THE CHARACTERISATION OF TURKEY RHINOTRACHEITIS VIRUS FROM CHICKENS AND THE DEVELOPMENT OF A SUITABLE VACCINE

by

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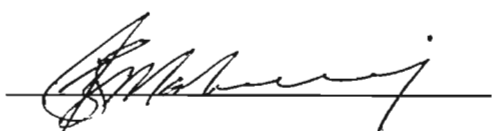
DECLARATION

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

Signed this 1st day of December 1994



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CONTENTS

ABSTRACT

Three turkey rhinotracheitis virus-like (TRTV-like) isolates were isolated from chickens with swollen heads. All grew well via the yolk sac (y/s), chorioallantoic membrane (CAM), tracheal organ culture (TOC), chicken embryo fibroblast (CEF), and Vero cell routes. Affected embryos were stunted and severely congested. No pathological alterations were detected in allantoic sac (a/s) inoculated embryos. The CEF and Vero cells required trypsinisation for five consecutive passages before any visible cytopathic effects (CPE) were observed. Intra-cytoplasmic eosinophilic inclusions were observed in Vero and CEF monolayers. Only isolate 652/93 caused 100% ciliostasis in TOC. The other two isolates were able to cause a maximum of only 70-80% ciliostasis. The isolates were inactivated by chloroform treatment and exposure to 56°C for 1 h. Long term storage could be achieved at -70°C or in liquid nitrogen but not at 4°C or at -20°C. The isolates did not agglutinate chicken red blood cells and were found to contain genomes of RNA. They were able to elicit TRTV antibodies in specific pathogen free (SPF) birds as measured with the Pathasure enzyme linked immunosorbent assay (ELISA) kit. They could also be neutralised by TRTV-specific antisera.

Electron microscopy of infective allantoic fluid (A/F) revealed particles of 100-300 nm in diameter with a helical nucleocapsid component approximately 15 nm in diameter and a fringe of approximately 12 nm long spikes. The processes of VLP development and maturation in TOC's and Vero cells were similar with

accumulations of virus-like nucleoprotein close to the plasma membrane, forming the nucleocapsid. Virus-specified spikes were then inserted into the plasma membrane after which the VLP budded from the plasma membrane, incorporating this membrane with spikes as its own.

Nine viral polypeptides with molecular weights of 200kDa, 83kDa, 53kDa, 40kDa, 37kDa, 28kDa, 19kDa and 15kDa were detected by SDS-PAGE in samples of the three isolates. The 83kDa and 53kDa polypeptides were also detected by western blotting using TRTV-specific antisera. Both, a TRTV and a 652/93 isolate non-radioactive DNA probe, appeared specific for TRTV and TRTV-like isolates. The 652/93 probe detected 652/93 virus in SPF chickens for 19 days post-inoculation.

A vaccine produced in SPF eggs using the attenuated 652/93 isolate, was able to protect vaccinates against virulent virus in laboratory challenge studies. In field trials, birds vaccinated at day-old or at day-old and again at 14 days, showed slightly improved performance compared to non-vaccinated birds. However, this benefit was not statistically significant. It is suggested that other environmental and disease factors mask the benefit provided by the vaccine in field trials.

The three TRTV-like isolates appear to be chicken strains of TRTV and vaccination with an autogenous vaccine appears to afford some benefit to vaccinates.

CHAPTER 1

REVIEW OF TURKEY RHINOTRACHEITIS AND SWOLLEN HEAD SYNDROME

1.1. The Poultry Industry in South Africa

Chicken meat is a relatively inexpensive source of protein for millions of South Africans and the country's broiler industry is well geared to adequately supply the increasing demand for chicken meat. The demand for white meat is further stimulated by the increasing awareness of consumers that red meat as a staple food, is unhealthy.

South Africa has an extensive poultry industry responsible for approximately 1% of the world poultry production and is the largest poultry meat producer in Africa, producing approximately 394 000 metric tons per annum, which comprises 22% of Africa's total production (FAO, 1992). The production of poultry in South Africa has steadily increased at an estimated rate of 10 000 metric tons per annum from 1989 to 1991 (FAO, 1992). World production of poultry meat represented 21.3% of all meat in 1986 and, in the United States of America (USA), chicken consumption had overtaken that of pork by 1986 and of beef in 1988 (Jordan, 1990). To maintain the continued growth of the poultry industry, it is essential that successful disease control be achieved by recognition, treatment and prevention of the diseases.

The only poultry production of economic significance in South

Africa, is chicken. Production of other poultry is mainly at an informal level.

1.2. Symptoms and Pathology caused by Turkey Rhinotracheitis Virus and the Swollen Head Syndrome Virus

The Afrikaans word for swollen head syndrome (SHS), "dikkop", aptly describes the condition frequently seen in turkeys and chickens suffering from a severe respiratory disease.

"Dikkop", or SHS, was first described in turkeys by Buys and Du Preez (1980) in South Africa, and later in Great Britain by many researchers (Anon, 1985; McDougall and Cook, 1986; Naylor and Jones, 1993; Wilding, Baxter-Jones and Grant, 1986; Wyeth, Gough, Chettle and Eddy, 1986). However, Morley and Thomson (1984), described a swollen head condition in broilers in South Africa that was seen from as early as 1972. According to Box (1989), turkey rhinotracheitis (TRT) was present in Europe from 1981 but was not linked to the disease previously described in South Africa. Initially, outbreaks of the disease were sporadic, but later became epidemic (Box, 1989).

Various names had been used to describe the disease in turkeys, including TRT, turkey coryza and acute respiratory syndrome (Wyeth, 1990). The names "TRT" and "SHS" are currently used to describe the disease in turkeys and chickens respectively.

The symptoms of TRT are consistent irrespective of the geographi-

cal region of the disease outbreak. Symptoms associated with TRT are sneezing, coughing, snicking, rales and a watery nasal discharge which thickens with progression of the disease and becomes turbid and purulent, eventually blocking the nares (Buys, Du Preez and Els, 1989a; Collins, Gough, Lister, Chettle and Eddy, 1986; Gough, Collins and Hancock, 1988b; Naylor and Jones, 1993; Wyeth, 1990). Other clinical signs of disease include depression, change of voice, gasping, torticollis, sub-mandibular oedema, swollen infra-orbital sinuses, and foamy occular discharge (Jordan, 1990; Wyeth and Alexander, 1989). Birds are eventually forced to breathe with open mouths (Buys et al., 1989a). The clinical symptoms appear to be more severe during the colder months of the year (Jordan, 1990).

Post mortem examinations reveal sinusitis, conjunctivitis, rhinitis, tracheitis, and pericarditis (Naylor and Jones, 1993; Wyeth, 1990). Jones, Williams, Baxter-Jones, Savage and Wilding (1988), found after experimental inoculation of turkeys with a TRT virus (TRTV), abnormalities in the oviducts 2-12 days post-inoculation (pi). This included the deposition of inspissated albumen and yolk material in the abdominal cavity. The virus did not appear to replicate in the lungs or air sacs. However, virus was detected in the epithelium of the uterus on day 7 pi and in all other regions of the oviduct on day 9 pi. Virus was also isolated from the middle magnum and vagina on day 9 pi.

Microscopic changes may occur in the mucosa of the nares and trachea between 4-10 days after infection (Jordan, 1990). The

first abnormality is a focal loss of the cilial layer at 2 days pi (Jones, Baxter-Jones, Wilding and Kelly, 1986). Jones et al. (1986) demonstrated changes in the trachea following experimental inoculation of turkeys with virulent virus. There was focal discontinuity at day 2 followed by extensive loss of cilia by day 4 together with extrusion of epithelial cells. Vacuolation and cell debris, together with infiltration of heterophils and lymphocytes, within the epithelium, were observed. Recovery occurred after approximately 10 days pi, and the tracheal epithelium returned to normal (Jones et al., 1986).

TRT infection is confined to the upper respiratory tract (Cook, Ellis and Huggins, 1991; Cook, Kinloch and Ellis, 1993b; Jones, 1993; Jones, Baxter-Jones, Savage, Kelly and Wilding, 1987). Jones et al. (1986), and Baxter-Jones, Wilding and Grant (1986), detected virus in the tracheas of experimentally infected poults 2-4 days pi. According to Gough et al. (1988b), no generalised viraemia occurs, confirming that replication of the virus is localised in the respiratory tract. Cook et al. (1991) recovered large amounts of TRTV from the respiratory tract of turkey poults for 5 days after experimental eyedrop inoculation and they found that small amounts of virus could be shed up to 14 days after inoculation.

The disease of TRT is characterised by a rapid onset and very rapid spread through a flock. Morbidity is often 100% within 24 hrs of the first signs of the disease (Buys et al., 1989a; Wyeth, 1990). Mortality may vary from 1-50%, but could reach 90% in some

cases (Gough et al., 1988b; Wyeth, 1990). Mortality rates may be increased by the presence of other pathogens, poor hygiene or ventilation, overstocking, and cold damp weather (Jordan, 1990; Naylor and Jones, 1993). Layers often suffer a drop in egg production of up to 70%, which is of sudden onset and lasts for 10-21 days (Peleteiro, 1991).

