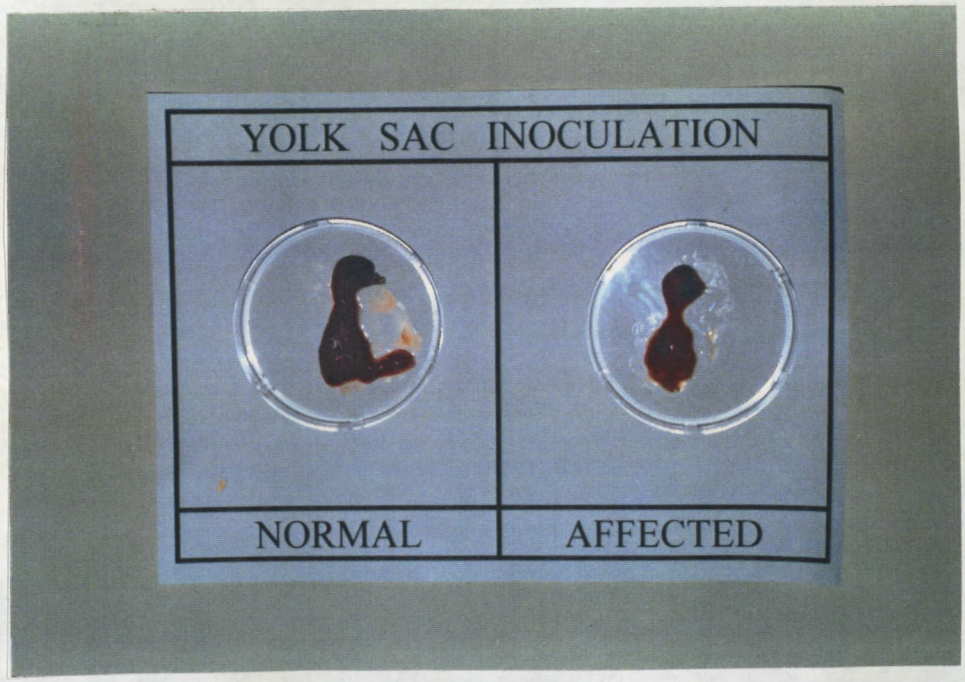

Plate 1: Yolk sac inoculation of SPF egg. Normal (left) and Entero-like virus-affected (right) embryo. Affected embryo appears stunted and congested.

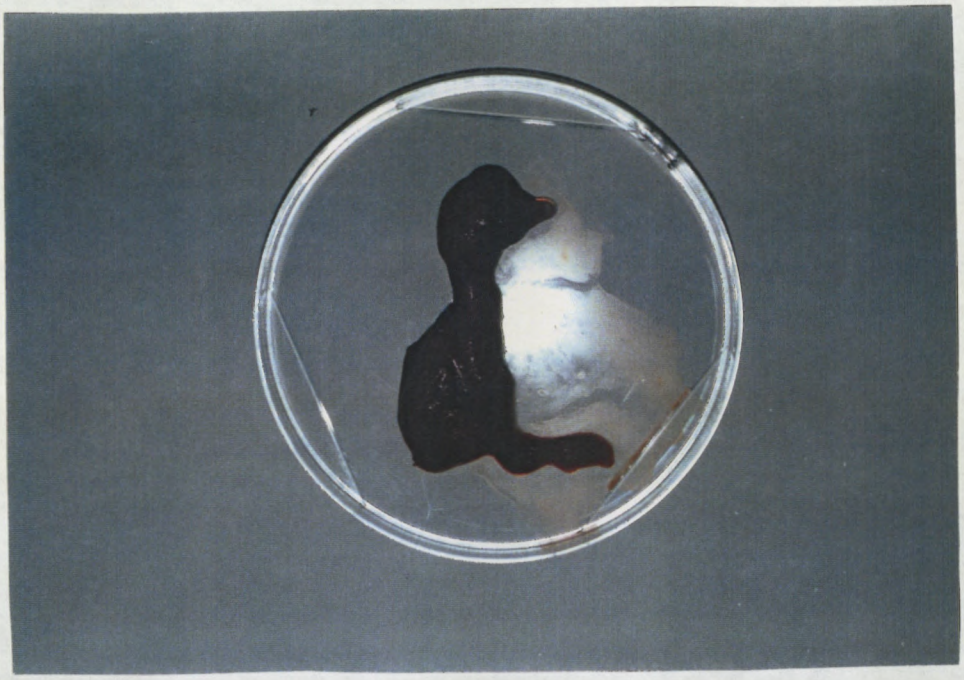

Plate 2: Normal Embryo (Enlarged Plate), yolk sac inoculation of SPF egg.

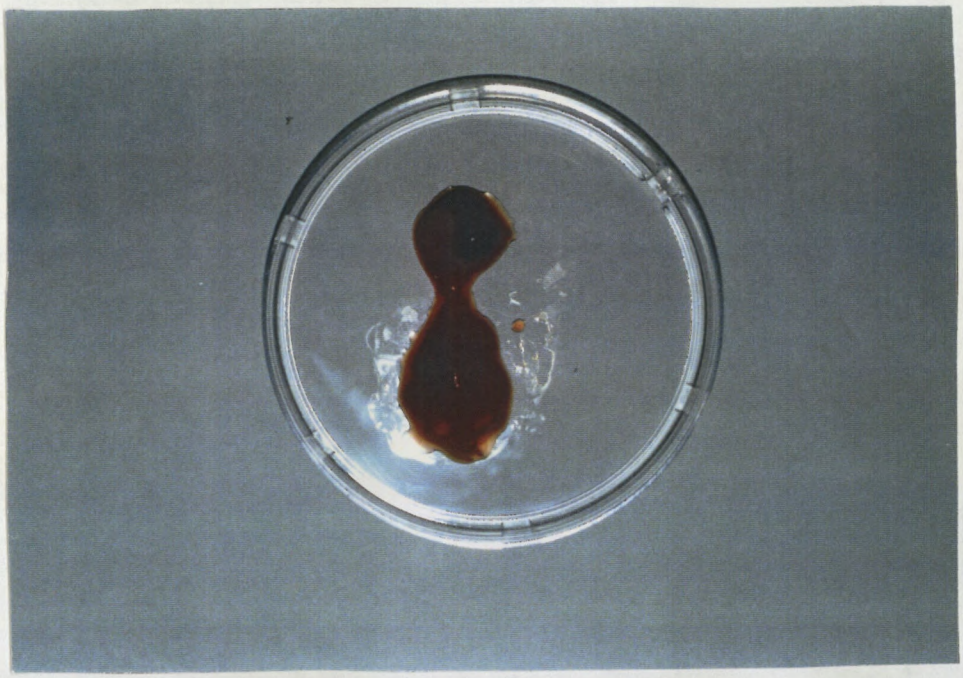

Plate 3: Embryo infected with Entero-like virus (Enlarged Plate), yolk sac inoculation of SPF egg. Embryo displaying severe stunting and haemorraging.