The disease originally occurred in older turkeys between 9-20 wks of age (Wyeth, 1990), but Gough et al. (1988b) showed that TRTV could affect flocks from as early as one day of age, despite the fact that they had maternal antibodies. It therefore appears that maternal antibodies cannot protect poults in the face of a severe TRT outbreak. According to Wyeth (1990), mortality is usually higher in younger turkeys and affected birds take approximately 3 weeks to fully recover.

An outbreak of TRT in South Africa in 1978, completely destroyed the country's turkey industry (Stuart, 1986), and there are at present no large scale commercial producers of turkeys. There were also devastating effects of TRT in the United Kingdom (UK) (Gough et al., 1988b).

The involvement of a TRTV-like virus in SHS, was confirmed by histology, electron microscopy, serology and challenge studies (Gaudry, 1991; O'Brien, 1985; Picault, Giraud, Guittet, Bennejean, Lamande, Toquin and Gueguen, 1987; Vanmarcke, 1989). TRTV has been isolated from chickens with clinical SHS (Buys, Du Preez and Els, 1989b; Jones, Naylor, Bradbury, Savage,

Worthington and Williams, 1991; Wyeth, Chettle, Gough and Collins, 1987), and Picault et al. (1987) were able to reproduce TRT in turkeys with an isolate from chickens with SHS. Swollen-head syndrome causes extensive losses in the chicken industry and the syndrome appears to be more predominant in broilers, than in layers (Gaudry, 1991). According to Shane (1992; 1993), SHS in broiler chickens is an emerging condition with a complex aetiology.

The symptoms of SHS vary from broilers to layers or breeders, the last two groups showing a similar picture (Anthony, 1992; Peleteiro, 1991). In broiler chickens, SHS may result in only 1-2% of the birds exhibiting pronounced lesions and symptoms (Buys, 1990; Cambridge Veterinary Sciences, 1989; Pattison, Chettle, Randall and Wyeth, 1989). However, according to Peleteiro (1991), morbidity is usually high, ranging from 1-90%, depending on the general condition of the flock. Mortality is generally low, rarely exceeding 10%. The disease also has a rapid onset (Anthony, 1992).

SHS in broilers usually occurs between 20-30 days, but it has been observed in birds 15 days of age (Naylor and Jones, 1993; Shane, 1992). Flocks initially appear depressed, with mild sneezing and coughing, followed by a nasal discharge (Anthony, 1992; Buys et al., 1989b; Peleteiro, 1991; Shane, 1992). The first symptoms of SHS in broilers are often disregarded, as they appear as mild respiratory distress mainly at night. However, within 48 hours, the birds show hyperlacrimation, congestive

conjunctivitis, oedema of the eyelids and perioccular oedema. The oedema extends to the base of the head and the inter-mandibular space and wattles. Affected birds show an abnormal "almond eye" appearance (Anthony, 1992; Peleteiro, 1991; Shane, 1992). A nasal discharge that may last for 4 days, often occurs (Dr S.S. Buys, Early Bird, 1992 pers. comm.). Pericarditis, perihepatitis, rhinitis, and tracheitis, are common features (Cambridge Veterinary Sciences, 1989; Peleteiro, 1991).

In the acute form of the disease, birds with nervous symptoms such as torticollis, incoordination and opistotonus, are observed (Anthony, 1992). The main problem in broilers, is that there is a decrease in food intake, resulting in poor growth performance (Buys, 1990; Naylor and Jones, 1993; Peleteiro, 1991). Birds that do not eat or drink, die of dehydration and exhaustion (Shane, 1992). Recovered birds often have airsacculitis, due mainly to Escherichia coli (Anthony, 1992; Buys, 1990). The disease in broilers, runs its course in 7-10 days (Peleteiro, 1991).

In commercial layers and broiler breeders, SHS mainly affects the birds at the peak of production, or just before they enter the production stage (Naylor and Jones, 1993; Peleteiro, 1991). According to Vanmarcke (1989), floor reared broiler breeders show the most clear and intensive symptoms. Prior to the appearance of the obvious clinical signs, birds appear depressed, and there is a slight increase in mortality. A decrease in food intake may occur. Morbidity at this stage is generally low between 1 and 5% (Anthony, 1992). The disease has a rapid onset and can cause a

drop in egg production of between 5-40% (Peleteiro, 1991; Shane, 1992). There may be a drop in the percentage hatchability (Cambridge Veterinary Sciences, 1989). Chicks from eggs laid during this period also perform poorly (Peleteiro, 1991). Egg production returns to normal after 2-3 wks of the onset of SHS.

Apart from oedema of the head, pin-point haemorrhagic lesions occur in the palatine cleft and mucosae of the upper respiratory tract. From these lesions, bacterial complications occur with inflammation of the surrounding tissue. These cause the head and sinus swelling (Pattison et al., 1989). The secondary bacterial infections in the brain are responsible for the nervous disorders (Vanmarcke, 1989).

Haemorrhages may occasionally occur in the pericardial fat (Eleazer, 1991). According to Peleteiro (1991), the kidneys occasionally become inflamed and congested, and disturbances of the ovaries also occur. Involution of the ovaries with surrounding moist inflammatory lesions and released yolk free in the peritoneum, were found (Pattison et al., 1989; Peleteiro, 1991). Some birds have a foul smelling greenish diarrhoea with nervous symptoms similar to that of affected broilers (Eleazer, 1991; Naylor and Jones, 1993).

The disease runs its course in layers and breeders in 4-6 wks but although the mortality and morbidity may be low, affected birds often die (Anthony, 1992).

1.3. Global Distribution of TRT and SHS

TRT and SHS have been reported from many countries where intensive rearing of turkeys and chickens for meat occurs (Lister and Alexander, 1986). Although SHS was initially observed in turkey producing areas, it was recently found in areas where no turkeys were grown (Anthony, 1992).

TRT was first seen in turkeys in South Africa in 1978 by Buys and Du Preez (1980). In 1978, a virulent outbreak of a similar disease occurred in Israel (Weisman, Strengel, Blumenkranz and Segal, 1988), and spread to Germany in 1980 (Stuart, 1986). The disease was then seen in The Netherlands and France in 1981 (Alexander, Borland, Bracewell, Chettle, Gough and Lister, 1986a; Alexander, Gough, Wyeth, Lister and Chettle, 1986b; Giraud, Bennejean, Guittet and Toquin, 1986; Giraud, Le Gros, Guittet, Bouquet, Toquin and Bennejean, 1987; Wyeth, 1990). In June 1985, an acute, highly contagious respiratory disease of turkeys was reported in England and Wales (Anon, 1985). Many subsequent reports on the occurrence of the virus have been made from the UK (Gough et al., 1988b; McDougall and Cook, 1986; Wilding et al., 1986; Wyeth et al., 1986). However, according to Baxter-Jones (1992), TRT could have been present in Europe since the early 1970's. According to Andral, Louazis, Trap, Newman, Bennejean and Gaumont (1985), all European countries experienced a similar type of disease. TRT has also been reported from Hungary, Italy, Northern Ireland, Greece, Spain and Mexico (Eleazer, 1991; O'Loan, Allan, McNair, Mackie and McNulty, 1990;

Wyeth, 1990). An extensive study of Australian turkey flocks failed to find any positive sera (Bell and Alexander, 1990).

Strict quarantine procedures enforced by the USA, prevented the early occurrence of TRT in that country (Pearson, Senne, Panigrahy and Mixson, 1993). However, Grow (1992) confirmed the presence of TRT in North Carolina.

SHS has been reported from many countries including England, France, Portugal, Spain, Germany, Belgium, Italy, Saudi Arabia, Kuwait, South Africa, Taiwan, Mexico, Mauritius, Argentina, Canada, The Netherlands, Israel and Peru (Buys et al., 1989b; Eleazer, 1991; Goren, 1985; Lu, Shien, Tsai, Tseng, Lee and Lin, 1994; O'Brien, 1985; Pages, Ramis and Majo, 1992; Perelman, Meroz and Samberg, 1988; Picault et al., 1987; Shane, 1992; Zellen, 1988). Chicken sera from Hungary, Morocco, Malaysia, Philippines and the Dominican Republic, tested at Rhone Merieux in France, confirmed the presence of SHS in these countries (Gaudry and Le Gros, 1993; Vanmarcke, 1989). The syndrome was first observed in South Africa in the early 1970's by Morley and Thomson (1984). The prevalence of the disease increased since then with the expansion and intensification of the country's broiler industry.

SHS remains a problem in most countries, especially in areas of high poultry density (Shane, 1992). As is evident, TRT and SHS are present virtually throughout the "poultry world".