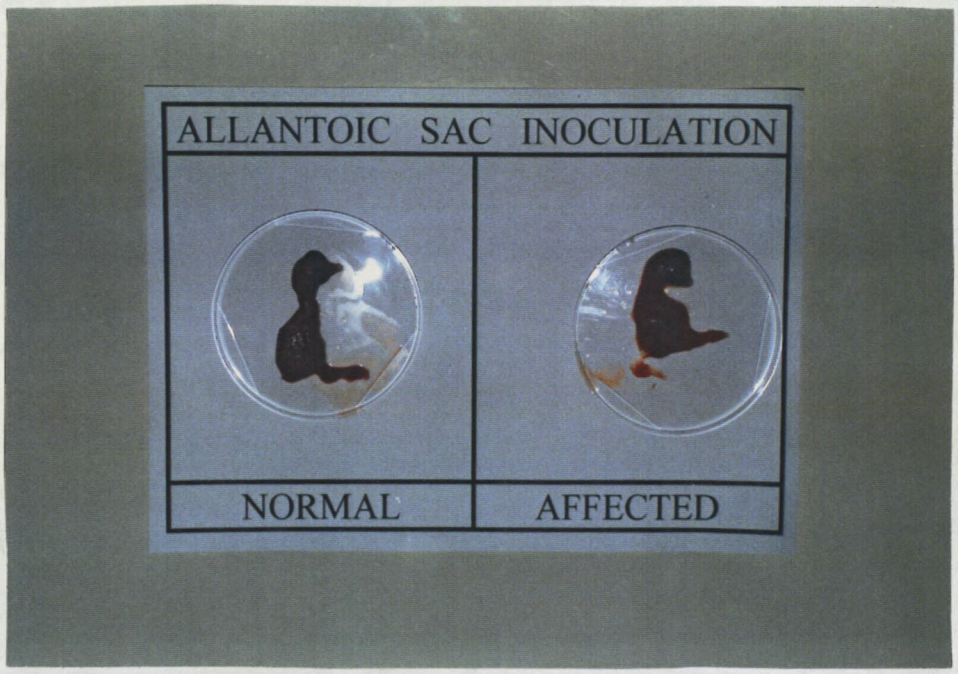

Plate 4: Normal embryo (left) and Entero-like virus-affected embryo (right), allantoic sac inoculation of SPF egg. Affected embryo appears pale, stunted and odematous.

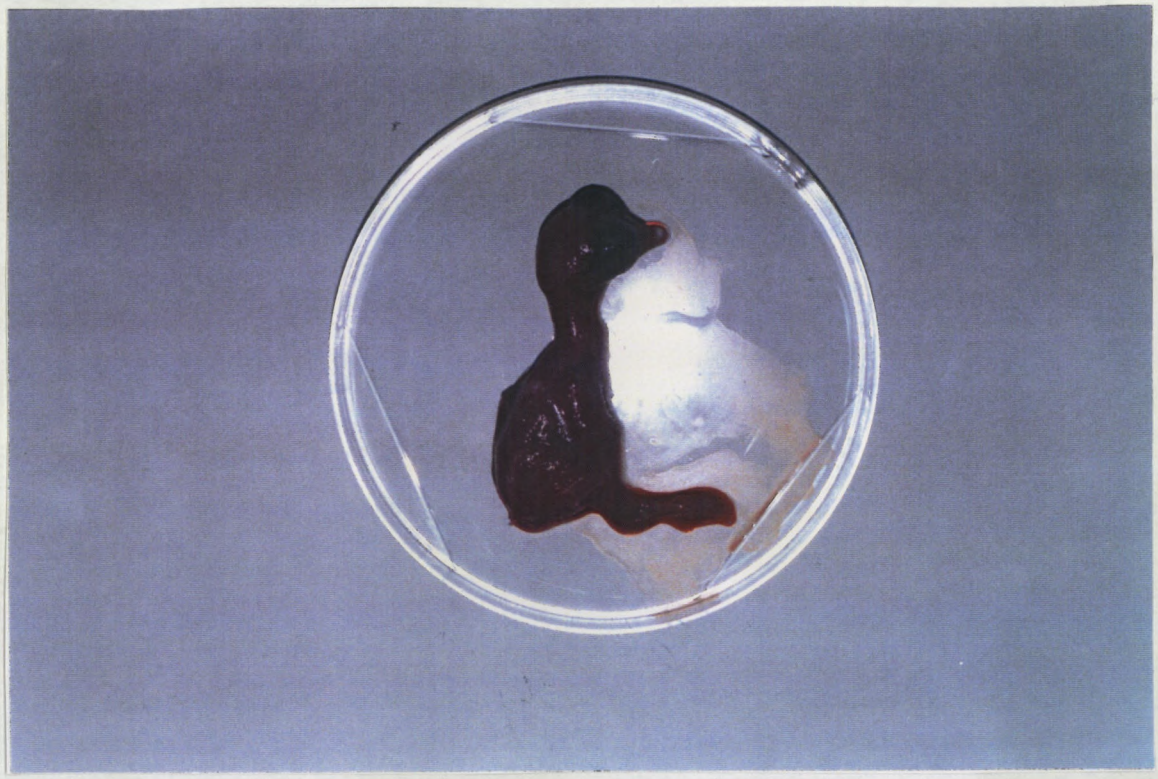

Plate 5: Normal embryo (Enlarged Plate), allantoic sac inoculation of SPF egg.

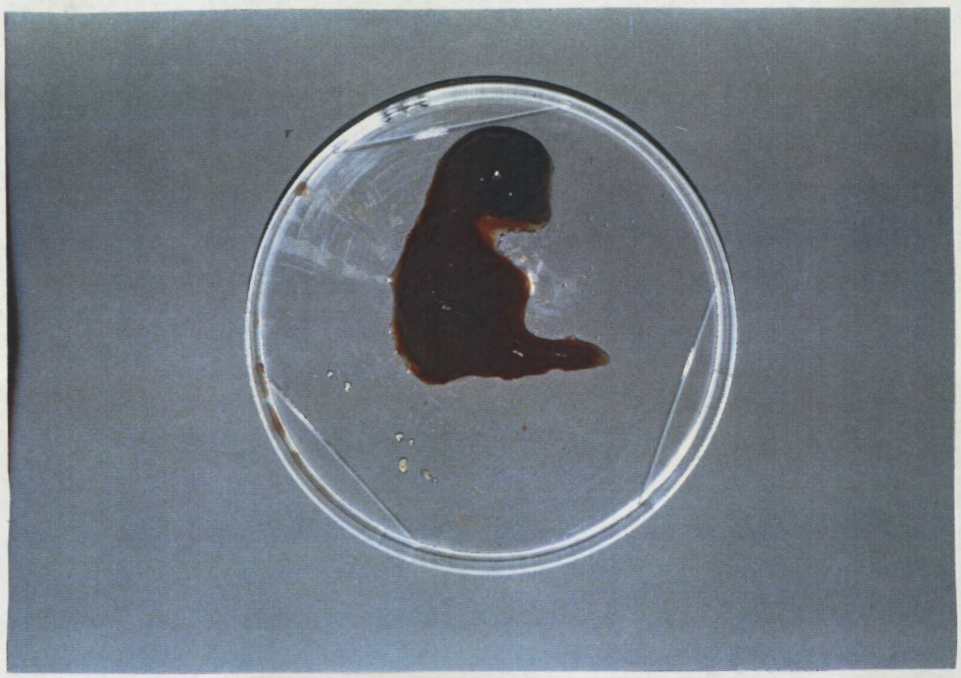

Plate 6: Entero-like virus-infected embryo (Enlarged Plate), allantioc sac inoculation of SPF egg. Affected embryo appears pale, stunted and oedematous.

4.4 Transmission Study

The purpose of the transmission study was to reproduce the disease in SPF and commercial birds. The first SPF group that was inoculated intracerebrally with 0.05 ml of a 10^{-1} dilution of challenge material displayed 60 percent mortality within 5 days of inoculation with the remaining 40 percent being very depressed. The survivors displayed lack of movement with both legs outstretched laterally consequently suggesting the possibility of "paralysis syndrome". The inability of these birds to move resulted in inaccessibility to feed and water. The survivors were very stunted and significantly under weight when compared to the control group (Group two). The control group showed no clinical symptoms of the "paralysis syndrome". The average live mass of the control group at 14 days was 188 grams whilst the average weight of the challenge group (Group one) survivors, at 14 days was 148 grams.

Post mortem analysis on the challenge group displayed severe dehydration of the internal organs with moderate swelling of the kidneys, possibly due to the inability of the affected birds to drink water. Brain and kidney samples were taken separately (aseptically) from the control group and challenge group. These samples were tested for the presence of bacteria, fungi and viruses. The control group produced negative results whilst the challenge group was negative for bacteria and fungi, but positive for a virus. The virus caused death, severe stunting, haemorraging, oedema and necrotic liver lesions of SPF embryos when inoculated via the yolk sac route. The embryo pathology and the death pattern was similar to that of isolate H02283/94.

The third SPF group that was inoculated orally with 0.5 ml of challenge material displayed no mortality. This group developed severe depression after 6 days with symptoms ranging from moderate paralysis, outstretched legs and stunted growth. The control group (Group four) was unaffected with the average weight being 180 grams and the challenge group (Group three) average weight being 142 grams. Brain and kidney samples were tested as per group one and two. The challenge group (Group three) was negative for bacteria and fungi but produced a virus that displayed similar embryo pathology to Group one. The control group (Group four) was negative for bacteria, fungi and viruses.

The first commercial Hubbard group that was inoculated intracerebrally with 0.05 ml of a 10^{-1} dilution of challenge material displayed no clinical symptoms of the paralysis syndrome. The control group also showed no symptoms. The average weight of the challenge group after 14 days was 380 grams with the average weight of the control group being 382 grams. Post mortem analysis of both these groups showed no evidence of any disease. Brain and kidney samples from both these groups were negative for bacteria, fungi and viruses.

The commercial Hubbard group that was inoculated orally with 0.5 ml of challenge material also showed no clinical symptoms of paralysis. There was no difference between the challenge and the control groups with the average weight of the control group being 375 grams and the average of the challenge group was 372 grams.

The disease ("Paralysis syndrome") was successfully reproduced in SPF birds but

not in the commercial Hubbard birds.

4.5 CELL CULTURE

4.5.1 Growth in CEF

There were no visible CPE on the first, second and third passages. The forth and fifth passages displayed poor cell growth when compared to unchallenged controls. This poor growth suggests viral CPE but upon Haematoxylin and Eosin staining, the monolayers displayed no inclusions or cytopathic change.

4.5.2 Growth in CEK

There was no visible CPE in all the passages.

4.5.3 Growth in Vero Cells

There were no visible CPE in all the passages and there were no abnormalities when viewed after Haematoxylin and Eosin staining.

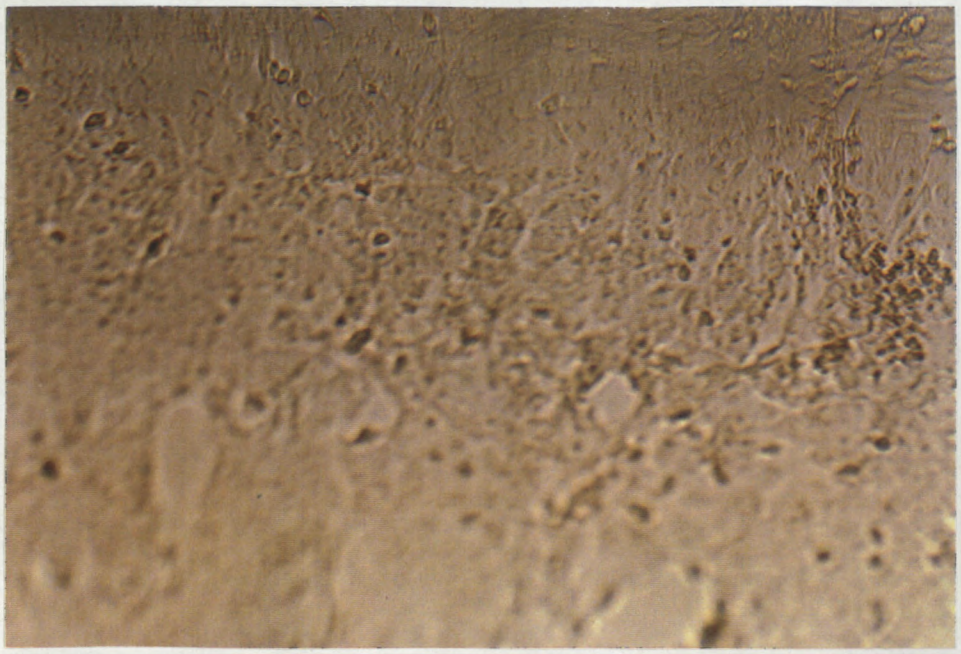

Plate 7: CEK (fifth passage) challenged with isolate H02283/94 displaying no visible CPE after H & E staining.

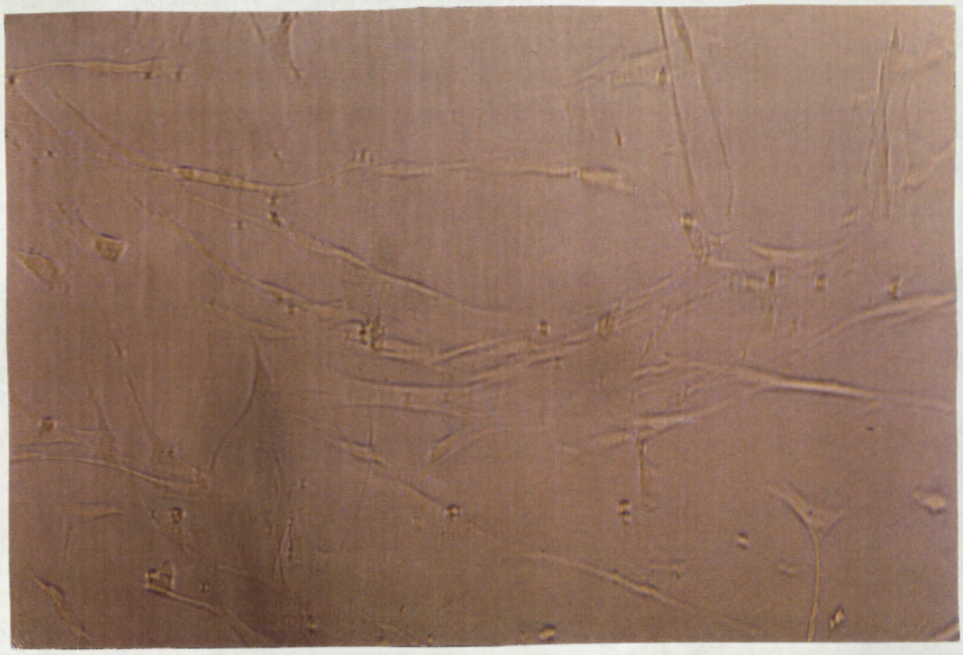

Plate 8: CEF (third passage) challenged with isolate H02283/94 displaying poor growth of monolayer, suggesting viral activity.