1.4. Isolation and Propagation

The viruses responsible for TRT and SHS, are very elusive. Successful isolation requires that tissues from the upper respiratory tract be submitted from early in the course of the disease (Jordan, 1990). Once clinical signs are apparent in the flock, the chances of isolation are considerably reduced (Baxter-Jones, 1992). According to Jordan (1990), it is difficult to isolate the virus beyond 6-7 days after infection (3-4 days after the onset of clinical signs).

Some researchers have been successful in the isolation of TRTV from chickens with SHS(Buys et al., 1989b; Jones et al., 1991; Picault et al., 1987; Wyeth et al., 1987). However, it is more difficult to isolate the virus from chickens, than it is from turkeys (Baxter-Jones, 1992). According to Buys (1990), the best samples for virus isolations are from birds with a distinct nasal discharge.

TRTV or SHS virus has most consistently been isolated using tracheal organ culture (TOC) of chickens or turkeys (Buys et al., 1989a; Giraud et al., 1986; Gough et al., 1988b; Jones et al., 1986; McDougall and Cook, 1986; Wyeth et al., 1986). Picault et al. (1987) and Jones et al. (1991) used chicken TOC's to isolate TRTV from chickens. Ciliostasis usually occurred within 1 wk of inoculation.

Embryonated chicken eggs have also been used for the isolation

of TRTV and SHS viruses. The viruses caused congestion and haemorrhages on the embryos when the yolk sac (y/s) route of inoculation was used (Buys et al., 1989a,b; Gough et al., 1988b; Wyeth, 1990). Buys et al. (1989a) were able to grow TRTV on the chorio-allantoic membrane of embryonated specific pathogen free (SPF) eggs. However, when the TRTV or SHS virus was inoculated via the allantoic sac (a/s) route, there was a lack of embryo pathology, but the virus did replicate as evidenced by the visualisation of virus particles with the electron microscope (Buys et al., 1989b; Cook and Ellis, 1990).

The TRT and SHS viruses were able to grow in Vero cell monolayers, where they caused cytopathic effects (CPE) consisting of cytoplasmic eosinophilic inclusions and polykaryocytes (Buys et al., 1989a,b; Giraud et al., 1986). Gough et al. (1988b) were unable to grow the virus in chicken embryo liver cells (CELC), but Williams, Savage and Jones (1991a) successfully cultivated the TRTV in CELC. TRTV and SHS virus were also adapted to grow in chicken embryo fibroblast (CEF) cells where they caused the formation of syncytia and eosinophilic inclusions (Naylor and Jones, 1993).

1.5. Transmission

The methods of spread of the virus responsible for TRT and SHS, are still uncertain.

Although there is no evidence of transovarial transmission of the

virus, Jones et al. (1988), speculate that the replication of the virus in the oviduct, although for a short period, could cause some of the virus to be shed in the eggs which develop during this period. However, they found the ovary to be free of virus. Jones et al. (1988) believed that if transmission did occur by this method, then it was most likely a temporary occurrence of a low level and of little importance in the spread of the infection. It is now generally accepted that the transmission is horizontal (Peleteiro, 1991).

In the initial TRT outbreak in the UK in 1985, the disease spread within 24 h through the entire site, and during the next few weeks, it spread to other parts of the UK. Within 4 wks, 9 other outbreaks were reported. Within 3 months, 72 outbreaks occurred (Gough et al., 1988b; Lister and Alexander, 1986; McDougall and Cook, 1986; Wilding et al., 1986; Wyeth, 1990; Wyeth et al., 1986). The primary wave of infection affected turkey flocks of all ages, and the disease became enzootic. Thereafter, successive waves of infection occurred, affecting only those farms that had been depleted and restocked with susceptible poults. The frequency of the secondary waves of infection appeared to be dependent upon the density of the turkey population (Jordan, 1990).

In high density areas, most flocks experience disease during the first 16 wks of age, and in low density areas, secondary waves of infection may only be rarely encountered (Gaudry and Le Gros, 1993; Jordan, 1990).

The rapidity of spread of the virus, suggests that airborne transmission is the primary method, followed by the movement of vehicles, personnel, and feed, from contaminated to "clean" sites (Jordan, 1990; Shane, 1992). Poor biosecurity practices are important factors in the spread of disease (Gaudry and Le Gros, 1993).

Cook et al. (1991), showed that TRTV is highly contagious. A single infected bird within a group was able to rapidly infect all others in that group. This only occurred for the first nine days after infection. Seronegative sentinel birds placed in a flock previously exposed to TRTV, did not develop antibodies to TRTV. This suggests that TRTV is rapidly excreted from infected birds (Jordan, 1990). This was confirmed by D.K. Thomson (Rainbow Farms, 1993 pers. comm.).

The close relationship between TRTV and SHS virus explains why both diseases spread more rapidly in geographic areas with a high concentration of turkeys or chickens. Since their first appearance, TRT and SHS have evolved in a very irregular fashion characterised by periods of persistence in some countries and sudden disappearance in others (Peleteiro, 1991).

1.6. Host Range

The virus of TRT originally affected turkeys, but has since adapted to other avian species (Buys 1990, Gough et al., 1988b; Jones et al., 1987; Picault et al., 1987). According to Gaudry

and Le Gros (1993) the prevalence of TRT facilitated the adaptation of the virus to hosts different from the initial turkey.

Picault et al. (1987), showed that the TRTV and SHS virus were related, by reproducing TRT in turkeys with a homogenate from chickens affected with SHS. They were also able to reproduce the disease in guinea fowls.

Gough, Collins, Cox and Chettle (1988a), experimentally inoculated turkeys, chickens, Pekin ducklings, goslings, pheasants, guinea fowls and pigeons, with a TRTV. Only the turkeys showed obvious symptoms of infection. No clinical signs were detected in the other groups of birds, except for the pheasants, which had a transient conjunctivitis. Virus was reisolated only from the turkeys and chickens. This is in contrast to the results of Picault et al. (1987), who were able to reproduce the disease in guinea fowls. However, Gough et al. (1988a) detected antibodies against TRTV in sera from the turkeys, chickens, pheasants and guinea fowls. All other groups were negative for antibodies against TRTV. This suggests that the turkeys are the most susceptible to TRTV. However, these results were obtained under laboratory conditions, and may not represent the situation in the field, where many other factors contribute to the severity of the disease.

Jones et al. (1987), were able to induce mild clinical signs of infection in SPF chicks inoculated with TRTV. Cook, Dolby, Southee and Mockett (1988), suggest that TRTV could infect

chickens without necessarily being responsible for clinical disease.

In South Africa, a difference in clinical pathotypes, has been described (Buys et al., 1989b). The chicken SHS virus caused symptoms in both chickens and turkeys, but the turkey TRTV produced clinical disease only in turkeys (Buys et al., 1989b; Cook, 1993; Cook et al., 1993b). This indicates that the pathogenicity of the virus can evolve rapidly (Gaudry and Le Gros, 1993). According to Buys (1990), the TRTV from turkeys appears to be more host specific than the virus isolated from chickens. Further work on the SHS and TRT isolates of Buys et al. (1989b) by Cook et al. (1993b), showed that although the SHS isolate caused symptoms in both chickens and turkeys, the symptoms were more severe in the species from which the isolate was made. However, the symptoms observed were often only in a small proportion of the inoculates. They also found little difference in the clinical signs which the chicken isolate caused in seven different inbred chicken lines. Buys et al. (1989b), believed that the TRTV from turkeys could adapt itself to infect chickens. According to them, the viruses isolated in South Africa underwent changes with regard to their growth characteristics.

Many clinicians initially believed that the two diseases, TRT and SHS, were unrelated, but it is now accepted that the virus responsible for TRT is also involved in SHS of chickens (Cook et al., 1993b)

1.7. Interactions with other Agents

According to Shane (1992; 1993), a complex of factors is required to initiate TRT and SHS. This is especially true for SHS in broiler flocks. Some of the factors include immunosuppression associated with infectious bursal disease, chicken anaemia agent, Marek's disease, and reovirus; exposure to respiratory viruses or other unidentified agents, the presence of pathogenic bacteria such as pathogenic serovars of E. coli, environmental stress which exacerbates the incidence and severity of SHS in broilers, and the administration of live vaccines like Newcastle disease virus and infectious bronchitis virus, especially by the aerosol route (Gough et al., 1988b; Jordan, 1990; Shane, 1992; 1993).

Most specialists stress the importance of environmental factors in the onset of the disease (Anthony, 1992; Gough et al., 1988b; Jordan, 1990; Peleteiro, 1991). These include the stocking density of birds in the house, aeration of the house, levels of ammonia in the air, dust in suspension, poor general hygiene, and cold damp weather. In many TRT outbreaks, the respiratory disease was attributed to TRTV, but the high mortality which resulted in many cases, was attributed to the combination of factors, including secondary bacterial invasions, as listed earlier (Gough et al., 1988b; Jordan, 1990).