4.5.4 Titration of the virus

The titration of the H02283/94 isolate via the yolk sac and allantoic sac routes is depicted in Table3.

Table 3: Titration of the virus via the yolk sac and allantoic sac routes

ROUTE	AFFECTED/TOTAL PER DILUTION					EID_{50}/ml
	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
Yolk sac	5/5	5/5	5/5	2/5	0/5	$10^{6.6}$
Allantoic sac	3/5	0/5	0/5	0/5	0/5	$10^{3.8}$

Table 3 clearly shows that the virus grew better in the yolk sac than in allantoic sac.

4.6 Physical and Chemical Characteristics of the Virus

4.6.1 Haemagglutination Test (HA)

All passages of isolate H02283/94 was found to be free of haemagglutinating activity.

4.6.2 Heat Stability

The titres of isolates H02283/94 and the CELO virus (heat resistant control) were virtually unaltered when exposed to 56°C for 1 hour. However, the H_{120} IBV was completely inactivated when exposed to 56°C for 1 hour (Table 4).

Table 4: Titres of controls and isolate H02283/94 exposed to 56°C for 1 hour

SAMPLE	TREATMENT	TITRE (EID_{50}/ml)
H02283/94	Untreated	$10^{6.6}$
H02283/94	56°C 1 hour	$10^{6.4}$
H_{120} IBV	Untreated	$10^{6.8}$
H_{120} IBV	56°C 1 hour	0
CELO Virus	Untreated	$10^{6.4}$
CELO Virus	56°C 1 hour	$10^{6.2}$

4.6.3 Chloroform Sensitivity Test

The chloroform sensitive control H_{120} IBV failed to cause any embryo pathology or mortality when treated with chloroform, whereas the untreated sample caused 100 percent embryo mortality in both the 10^{-1} and 10^{-2} dilutions. The chloroform sensitive control was therefore inactivated by chloroform. Both the chloroform treated and untreated samples of the isolate H02283/94 and the CELO virus (chloroform resistant control) caused 100 percent embryo mortality in the 10^{-1} and 10^{-2} dilutions. The chloroform resistant CELO virus and the H02283/94 isolate was not affected by chloroform and therefore caused embryo mortality when inoculated into SPF embryonated eggs.

4.6.4 Nucleic Acid Determination

There was no difference in the titre of isolate H02283/94 and the NDV (RNA positive control), in the presence or absence of IDUR. However, the titre of the positive control sample (CELO virus) dropped from $10^{6.4}$ to $10^{3.2}$ EID_{50}/ml. Refer to Table 5 for results.

Table 5: Result of the nucleic acid determination test of isolate H02283/94

SAMPLE	TREATMENT	TITRE (EID_{50}/ml)
H02283/94	Untreated	$10^{6.6}$
H02283/94	IUDR Treated	$10^{6.6}$
NDV Clone 30	Untreated	$10^{9.4}$
NDV Clone 30	IUDR Treated	$10^{9.4}$
CELO Virus	Untreated	$10^{6.4}$
CELO Virus	IUDR Treated	$10^{3.0}$

4.6.5 Serum Neutralisation Test

Both the pre-bleed and the 4 week post challenge sera of isolate H02283/94 were negative for antibodies to NDV, IBV, Reo virus, IBDV, TRTV, MG, MS, AE and BWD. The results of the neutralisation test using monospecific (H02283/94) antisera is shown in Table 6.

Table 6: **Result of the neutralisation test against monospecific antisera to isolate H02283/94**

ISOLATE	VIRUS TITRE (EID_{50}/ml)	NEUTRALISING TITRE (EID_{50}/ml)	NEUTRALISATION INDEX
H02283/94	$10^{6.6}$	$10^{2.4}$	4.0
IBV H_{120}	$10^{6.8}$	$>10^{5.2}$	<1.6
CELO Virus	$10^{6.4}$	$>10^{5.2}$	<1.2
NDV Clone 30	$10^{9.4}$	$>10^{8.6}$	0.8

The H02283/94 isolate was significantly neutralised by the monospecific antisera of the above isolate. However, the negative IBDV, CELO virus and NDV controls were not neutralised by monospecific antisera to the H02283/94 isolate.

4.6.6 SDS PAGE Analysis of the Protein of the H02283/94 isolate

Plate 9 shows the result obtained after CBB staining of the SDS-PAGE gel. The molecular weights of the protein bands in the test sample (lane three), the normal control (lane two) and the high range molecular weight marker (lane one) were determined from the standard graph (Fig. 6). Table 7 depicts the molecular weights of the various bands obtained from the test and control samples. r, normal control and isolate H02283/94.

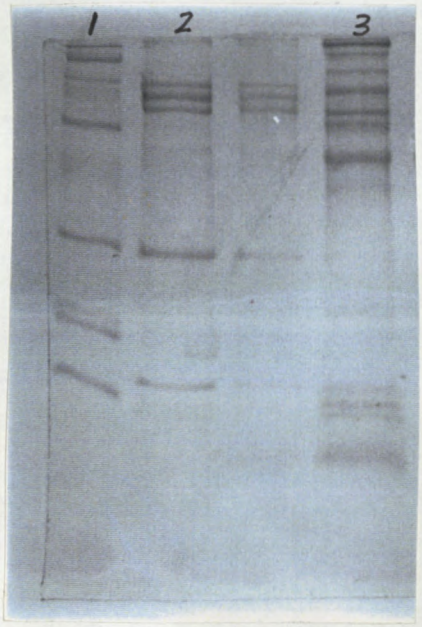

Plate 9: SDS PAGE electrophoresis gel of normal allantoic fluid and allantoic fluid containing isolate H02283/94.

Lane one = High range molecular weight marker.

Lane two = Normal control.

Lane three = Sample H02283/94 (affected allantoic fluid).

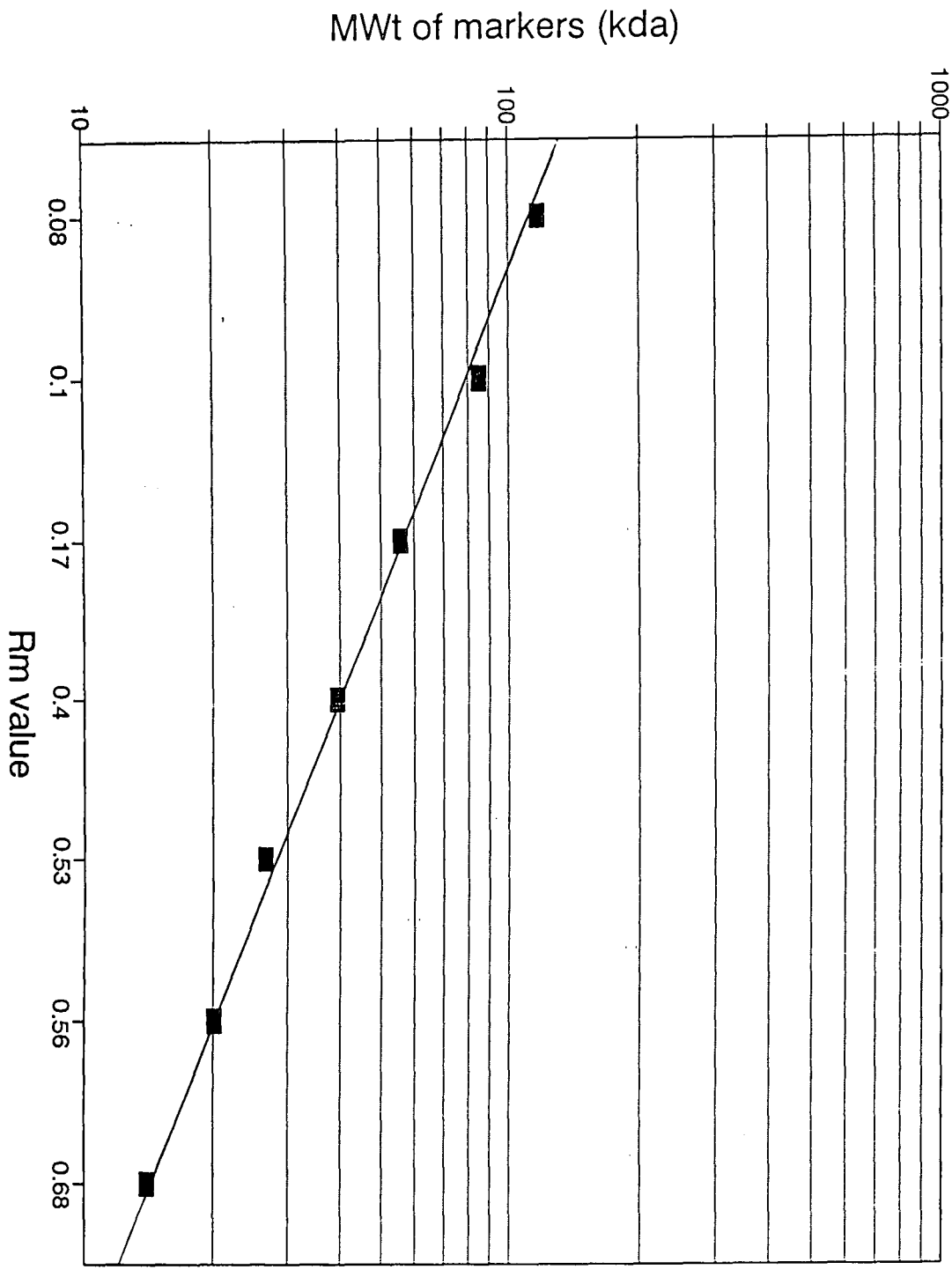

Figure 6. Molecular weights of marker against their respective Rm values

Table 7: The R_m values and resultant molecular weights (M_r) of the protein bands of isolate H02283/94 and normal allantoic fluid obtained using the standard graph

NORMAL ALLANTOIC FLUID		H02283/94	
R_m	M_r (kDa)	R_m	M_r (kDa)
-	-	0.065	93.16
0.07	92.44	0.07	92.44
0.1	88.16	0.1	88.16
0.13	83.87	0.13	83.87
-	-	0.15	81.82
-	-	0.22	71.01
-	-	0.25	66.73
-	-	0.27	63.87
0.41	43.87	0.41	43.87
0.65	9.58	0.65	9.58
-	-	0.66	8.15
-	-	0.68	5.29
-	-	0.71	1.01

4.7 Electron Microscopy

The total magnification of 150 000 times was used. The bar scale on the micrographs was 15 mm which is equivalent to 100 nm.

The electron micrographs (Plate 10) illustrate typical grouping of virus particles. The diagnosis of the virus particles in allantoic fluid was plagued by the overwhelming presence of structureless electron transparent particles of varying diameter between 10 nm and 200 nm. These particles probably originated from embryonic material consisting of lipid and proteinaceous components.

The virus particles can be distinguished from their background due to their characteristic aggregation, higher contrast, uniform size and isometric shape. The size varies on average between 28 nm and 32 nm on the micrographs due to the drying and flattening during preparation. These diameters correspond to the accepted size for Picornaviridae (Lennette and Scamidt, 1979). On the micrographs, some particles exhibit a hexagonal shape (Plate 11) which indicates an icosahedral symmetry. The virions have no envelope and the electron micrographs revealed no surface projections, with the surface being almost featureless although some fine, flat surface structures can be discerned in the negative contrast of the virus particles. No other virus like particles were observed in the sample.

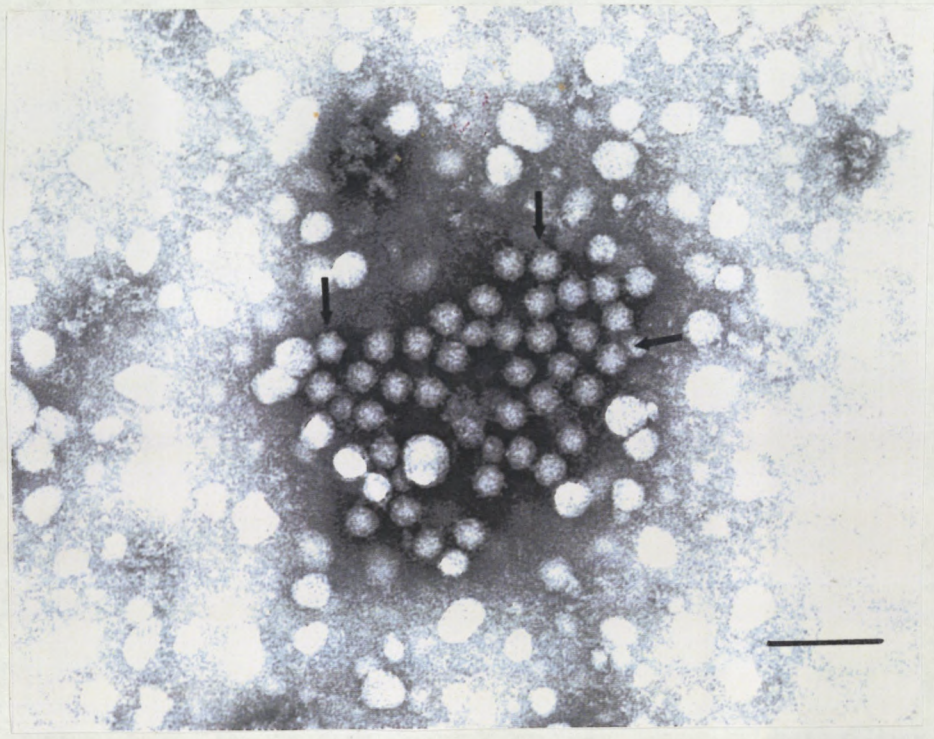

Plate 10: Electron micrograph of negatively stained Entero-like virus particles.