Cook et al. (1991), were able to recover virulent TRTV from the internal organs and intestinal tract of birds only if bacteria (Bordetella avium, and a Pasteurella-like organism) were adminis-

tered simultaneously. They suggest that the bacteria caused damage to the upper respiratory tract tissue, thereby permitting penetration of the virus. TRTV replication which is limited mainly to the ciliated epithelium of the upper respiratory tract, causes ciliostasis, resulting in suppression of an important mechanical barrier to bacterial and viral infections. The virus is in itself not immunosuppressive, but it reduces the capability of non-specific immunity (Gaudry and Le Gros, .1993). General immunity may not be of great help against infection, as compared to local immunity (Cook et al., 1991; Gaudry and Le Gros, 1993; Williams, Savage, Worthington and Jones, 1991b).

1.8. Physicochemical Properties

The agents responsible for TRT and SHS, have been identified as extremely pleomorphic viruses ranging in size between 40-1000nm (Buys et al., 1989a;b; Collins and Gough, 1988; Gough et al., 1988b; Picault et al., 1987; Wyeth et al., 1986). Virions were either filamentous or spherical; spherical forms ranging in size from 40-200nm in diameter, while filamentous forms differed widely in length with some as long as 1000nm. The virions possess a fringe of closely spaced projections 13-14nm long (Collins and Gough, 1988; Giraud et al., 1987; Gough and Collins, 1989; Wyeth, 1990). The internal helical nucleocapsid has a diameter of 14nm with a pitch of 7nm.

Ultra-thin sections of Vero cells, showed viral material in electron-dense accumulations in cytoplasmic inclusions (Giraud

et al., 1987; Buys, 1990). Virions budded from the cell through a modified outer-cell membrane. Pages et al. (1992) observed numerous spherical virions with diameters ranging from 100 - 200 nm in Vero cells, but they did not see any filamentous forms.

The viruses contain lipids and they lack haemagglutinating and neuraminidase activity (Buys et al., 1989a;b; Giraud et al., 1987; Wyeth, 1990). The viral genome consists of RNA of approximately 15000 bases (Dr D. Cavanagh, Institute for Animal Health UK, 1993 pers. comm.). These properties are characteristic of the Pneumovirus genus. Collins and Gough (1988), confirmed, using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the viral polypeptides and electron microscopy, that TRTV is a Pneumovirus. Also, the morphological and biological properties of TRTV resembled those of the human and bovine respiratory syncytial viruses (RSV), which are Pneumoviruses (Cavanagh and Barrett, 1988; Collins and Gough, 1988; Gough and Collins, 1989; Wyeth et al., 1986). Yu, Davis, Barrett, Binns, Boursnell and Cavanagh (1991), sequenced the fusion glycoprotein of TRTV and found a close identity with RSV while Cavanagh and Barrett (1988) found the mRNA profile to be similar to RSV. However, Yu, Davis, Brown and Cavanagh (1992) and Yu, Davis, Li and Cavanagh (1992) showed that RSV and TRTV had different gene orders.

Giraud et al. (1987), using SDS-PAGE, detected five viral proteins of molecular weights 94kDa, 83kDa, 58kDa 50kDa, and 35kDa. The 58kDa protein was believed to be a glycoprotein.

However, Collins and Gough (1988) and Gough and Collins (1989), reported the presence of seven viral polypeptides of 200kDa, 84kDa, 54kDa, 42kDa, 37kDa, 31kDa, and 14kDa. The 84kDa and 54kDa polypeptides were found to be glycosylated, which confirmed their viral specificity. The 42kDa and 31kDa polypeptides were tentatively designated as the "N" and "M" proteins respectively.

Ling and Pringle (1988), identified 8-10 TRT-virus-specified polypeptides with molecular weights of 129kDa, 83kDa, 57kDa, 45kDa, 38kDa, 35kDa, 30kDa, 22kDa, 19kDa, and 15kDa. They suggested that the 22kDa polypeptide could be a non-structural protein or a minor structural polypeptide. They also believed that the 57kDa polypeptide was a disulphide bonded dimer of the 45kDa and 15kDa polypeptides. They found that the 57kDa, 45kDa, and 15kDa polypeptides were glycosylated. Ling and Pringle (1988) attribute the differences in molecular weight estimations between their work and that of Collins and Gough (1988), to the different gel conditions and the different range of reference marker proteins employed.

The SDS-PAGE profiles of all TRTV isolates tested by Gough and Collins (1989), were found to be identical. The buoyant density of TRTV was found to be between 1.2 - 1.22g/ml (Collins and Gough, 1988; Gough and Collins, 1989).

1.9. Vaccine Development

Many researchers have attempted to develop vaccines against TRTV.

The methods employed to achieve this goal often differed. Some vaccines comprise egg passaged material, while others comprise Vero cell passaged material (Cook, Ellis, Dolby, Holmes, Finney, and Huggins, 1989a; Cook, Holmes, Finney, Dolby, Ellis and Huggins, 1989b; Gaudry, 1991; Gaudry and Le Gros, 1993; Giraud et al., 1987; Williams et al., 1991a;b).

According to Cook et al. (1991), the most urgent need is for a vaccine to protect poults against infection during the rearing stage when the stocking density is highest and when risk of secondary infection with pathogenic bacteria is greatest. This also applies to broilers, where the birds are subjected to enormous stress.

Buys et al. (1989a) found that their strain of TRTV was attenuated within three passages in Vero cells and poults vaccinated with this material, resisted subsequent challenge with virulent TRTV. They also found that the 17th chorio-allantoic membrane (CAM) passaged virus was sufficiently attenuated and afforded good protection against challenge with a virulent TRTV. Approximately 75% of the birds vaccinated with this material, were protected.

Giraud et al. (1987) used Vero cells to propagate their strain of TRTV. They found the 39th Vero cell passaged material to be suitable for a live vaccine. This vaccine offered good protection against experimental challenge. However, inactivated vaccine prepared with the 19th Vero cell passaged virus, was ineffective

and offered little or no protection against challenge infection.

Williams et al. (1991a) tested three preparations of a strain of TRTV, passaged differently, for their ability to protect poults against challenge with virulent TRTV. They used the 98th turkey-TOC passage, 28th CEF passage and 17th Vero cell passage material. The TOC preparation was still virulent after 98 passages, while the CEF passage material was over-attenuated and failed to protect poults. However, the Vero cell passage material was sufficiently attenuated and able to offer protection against clinical disease following experimental challenge. The TOC passaging is believed to closely simulate the bird to bird passage in the field resulting in little or no attenuation of the virus. However, birds inoculated with the TOC material were refractile to virulent challenge, suggesting that birds become immune to reinfection. Williams et al. (1991a;b) found that although birds were protected against virulent virus after vaccination, there was a very poor seroconversion against TRTV.

Further work by Williams et al. (1991b) showed that poults could be protected for at least 22 wks post vaccination and the vaccine was safe at ten times its normal dose. Attempts to back passage the virus from bird to bird, were unsuccessful.

A TRTV vaccine available from a commercial vaccine producer (Pittman-Moore), was able to protect turkeys from virulent TRTV with marked improvements in the performance of vaccinates compared to non-vaccinated control birds (Box, 1989). However,

this vaccine is costly, which prevents its widespread application for broilers.

Cook et al. (1989b) found that humoral antibody was not a good indicator of protection against TRTV infection, especially in older birds. Also, they found that maternally derived antibody may limit the effects of virus multiplication in a proportion of the birds, and the serological response to an attenuated TRTV in these birds could be very poor with many birds showing no detectable antibodies. They state that cellular immunity may be more important than humoral antibodies in the resistance to TRTV infection.

Cook and Ellis (1990) attenuated TRTV for vaccine use by alternate passaging in 9-day-old embryonated chicken eggs and chicken TOC. After 30 cycles of alternate passages, the virus caused only mild transient respiratory signs in turkeys and protected birds against challenge with a virulent TRTV 21 days later. This attenuated strain was stable with no reversion to pathogenicity after 12 turkey-turkey passages. The poult passaged and pre-passaged strains caused only mild respiratory signs, even when bacteria known to be associated with severe outbreaks of TRT in the field, were included in the inoculum (Cook et al., 1989a).

Cook et al. (1989a) used the severity of damage on the trachea as well as clinical signs, to determine the extent of attenuation. They found a good correlation between the degree of

deciliation of the trachea, and the clinical signs observed. The attenuated strain and the tenth poult-passaged virus, induced minimal damage to the ciliated epithelium. This attenuated strain was shown to be effective in protecting poults against experimental challenge with TRTV for up to 14 wks after vaccination. Immunity developed within six days of vaccination (Cook et al., 1989b). Poults free of TRTV antibodies and those hatched from TRTV-immune dams, were both equally well protected but the serological response of the latter was poor with many poults showing no detectable serum antibody response. Cook et al. (1989b) found that one ciliostatic dose 50% (CD_{50}) of vaccine was effective in protecting poults against challenge.