Bar = 100 nm.

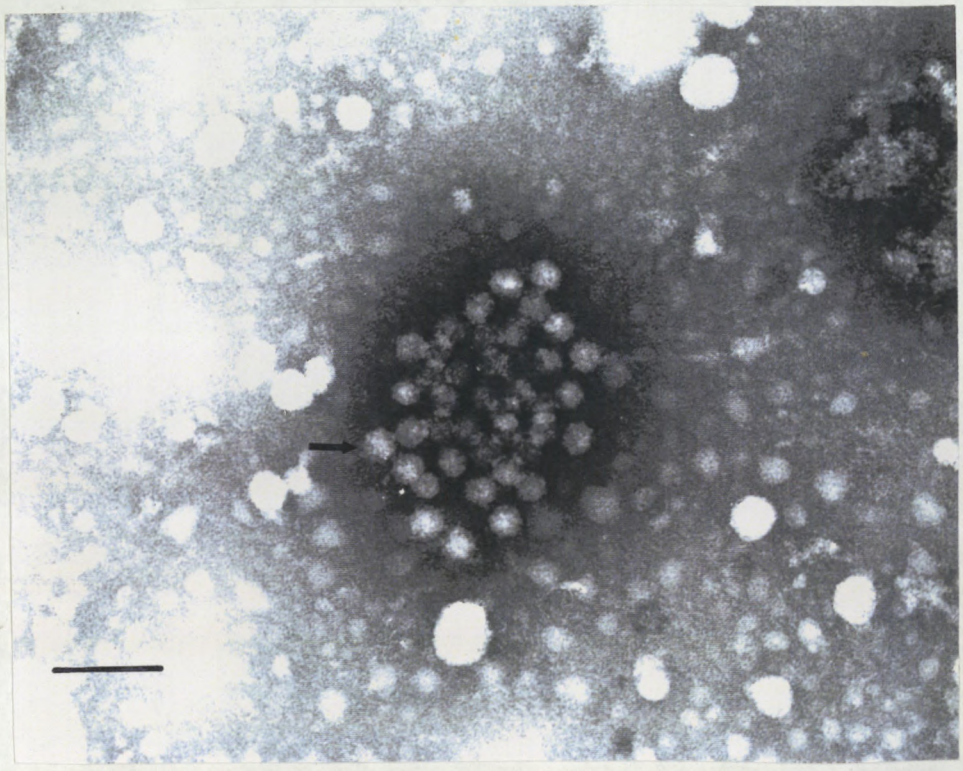

Plate 11: Electron micrograph of negatively stained Entero-like virus particles.

Bar = 100 nm.

4.8 Vaccine Production

4.8.1 Sterility Tests

There was no growth of microorganisms in any of the agars or broths.

4.8.2 Purity Tests

The virus was neutralised by monospecific antisera against the H02283/94 isolate. The neutralisation index was 4.2.

4.8.3 Safety Tests

The chick embryo safety test showed no signs of embryo pathology, thus indicating the absence of any viable or residual viruses. The degree of vaccination reaction was moderate with no abnormal reactions, necrosis, haemorraging or pus formation. The birds appeared healthy throughout the duration of the trial.

4.8.4 Serological Tests for Externous viruses

The SPF birds inoculated with the vaccine was serologically negative for the presence of antibodies against the various organisms tested. The results are shown in Table 8.

Table 8: **Antibody response of chickens pre and post vaccination**

ORGANISM	ANTIBODY TITRES*	
	PRE-INOCULATION	POST-INOCULATION
NDV	0	0
IBV	49	50
IBDV	68	70
Reo Virus	72	70
AEV	22	20
MG	Negative	Negative
MS	Negative	Negative

* = ELISA index values

NB. Index value > 100 is Positive.

4.8.5 Evaluation of Efficacy of the Vaccine in Field Trials

The result of the serum neutralisation test against H02283/94 isolate is shown in Table 9.

Table 9: Serum neutralisation results of vaccinated and non vaccinated

SERA	VIRUS TITRE (EID_{50}/ml)	NEUTRALISING TITRE (EID_{50}/ml)	NEUTRALISATION INDEX
Site A* Pre-bleed	$10^{6.8}$	$10^{5.0}$	1.0
Site A* weeks post-inoculation	$10^{6.8}$	$<10^{1.2}$	>4.8
Site B*	$10^{6.8}$	$10^{5.0}$	1.0

A* = Vaccinated Site

B* = Non-vaccinated Site

Four weeks post vaccination, there was a significant increase in the neutralisation index to isolate H02283/94. The pre-bleed and the non-vaccinated sera remained negative to isolate H02283/94. This indicated that the vaccinated flock sero-converted significantly to isolate H02283/94. The progeny from the vaccinated flock were monitored for any possible outbreak of the "paralysis syndrome". There were no outbreaks of the disease in any of the full house placements of the vaccinated flock. There were two further outbreaks of the disease on separate farms. One of the affected broiler farms had a part

placement of 20 percent from the vaccinated parent flock whilst the other affected broiler flock consisted of no chickens from the vaccinated parent flock.

CHAPTER 5

Discussion

When the disease was observed in Hammarsdale an Avian Encephalomyelitis (AE) virus outbreak was suspected (Morley, pers. comms. 1994). The reason for suspecting A.E. was due to the age susceptibility of the disease (5 to 18 days) and the clinical symptoms displayed by the affected birds, especially the fine head tremor. The serum sample from affected birds tested by Mr. D.N. Bhoora (Rainbow Laboratory) for antibodies to A.E. was negative. This eliminated A.E. virus as a possible causative agent. The post mortem and the histopathology analysis were inconclusive. This resulted in the search for the causative agent and implementation of possible control measures.

The results demonstrate that there were no pathogenic bacteria and fungi isolated from the affected birds. The feed and water from affected houses did not reproduce the disease symptoms when fed to SPF birds. The feed did not contain toxic levels of any significant toxins, thus eliminating the possibility of any toxin poisoning.

A virus, however, was isolated from the brain and kidneys of severely affected birds. Although care was taken during sampling for virus isolation, there is always the possibility that the virus isolated could have been a possible contaminant. The virus was therefore inoculated orally and intracerebrally into SPF and commercial Hubbard chicks. The disease symptoms were successfully reproduced in the SPF group, with the causative agent being re-isolated from

the brain and kidneys of affected birds. This satisfied the criteria for Koch's postulates. The SPF birds that were challenged intracerebrally were more severely affected with 60 percent mortality, whilst the orally challenged group had no mortality but did display moderate symptoms of the disease. A possible reason for the above result is because the orally challenged birds absorbed reduced amounts of the virus into the blood stream consequently decreasing the chances of the virus passing the blood-brain membrane barrier, thus displaying moderate symptoms of the disease. However, the direct inoculation into the brain allowed for the concentration of the inoculum in the brain thus producing a more severe reaction (Rosenwald, et al., 1959).

The commercial Hubbard group did not display any symptoms of the disease when inoculated orally or intracerebrally. The possible reasons for this could be because the Hubbard broiler chicks may have had high levels of circulating maternal antibodies. This is possible if the parent flock was exposed to the entero-like virus, thus producing antibodies to the virus and providing cross protection to the progeny (Calnek et al., 1997).

The virus grew optimally in the yolk sac of SPF embryonated eggs (Table 3). The first passage of the original brain and kidney material showed no embryo pathology.

This often happens when a virus is removed from it's original host and inoculated into a different host (SPF embryonated eggs). The virus sometimes requires a few passages to adapt to the new host which is possibly the reason

for no embryo pathology in the first passage. The virus, however, gradually adapted to the SPF embryonated eggs showing 20 percent embryo pathology during the second passage and showing 100 percent embryo mortality by the fifth passage.

The original material displayed no embryo pathology in both the first and second passages when inoculated via the allantoic sac. The reason for this is that the yolk sac route is recommended for the primary isolation of the Picornaviruses, because the yolk sac cell lining closely resemble that of the enteric cell lines in adult chicken (Freeman and Vince, 1974). The fifth yolk sac material did, however, adapt gradually to the allantoic route of inoculation with the embryo pathology (pale, oedematis, haemorraghic and necrotic livers) being inconsistant (Plate 1). The CAM route showed slow adaptation of the original material, with 100 percent embryo pathology (oedema, haemorrage and liver necrosis) seen by the third passage. This pattern of embryo pathology is indicative of entero-like viruses previously isolated at the Rainbow Farms laboratory (Maharaj, pers.comms. 1994).

Table 3 clearly shows that the virus grew better in the yolk sac than in the allantoic sac. This is indicative of the Entero-like viruses which grows best in yolk sac, possibly because the yolk sac closely resembles that of the enteric cell lines in chicken.

The virus appears to grow poorly in CEF, CEK and Vero cells (Plate 7, 8).

Enteroviruses when inoculated into CEF cell lines usually display CPE that is indicative of displaced nucleus that is condensed and basophilic and the monolayer gradually disassociates with cell fragments and cell cytoplasmic projections. It appears that the virus grows best in embryonated eggs (Plates 2 and 6).

The virus does not agglutinate chicken red blood cells, and is resistant to both chloroform and heat at 56°C for 1 hour (Table 4). It is resistant to the thymine analog 5-iodo-2'-deoxyuridine , thereby classifying the virus as an RNA virus (Table 5). The H02283/94 isolate was neutralised by monospecific antisera to itself, but was not neutralised by monospecific antisera to IBDV, NDV and CELO virus (Table 6). The above physio-chemical profile of the virus suggests the possibility of an entero-like virus.

The differentiation and characterisation of an Enterovirus by Computer-Assisted Viral Protein Fingerprinting was done by Holland, et.al, 1998. Four characteristic structural genes were identified as (viral protein) VP1, VP2, VP3 and VP4. The molecular weights were 30 kDa, 27 kDa, 24 kDa and 7 kDa. These molecular weights did not correspond with that of isolate H02283/94. Further analysis involving Western Blotting and reverse transcription - polymerase chain reaction (PCR) is planned on isolate H02283/94.

The electron micrograph (Plate 10) indicates a high contrast uniform size (28 nm to 32 nm) and an isometric shape. Some particles exhibited a hexagonal

shape which indicates an icosahedral symmetry (Plate 11). The virion had no envelope and revealed no surface projections, with the surface being almost featureless although some fine flat surface structures could be discerned. The diagnostic features displayed by electron microscopy is adequate to classify the virus into the Picorna viridae family.

There appeared to be a good antibody response in the parent flock that were vaccinated with the KOE vaccine of the entero-like virus (Table 9). The non vaccinated flocks had no antibodies to the entero-like virus (Table 8). There were no outbreaks of the "paralysis syndrome" in any full house placements of the vaccinated flock. The two outbreaks that did occur could have been due to a horizontal field challenge of the entero-like virus. The broiler farm consisting of 30 000 chicks (full house placement) from the non vaccinated flock had little or no antibodies to the entero-like virus, consequently resulting in the disease outbreak. The other farm with 20 percent part placement of the vaccinated parent flock also had a horizontal field challenge, but the virus could have only affected the chicks that belonged to the parent flock that were non vaccinated.

Since the last "paralysis syndrome" outbreak in May 1996, no subsequent outbreaks have occured, although only one house of parent flock birds out of a total of 96 houses were vaccinated. It is believed that due to the integrated poultry structure in Rainbow poultry farms and the close proximity of the farms with movement of equipment and personnel from farm to farm, the virus could have been propagated in the parent flocks. The exposure of the parent flocks to

the entero-like virus may have resulted in significant sero-conversion of these flocks. This could have resulted in vertical transmission of antibodies to the progeny which may have provided sufficient protection of the broiler flocks to a possible field challenge.

'Paralysis syndrome' is a novel disease and closely resembles 'spiking mortality syndrome' seen in America. Both these diseases appeared in the early 1990's and were researched at the same time. The control measures for 'spiking mortality syndrome' was published recently and was therefore not implemented during the 'paralysis syndrome' outbreak.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

This study has shown that an entero-like virus is the most likely causative agent of the "paralysis syndrome". The lack of its recognition in prior studies is due to the fact that "paralysis syndrome" is a novel disease and that an entero-like virus has never been isolated from poultry which displayed the classical symptoms of the disease.

A clinically similar disease, "spiking mortality syndrome" (SMS) was seen in America in the early 1990's (Brown, pers. observations. 1991). The most striking similarities of both the diseases is the severe hypoglycaemia, growth retardation and significant size variation which refers to the uniformity of the flock.

The hypoglycaemia in SMS appears to be caused by a virus rather than a bacteria, since relative blood glucose levels in chicks inoculated with bacteria-free filtrate were almost identical to that of chicks challenged with unfiltered material (Davis et al., 1994).

Feed-related causes of SMS such as mycotoxicosis, marginal dietary energy requirements, and unknown toxins perhaps associated with animal or poultry byproducts were ruled out since the uninoculated chicks receiving the same diet were unaffected.

The control of SMS has been attributed to two factors. The first factor aims at preventing an outbreak of the disease by controlling the darkling beetle (Alphitobius diaberinus) population. The second factor deals with managing an outbreak by introducing a controlled lighting program.