Gaudry (1991) and Gaudry and Le Gros (1993) found differences in the vaccination results obtained in the field and in the laboratory. The vaccine response in birds at the laboratory, appeared good when evaluated by seroconversion and challenge studies, but in the field, there was often no seroconversion even after two vaccinations, and when there was a seroconversion, it was low and of a shorter duration than that obtained in the laboratory. This demonstrates the major role of the farm environment in the evolution of the disease (Gaudry, 1991).

Broiler breeders given a live attenuated vaccine followed by a killed oil emulsion vaccine (KOE), were well protected against virulent challenge (Gaudry and Le Gros, 1993). The KOE vaccine provided a high level of enzyme linked immunosorbent assay (ELISA) and serum neutralisation (SN) antibodies, especially when

used as a booster vaccine after live vaccination (Gaudry, 1991). However, the quality of live vaccination remains a problem, as is the situation for other members of the Pneumovirus genus.

Although there are a few vaccines licensed for use against TRT in turkeys, there are no vaccines currently available, comprising a chicken pneumovirus isolate, for use against SHS in chickens. Many commercial chicken producers have implemented vaccination programs using the turkey TRT vaccine. According to Gaudry (1991), the solution to vaccination against TRT and SHS will not come from the laboratory, but from extensive field trials.

1.10. Serology

Initial attempts to develop a TRTV diagnostic test were hampered by the difficulty in growing the virus. The first successful tests employed SN (Baxter-Jones et al., 1986) and indirect immunofluorescence (IIF) (Baxter-Jones, Cook, Frazier, Grant, Jones, Mockett and Wilding, 1987), but ELISA tests were developed by Grant, Baxter-Jones and Wilding (1987), Chettle and Wyeth (1988), and O'Loan, Allan, Baxter-Jones and McNulty (1989). Cook et al. (1988) demonstrated by ELISA and SN, the presence of TRTV antibodies in commercial chickens after the initial appearance of TRT in turkeys in the UK in 1985. Sera prior to this, were negative for TRTV antibodies. Apparently healthy flocks also had TRTV antibodies, indicating an infection at some stage. Cook et al. (1988) suggested that TRTV could infect chickens without necessarily being responsible for clinical disease.

Antibodies to TRTV can be detected by ELISA within 6-10 days of experimental infection, and it reaches a peak at 14 days post-infection (Cambridge Veterinary Sciences, 1989). Jones et al. (1991) detected antibodies to TRTV in experimentally inoculated chicks and poults, 13 and 16 days pi respectively. Antibodies could persist for a minimum of 26 wks after initial detection (Baxter-Jones, Grant, Jones and Wilding, 1989).

Baxter-Jones et al. (1989) compared SN, IIF, and ELISA for the detection of antibodies against TRTV and found significant correlation between the three tests. However, O'Loan et al. (1990) found IIF to be the most sensitive, providing the widest range of antigens for antibody detection. The SN tests are type specific and the sensitivity of the ELISA, although more sensitive than the SN test, depends largely on the conservation of different epitopes during antigen production (O'Loan et al., 1990). Jones et al. (1986) and Baxter-Jones et al. (1986) used IIF to demonstrate the presence of TRTV in the ciliated epithelium of the tracheal cells. O'Loan and Allan (1990) used a streptavidin-biotin-immunoperoxidase staining technique to detect TRTV in formalin and Bouins-fixed paraffin embedded tissue. They believed that this method would enable detailed pathological studies of TRT and SHS.

It has been shown that all TRTV isolates from the UK are antigenically related (Collins and Gough, 1988; Gough and Collins, 1989; Baxter-Jones et al., 1987). O'Loan, Curran and McNulty (1992) demonstrated antigenic relatedness between TRTV

isolates from Great Britain, France and South Africa using immuno-gold labelling of TRTV. However, Baxter-Jones (1992) found antigenic differences between TRTV isolates from different geographical locations, but he was unable to distinguish between chicken and turkey isolates. He believes that the antigenic variation could be due to the different passage histories of the isolates following primary isolation, or it may be due to separate routes of virus introduction into different countries. Cook, Jones, Ellis, Jing and Cavanagh (1993a) examined TRTV isolates from various countries and found using polyclonal antisera that although there was some diversity between the isolates, all the strains belonged to one serotype. The TRTV strains isolated in the UK in 1985 were closely related to isolates of TRTV made in South Africa in 1978 by Buys and Du Preez (1980). They also confirmed the findings of Baxter-Jones (1992), that one could not differentiate between chicken and turkey isolates of a particular country based on neutralisation studies with monoclonal antibodies. However, the chicken and turkey isolates from one country could be differentiated from that of another country (Cook, 1993).

Gaudry and Le Gros (1993) were able to differentiate nine Pneumovirus isolates from chickens or turkeys of various countries, into three groups; SHS strains, related TRT strains, and poorly related TRT strains. They found the SHS group to be more homologous than the TRT groups. A close antigenic relatedness was demonstrated between TRTV and SHS virus in Britain (Peleteiro, 1991; Wyeth et al., 1987). Based on the

serological and physico-chemical data, it is now accepted that TRT and SHS are induced by the same, or variant strains of the same virus.

1.11. Objectives of the Study

An attempt will be made to characterise the virus isolated from chickens and to determine its relatedness to TRTV from turkeys.

Due to the difficulty associated with the isolation of the rhinotracheitis virus from chickens, a quick, reliable and accurate diagnostic test is required for the diagnosis of infection with the chicken rhinotracheitis virus. A suitable and sensitive test is the PCR followed by probing with a non-radioactive probe. An attempt will be made to develop a DNA probe that will be able to detect the virus in chicken tracheas.

An attempt will also be made to develop a chicken rhinotracheitis virus vaccine capable of protecting birds against challenge with a field strain of the virus.

CHAPTER 2

ISOLATION AND PROPAGATION OF THE TRTV-LIKE AGENT

2.1. Introduction

Poultry grown in a high density environment are exposed to many potentially virulent and pathogenic organisms. Often, the symptoms observed in diseased birds are a result of infection with a combination of pathogens which are further complicated by environmental factors such as dust, ammonia and temperature. It is therefore difficult to state precisely the cause of the disease based solely on symptoms displayed by the birds. An accurate method of diagnosis is by nucleic acid detection using the polymerase chain reaction (PCR) (Saiki, Gelfand, Stoffel, Scharf, Higuchi, Horn, Mullis, and Ehrlich, 1988) or by isolation of the pathogen by conventional methods. These results should be used in conjunction with the symptomology and serological results (Metselaar and Simpson, 1982). The diagnosis can be further substantiated by inoculation of the isolated pathogen into birds and reproduction of the symptoms followed by re-isolation of the pathogen (Picault *et al.*, 1987). However, it is sometimes difficult to reproduce the disease symptoms in the absence of adverse environmental factors and secondary pathogens (Shane, 1993).

According to Jordan (1990), the diagnosis of TRT or SHS depends on the demonstration of the virus or by serological means. The

difficulty involved in the isolation of these viruses is well documented (Buys, 1990; Naylor and Jones, 1993; Wyeth et al., 1987). According to Baxter-Jones (1992) it is more difficult to isolate the virus from chickens, than it is from turkeys. Many host systems have been used for the primary isolation and subsequent propagation of the virus. The most commonly used routes are the a/s, y/s, Vero cell and TOC routes (Buys et al., 1989a,b; Cook and Ellis, 1990; Gough et al., 1988b; Williams et al., 1991a; Wyeth et al., 1986).

Investigations in determining the most convenient and successful methods for the isolation of TRTV-like viruses from chickens are described in this Chapter.

2.2. Materials and Methods

2.2.1. Sample material

Chickens displaying severe sinusitis with excessive sinus exudate and swollen heads, were selected for sampling. The birds were humanely euthanased with CO_2 gas and the infraorbital sinuses exposed by cutting with a sterile No. 22 scalpel blade just below the eye. Using a sterile bacterial loop, the sinus was swabbed and the loop streaked onto a 90 mm blood agar plate. The plates were incubated in 5% CO_2 at 37°C for 24 h. The sinus fluid and exudate were aseptically removed with a sterile 2 ml syringe fitted with a 15 gauge needle and ejected into sterile tryptose phosphate broth containing 10% glycerol and antibiotics. The sinuses were rinsed with this broth.

The sample was centrifuged at approximately 2000 g for 5 min in a Hereaus Hettich Universal II benchtop centrifuge to pellet the red blood cells and debris. The supernatant was aspirated and placed in a sterile Bijou bottle. A total of 37 separate samples were prepared in this way.

2.2.2. Allantoic sac inoculation

Ten Valo SPF eggs (Lohmann, Germany), incubated at 37°C for 10 days in a Buckeye egg setter incubator, were candled by holding them to a bright light and the air space of the embryonated fertile eggs marked with a pencil. Infertile and dead embryonated eggs were discarded.