Since chicks in the field are constantly pecking at litter in chicken houses from the day of placement, they could easily ingest or inhale a transmissable agent shed from a previous flock. This should emphasise the importance of biosecurity and proper cleanout, disinfection, and "down time" between flocks of broilers. Control of darkling beetles (Alphitobius diaberinus) is important since they have been shown to be vectors of enteric pathogens of poultry (Despins et al., 1994). The fact that SMS symptoms were reproduced in broilers when fed an homogenate of darkling beetle larvae indicates the presence of a transmissable agent. Therefore, by controlling the beetle population or ideally completely eliminating these beetles, one could prevent the outbreak of SMS from cycle to cycle.

Alteration in glucagon production or release could change chicken glucose levels drastically. Based on limited findings in field broilers with SMS, pancreatic glucagon is severely depressed. Glucagon elevates blood glucose (for short-term, immediate demands, due to fasting and or physical exertion) by glycogenolysis. Long-term glucose production occurs in the liver by gluconeogenesis, the break down of fats and proteins by the liver, (Guyton, 1976).

The fact that mildly hypoglycaemic chicks with SMS recover when left unstressed for several hours may be explained by a shift to gluconeogenesis by these chicks. This would allow a rise in blood glucose back to normal levels, even with fasting as long as the birds are not stressed.

To date no KOE vaccine was produced to control SMS. The suggested control measures with a controlled lighting program was recommended. In a controlled lighting program, when birds are in total darkness for 10 hours, they are likely not to eat. As a result, these chicks were fasted for 10 hours in addition to the 4 to 6 hours of experimental fasting. Fasting for this amount of time, 14 to 16 hours, allows for gluconeogenesis to become the primary pathway of glucose production. Therefore glycogenolysis, the glucagon-glycogen pathway, is by-passed as a source for plasma glycosis (Davis et al., 1995). This is sufficient to revert the affected birds back to normal glucose levels.

In the case of the "paralysis syndrome", controlled lighting was not tried and its impact on controlling the disease cannot therefore be discussed. In America the chicken houses are cleaned after every third or forth cycle, and this increases the likelihood of a transmissible agent being carried from flock to flock. In Rainbow Farms (PTY) LTD, the chicken houses are washed and disinfected after every cycle. An effective insecticide is also added during the terminal disinfection to ensure the termination of any insects including darkling beetles. This, therefore, virtually eliminates the chances of a transmissible agent being carried from cycle to cycle.

Albeit, strict measures are currently imposed to maintain biosecurity, one cannot further emphasise the importance of strict biosecurity with regards to disease control. Personnel must shower (with an antiseptic soap) and change into farm clothing when entering a farm. The movement of mortality trucks between farms must stop and the mortality must be treated on individual sites in pickling tanks. The pickling tank contents must be removed only when tanks are full, preferably one farm per day. Vehicles should be sprayed (especially tyres) with a suitable disinfectant before entering farms. The veterinarian and laboratory staff that move from farm to farm on a daily basis should plan their visits from young flocks to older flocks. This will reduce any possibility of disease transfer from older flocks to young flocks. Continued training and educating of farm personnel is very important especially with regards to biosecurity and disease control. No movement of feed, water, personnel and equipment between farms must be allowed. If equipment must be used on different farms eg. vaccinators, then these equipment must be disinfected or fumigated with formalin and potassium permanganate prior to entering the farm.

Stress could aggravate the disease, therefore it is recommended that vitamins and electrolytes (Fort Dodge) be administered to the birds for 3 to 5 days. This mixture of 100g sachet with 200 litres water may be repeated, orally, whenever necessary. The addition of sugar at a rate of 10 kg per 800 litres of water is a recommendation to address the short term sugar loss.

The administration of a KOE vaccine appears to provide sufficient protection to

the broiler flocks. This is an expensive and labour intensive exercise and should only be considered when all attempts to control the disease has failed.

The fact that there were no further outbreaks of the "paralysis syndrome" since May 1996, suggests that other cost effective control measures may be used. There is a global reluctance for the administration of a live attenuated virus in the field as a possible control measure. The reasons for the reluctance are founded, in that there is always the possibility of mutation and reversal to pathogenicity of the virus. Also the stress factors involved in administering a live virus in parent flocks may significantly affect egg production. There is also the possibility of vertical transmission of the virus from parent to the progeny, which could result in a disease outbreak in broilers.

Due to the fact that no further outbreaks were seen since May 1996, one can assume that the entero-like virus has been propagated in parent flocks before the birds got into lay, consequently inducing significant antibody response but not affecting egg production of the parent flocks. Therefore early contact of the parent flock with the entero-like virus would be an important prophylactic measure, ensuring adequate titres of specific maternal antibodies in broilers (Mendelson et al ., 1995). The use of an attenuated virus for vaccine purpose remains for discussion.

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APPENDICES

APPENDIX 1.

Nutrient Broth with Antibiotics

1.0 g Lab Lemco Powder

2.0 g Yeast Extract

5.0 g Peptone

5.0 g Sodium Chloride

920 ml Distilled Water

Autoclave at 121°C for 15 minutes and cool to 40°C before adding 1 vial penicillin and 1 vial streptomycin ensuring that the upper limits are 10 000 ug/ml for penicillin and 1.0 mg/ml for streptomycin. Shake well and aseptically dispense 9 ml into sterile universal bottles and store at 4°C. This medium is suitable for collecting post mortem material for virus isolation and also for decimal dilution for virus titration.

APPENDIX 2.

Antibiotic Solution

4.0 ml Penicillin (Stock 600 ug/ml), required upper limit 10 000 ug/ml.

0.5 ml Streptomycin (Stock 3,0 mg/ml), required upper limit 1.0 mg/ml.

2.0 ml Garamycin (Stock 40 mg/ml), required upper limit 2.0 mg/ml.

40 ml Sterile Distilled Water.

Do not autoclave, dispense in 2 ml aliquots and store at -20°C.

APPENDIX 3.

1 Molar Phosphate Buffered Saline (PBS)

8.0 g Sodium Chloride (NaCl)

1.60 g Disodium Hydrogen Ortho Phosphate (Na_2HPO_4)

0.51 g Potassium Dihydrogen Ortho Phosphate (KH_2PO_4)

1.0 l Distilled Water

pH 7.2

Autoclave at 121°C for 15 minutes and store at room temperature.

APPENDIX 4.

Alsevers Solution

20.5 g Glucose

4.2 g Sodium Chloride

8.0 g Trisodium Citrate

0.55 g Citric Acid

1.0 l Distilled Water

pH 6.1

Autoclave at 121°C for 10 minutes and store at 4°C. Alsevers solution is suitable for red blood cell preservation.

APPENDIX 5.

SDS-Gel Loading Buffer

50 mM Tris.HCL (pH 6.8)

100 mM Dithiothreitol

2% SDS

0.1% Bromophenol Blue

10% Glycerol

APPENDIX 6.

Tris-glycine Electrophoresis Buffer

25 mM Tris

250 mM Glycine (pH 8.3)

0.1% SDS

APPENDIX 7.

3% Glutaraldehyde Fixative in PBS

0.8 g Sodium Chloride

0.16 g Disodium Hydrogen Ortho Phosphate

0.051 g Potassium Dihydrogen Ortho Phosphate

3.0 ml Glutaraldehyde

100 ml Sterile Distilled Water