Using a steel needle, a hole was punched into the shell of each of five of the candled embryonated eggs, approximately 2 mm above the base of the air space. Approximately 0.2 ml of the sample was inoculated, without filtration, using a 2 ml syringe fitted with a 26 gauge needle, into the a/s of the five 10-day-old embryonated eggs. The inoculation holes were sealed with molten Paraclean wax (Klinipath) and the eggs incubated at 37°C.

The eggs were candled after 24 h. Embryos that died within 24 h of inoculation, were discarded as traumatics caused by the inoculation. The eggs were candled daily for 7 days. After incubation at 37°C for 7 days, the eggs were chilled at 4°C for 2 h. The allantoic fluid was harvested with a sterile disposable plastic Pasteur pipette into a sterile 25 ml McCartney bottle and

centrifuged at approximately 2000 g for 5 min in a Hereaus Hettich Universal II centrifuge. The supernatant was transferred to another sterile McCartney bottle.

The allantoic fluid was checked for haemagglutinating (HA) activity (refer to Chapter 3) and further work was done only on samples found to be HA negative. The HA activity of the positive samples was neutralised with Newcastle disease virus specific antisera. Samples causing stunting, curling or clubbing of the embryo were tested for the presence of Infectious Bronchitis virus (IBV) by the serum neutralisation (SN) test (see Chapter 3). Further passaging of IBV positive samples was terminated.

Approximately 0.2 ml of the HA and IBV negative allantoic fluid was re-inoculated into the a/s of each of five 10-day-old embryonated SPF eggs. This was repeated for 10 passages per sample.

2.2.3. Yolk sac inoculation

Six-day-old embryonated SPF eggs were candled and the centre of the air space of each of five fertile eggs was marked. Approximately 0.2 ml of the 1st a/s material was inoculated into the y/s of the five eggs using a 2 ml syringe fitted with a 21 gauge needle. The inoculation holes were sealed with Paraclean wax and the eggs incubated at 37°C for 24 h. The eggs were candled the following day and all dead embryos discarded. The remainder of the eggs were incubated at 37°C for 10 days or until

embryo mortality occurred. Embryos found dead when candling, were immediately chilled at 4°C for 2 h. The remainder of the embryos were terminated at 10 days post-inoculation by chilling at 4°C for 2 h. The 1st y/s passaged material from the dead and live embryos, were harvested as described earlier for the a/s material, and re-inoculated into the y/s. This was repeated until a consistent pattern of embryo mortality was achieved. In cases where the virus was unable to cause any consistent pathology in the embryo after 30 y/s passages, the egg passaging was terminated.

2.2.4. Chorio-allantoic membrane inoculation

Ten 12-day-old embryonated SPF eggs were candled for viability and a mark made on the side of each egg in an area free of blood vessels, approximately 1.5 cm below the air space. A hole was punched with a needle at this mark without piercing the shell membrane. Another hole was punched at the top of the air space and the egg placed horizontally in an egg tray with the first hole facing up. With the egg in the horizontal position, a rubber bulb was used to draw out the air from the air space. The negative pressure created, caused an artificial air space to form over the first hole.

Using a 2 ml syringe fitted with a 26 gauge needle, 0.2 ml of the 3rd y/s material of isolates causing pathology of the y/s-inoculated embryos (isolates 4916/91, 652/93 and 711/93), were inoculated onto the chorio-allantoic membrane (CAM) at the

artificial air space. Both the holes were sealed with molten Paraclean wax and the embryos incubated at 37°C for 24 h, after which they were candled for traumatics. The traumatics were discarded and the remaining eggs re-incubated for 7 days. The eggs were candled daily and all dead embryos were chilled for 2 h before harvesting of allantoic fluid (A/F) and CAM's. Embryos still alive on day 7 pi, were also chilled for 2 hrs and the A/F and CAM's harvested.

The CAM's and A/F from the dead and live embryos, were pooled and homogenised in a Waring blendor. The resulting homogenate was centrifuged (2000 g for 5 min). The supernatant was retained and filtered through a 0.22μ Sterivex-GS filter unit (Millipore) and then re-inoculated onto the CAM's of a further 10 12-day-old SPF eggs. This procedure was repeated for 10 passages.

2.2.5. Growth in chicken embryo fibroblast cell (CEF) monolayers

The embryos were removed from five 10-day-old SPF eggs and placed in a sterile Petri dish. The head and limbs were cut off and the embryos homogenised by passing through a 5 ml syringe into a sterile beaker. The homogenate was washed three times with Hanks balanced salts solution (HBSS) (Flow Laboratories) to remove red blood cells.

Approximately 30 ml of a 0.25% 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

15 min. The action of the trypsin was stopped by the addition of 2 ml bovine foetal calf serum (FCS) (Sterilab).

The trypsinised tissue was centrifuged at 1000 g for 15 min in a Hereaus Hettich Universal II centrifuge. The pelleted cells were resuspended in 5-10 ml of growth medium consisting of Eagles minimum essential medium (MEM) (Highveld Biologicals) with 10% FCS. The number of cells were counted in a haemocytometer and diluted to achieve a minimum concentration of 2 X $10^5 ml^{-1}$. The cell suspension was dispensed in 5 ml aliquots to 50 cm^3 tissue culture flasks (Greiner Labortechnik) and incubated at 37°C for 24 h, by which time a confluent monolayer of cells, viewed with a Leitz Diavert inverted microscope, was obtained.

The 3rd a/s or 4th y/s passaged material of the 37 samples were separately inoculated onto confluent monolayers. One monolayer to be used as a control, was inoculated with healthy allantoic fluid. Inoculation was performed by first washing the monolayer three times with HBSS and then introducing 1 ml of inoculum into the flask. Inoculation contact time was 1 h. The inoculum was discarded and the monolayer washed three times with HBSS. After the final wash, maintenance medium consisting of Eagles MEM without FCS was added to the flasks and the flasks incubated at 37°C for a maximum of 14 days. If no CPE was observed, the flasks were freeze-thawed once and the cell suspension centrifuged at 1000 g for 5 min in a Hereaus Hettich Universal II centrifuge. The supernatant was then inoculated onto a new monolayer. This procedure was repeated for a minimum of five passages per sample.

A slight variation of the method of Williams et al. (1991a) was employed in an attempt to adapt the virus, if any, for growth in CEF's. This was achieved by separately inoculating a monolayer with the sample, and trypsinising the monolayer 3 days later. Trypsinisation was performed by first discarding the MEM above the monolayer and then washing the monolayer three times with HBSS. The monolayer was washed with 0.25% trypsin and allowed to stand in contact with 5 ml of the trypsin solution for 60 sec. The trypsin was decanted and the monolayer allowed to stand for 90 sec. After 90 secs, the cells began disassociating from each other. The flask was then gently tapped to release all the cells and the trypsin action was stopped with the addition of 10 ml of growth medium. After incubation at 37°C for 24 h, the growth medium was replaced by maintenance medium. The trypsinisation procedure was repeated for a minimum of seven passages.

Monolayers inoculated with allantoic fluid from healthy non-virus inoculated 12-day-old embryonated SPF eggs, were trypsinised as described above and used as controls.

2.2.6. Growth in chicken embryo kidney cell (CEK) monolayers

Chicken embryo kidney cell monolayers were prepared as for CEF's, using 19-21 day-old SPF embryos or day-old SPF chicks. The kidneys were removed from the embryos or chicks and processed as for CEF's. Confluency of the monolayers was achieved after 48 hrs, at which time they were separately inoculated as described for CEF monolayers, with the 4th y/s passaged allantoic fluid of

the samples. A minimum of three passages in CEK monolayers were performed for each sample. Controls consisted of CEK monolayers inoculated with healthy allantoic fluid.

2.2.7. Growth in Vero cell monolayers

Vero cells from three different sources were used. Cells were obtained from King Edward VIII Hospital (KEH), Onderstepoort Veterinary Laboratory (OP), and strain E6 from Highveld Biologicals.

Upon receipt of the cells, the monolayer of cells was washed three times with 1M phosphate buffered saline (1M PBS pH 7.2). The monolayer was trypsinised by the addition of 5 ml of a 1:1 mixture of 0.25% trypsin and 0.1 mM EDTA (Highveld Biologicals). The solution was left in contact with the monolayer for 90 secs and poured off leaving approximately 0.5 ml on the monolayer. The flask with the monolayer was allowed to stand at room temperature for approximately 3 min during which time the cells detached from the flask. The detached single cells from each flask were resuspended in approximately 30 ml of growth medium and aliquots of 5 ml 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 24-48 h. The confluent monolayer was maintained with MEM containing no serum. The cell line was propagated by trypsinisation of uninoculated monolayers after

every 3-4 days. Confluent monolayers were separately inoculated with 3rd y/s passaged allantoic fluid of each sample. A control was maintained for each passage by inoculating a monolayer with healthy allantoic fluid. Five passages, each lasting 5 days, were performed with the sample and control materials.

The modification of the method of Williams et al. (1991a), used to adapt the isolates to CEF's, was also used to attempt to adapt the virus, if any, to Vero cell monolayers. Monolayers were separately inoculated with the 3rd y/s material of each sample and trypsinised 4 days later. This trypsinisation procedure was repeated for a minimum of seven passages, after which normal passaging was resumed. The above procedures were used for each of the three sources of Vero cells.

2.2.8. Growth in tracheal organ culture

Three 19-21 day old SPF chicken embryos were euthanased by cervical dislocation and the trachea aseptically removed and placed in a Petri dish containing HBSS. Sterile Whatman No. 1 filter paper was placed on the lid of another Petri dish. The trachea was then transferred to this filter paper and transversely cut into approximately 1 mm thick rings using a No. 22 scalpel blade. The tracheal rings were placed into another Petri dish containing HBSS. The rings were individually placed in sterile 15 ml tissue culture tubes (Greiner Labortechnik) containing 1 ml of MEM.

The tubes containing the tracheal rings were inserted into a roller drum in a 37°C incubator and allowed to rotate at approximately 1 rpm for 24 h. The rings were checked for cilial activity using a Zeiss Axioskop light microscope. Rings with good cilial activity were chosen for inoculation with the 4th y/s material of all 37 samples from which virus isolations were attempted. Two TOC rings were used for each sample. Two TOC's, for use as controls, were inoculated with healthy allantoic fluid. Inoculation was performed by first decanting the MEM from the tube containing the TOC and replacing it with 0.5 ml of the inoculum. After incubation for 1 h, the inoculum was discarded and replaced with 1 ml of MEM.

The rings were scored daily for loss of cilial activity, by viewing with a Zeiss Axioskop light microscope. A scoring system based on that of Cook, Darbyshire, and Peters (1976) was used. A score of 3 indicated a perfectly normal ring with all cilia beating whilst slight ciliostasis (10-40%) was recorded as a score of 2. A score of 1 indicated 50-80% ciliostasis and a score of 0 was reflective of 90-100% ciliostasis. A passage was terminated upon ciliostasis or when the combined score of the two controls deteriorated to a score of 2. A minimum of three blind passages were performed for each isolate before they were discarded as negative.

2.2.9. Titration of the virus

The titres of the three isolates that were made, were determined

in each of the growth systems, except the a/s, by diluting the virus in decimal dilutions from 10^{-2} to 10^{-6} in nutrient broth for eggs, and MEM for monolayers and TOC's, and inoculating the standard volume of each dilution via their respective routes (eggs, monolayers, and TOC's). Five units of each growth system were used per dilution. The infective dose 50% (ID_{50}) of the virus in each system was calculated according to a modification of the Spearman-Karber method (Villegas and Purchase, 1989) depicted below:

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

L = highest dilution with mortality or pathology

L1 = difference in consecutive dilutions

X = volume of inoculum used per egg or monolayer

2.3. Results

2.3.1. Sample material

Four novel Haemophilis paragallinarium isolates as described by Horner, Bishop and Haw (1992), and identified by Dr R Bragg (Onderstepoort Veterinary Laboratory) and Mr M.K Govender (Rainbow Farms, Pty Ltd), were made from four of the 37 samples. E. coli, identified by Mr M.K Govender, was isolated from the remaining 33 samples.

2.3.2. Allantoic sac inoculation

Seven samples caused severely congested embryos in the 1st a/s passage and the HA and SN tests on these samples confirmed the presence of Newcastle disease virus (NDV). Three samples caused

stunted, curled and clubbed embryos in the 1st a/s passage. Serum neutralisation (SN) tests on these isolates identified the isolates as the Massachusetts strain of IBV. All other samples caused no embryo mortality or pathology in all 10 a/s passages.

2.3.3. Yolk sac passage

There was no embryo mortality or pathology in the 1st and 2nd y/s passages, but in the 3rd passage, 50% of the embryos of three samples (sample 4916/91, 652/93 and 711/93) showed pathological changes at day 8 pi. Pathology consisted of pale and stunted embryos. The liver, kidneys and other internal organs appeared normal. By the 4th passage, there was 100% pathology and 80% mortality at day 8 pi. At this stage, the dead embryos were severely congested (Plate 2.1). There was 100% mortality by day 6 pi in all subsequent passages of these samples. All other samples processed failed to cause any pathology or mortality of the embryos.

2.3.4. Chorio-allantoic sac inoculation

The 3rd y/s material of sample 4916/91, 652/93 and 711/93 inoculated onto the CAM, caused 80% embryo mortality by day 6 pi. The affected embryos were congested and stunted. Embryos that were still alive at this point, were pale and stunted. All the internal organs appeared normal.

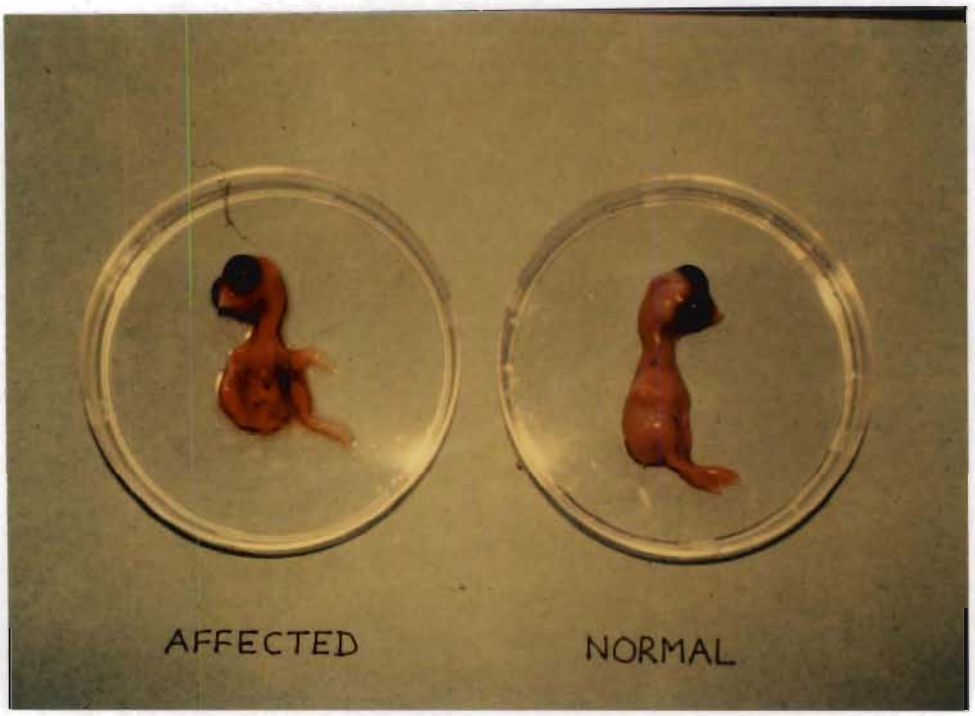


Plate 2.1. Normal (right) and TRTV-like virus-affected embryo. Affected embryo appears stunted and haemorrhagic.

2.3.5. Growth in CEF monolayers

Monolayers inoculated with the 3rd a/s and 4th y/s passaged material of all samples, failed to develop any CPE even after five passages in this system. However, when monolayers inoculated with the 3rd a/s or 4th y/s materials were trypsinised for five consecutive passages, a CPE developed on day 2 pi in the 5th passage of three samples, samples 4916/91, 652/93 and 711/93. The CPE, of all three samples, was characterised by the development of large syncytia (Plate 2.2). The control monolayer in each passage was normal with no CPE.

2.3.6. Growth in CEK monolayers

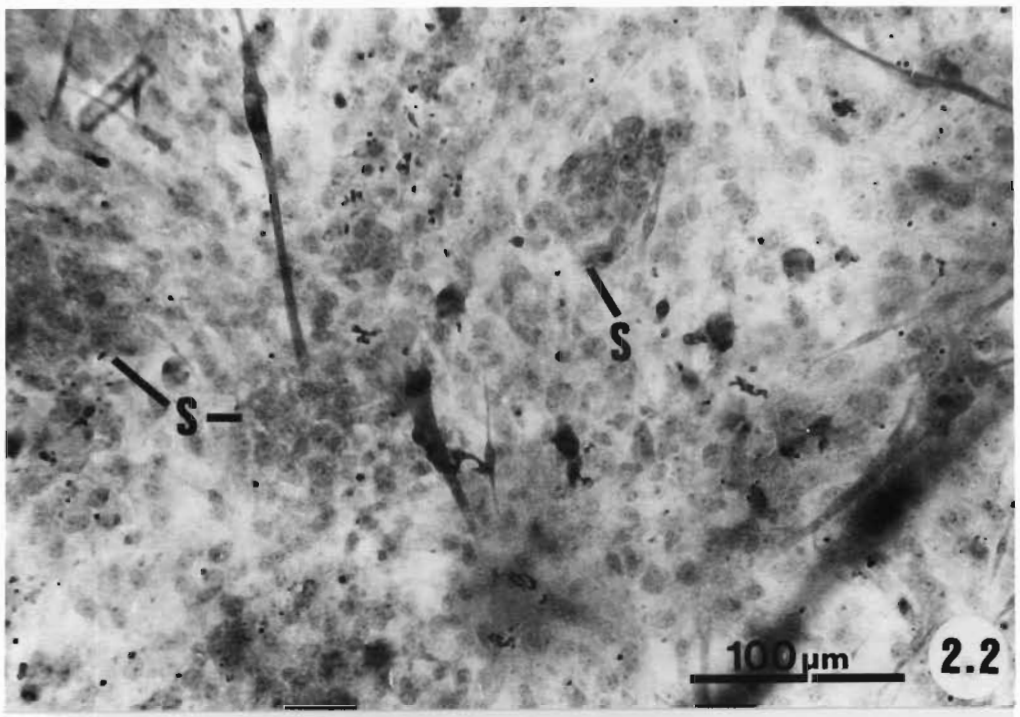
Only three samples, sample 4916/91, 652/93 and 711/93, caused CPE in CEK monolayers. In all cases, the CPE occurred in the third passage on day 4 pi and was characterised by the presence of a few loose cells floating in the medium above the monolayer. The number of loose cells did not increase substantially after day 4 pi or in subsequent passages. There was no evidence of CPE in any of the control monolayers.

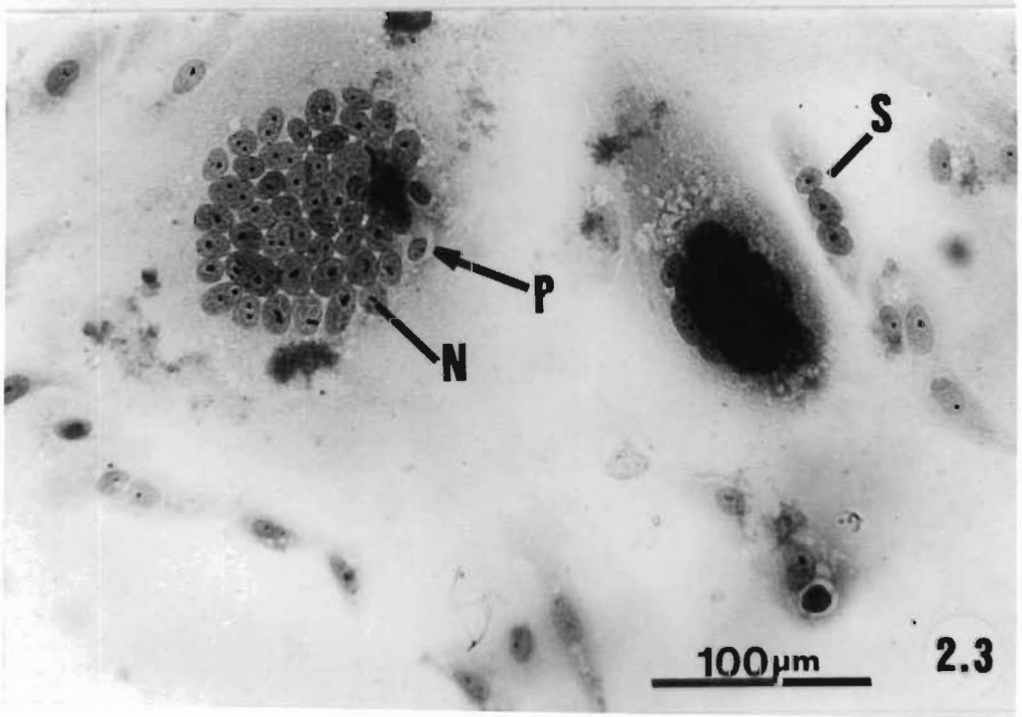
2.3.7. Growth in Vero cell monolayers

There was no detectable CPE in any of the three sources of Vero cells after five routine passages. However, after trypsinisation of the monolayers, CPE consisting of large syncytia and polykaryocytes (Plate 2.3), developed with samples 4916/91,

Plate 2.2. Typical large syncytia (S) formation in a CEF monolayer infected with TRTV-like virus.

Plate 2.3. The formation of large syncytia (S) and polykaryocytes (P) in a Vero cell monolayer. Numerous nuclei (N) occur within a giant cell.


S
S
100µm
2.2


S
P
N
100µm
2.3

652/93 and 711/93, on day 2 pi in the 4th passage of the OP and E6 strains of Vero cells. No CPE developed, even after 7 passages, in the KEH Vero cells.

Once CPE developed, only a further two passages were necessary to sufficiently adapt the virus to the cells to enable them to cause CPE without further trypsinisation.

2.3.8. Growth in tracheal organ culture

Ciliostasis only occurred after the second passage and with only three samples; sample 4916/91, 652/93 and 711/93. All other samples failed to cause ciliostasis after three blind passages and were discarded. Isolate 4916/91 caused 20% ciliostasis (score of 2) by day 7 pi during the 2nd passage which increased to 40% (score = 1-2) by day 5 pi during the 3rd passage and 70% (score = 1) by day 5 in subsequent passages. The amount of ciliostasis with this isolate did not increase beyond 70%, even after 15 TOC passages.

Isolate 652/93 caused 50% ciliostasis by day 7 pi in the 2nd TOC passage and 100% ciliostasis (score = 0) by day 5 pi in subsequent passages.

Ciliostasis (50%) caused by isolate 711/93 only became evident by day 7 pi during the 3rd passage. Subsequent passages caused only 70-80% ciliostasis observable by day 5 pi. The degree of ciliostasis did not increase beyond 70-80% even after 10 TOC

passages.

2.3.9. Titration of the virus

The results of the titrations of the three isolates in each growth system, are depicted in Tables 2.1, 2.2 and 2.3 below.

Table 2.1. Titration results of TRTV-like isolate 4916/91, in various growth systems

ROUTE	AFFECTED/TOTAL PER DILUTION					ID_{50}/ml
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
y/s	5/5	5/5	2/5	0/5	0/5	$10^{4.6}$
CAM	5/5	3/5	0/5	0/5	0/5	$10^{3.8}$
CEF	5/5	5/5	0/5	0/5	0/5	$10^{4.2}$
CEK	5/5	0/5	0/5	0/5	0/5	$10^{3.2}$
Vero	5/5	5/5	2/5	0/5	0/5	$10^{4.6}$
TOC	5/5	4/5	0/5	0/5	0/5	$10^{3.6}$

Table 2.2. Titration results of TRTV-like isolate 652/93 in various growth systems

ROUTE	AFFECTED/TOTAL PER DILUTION					ID_{50}/ml
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
y/s	5/5	5/5	5/5	5/5	3/5	$10^{6.8}$
CAM	5/5	5/5	5/5	1/5	0/5	$10^{5.4}$
CEF	5/5	5/5	5/5	5/5	0/5	$10^{6.2}$
CEK	5/5	5/5	5/5	0/5	0/5	$10^{5.2}$
Vero	5/5	5/5	5/5	5/5	0/5	$10^{6.2}$
TOC	5/5	5/5	5/5	0/5	0/5	$10^{5.2}$

Table 2.3. Titration results of TRTV-like isolate 711/93 in various growth systems

ROUTE	AFFECTED/TOTAL PER DILUTION					ID_{50}/ml
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
y/s	5/5	5/5	0/5	0/5	0/5	$10^{4.2}$
CAM	5/5	3/5	0/5	0/5	0/5	$10^{3.8}$
CEF	5/5	5/5	3/5	0/5	0/5	$10^{4.8}$
CEK	5/5	0/5	0/5	0/5	0/5	$10^{3.2}$
Vero	5/5	5/5	5/5	2/5	0/5	$10^{5.6}$
TOC	5/5	3/5	0/5	0/5	0/5	$10^{3.8}$

2.4. Discussion

The results demonstrate the difficulty associated with the isolation of an avian Pneumovirus from chickens. From a total of 37 samples processed, only three yielded a Pneumovirus. This could be due to several factors, the most important of which could be that the sampling may have been done at an advanced stage of the disease when the amount of virus in the bird had diminished to very low levels. Other researchers have reported that the time of sampling is critical for successful Pneumovirus isolation (Buys, 1990; Wyeth et al., 1987). When symptoms in the birds are pronounced, it may be very difficult to isolate virus (Cook et al., 1991). It may also be difficult to determine the correct time for sampling because in many cases, initial infection produces few, if any, clinical signs unless secondary factors exacerbate the disease (Naylor and Jones, 1993).

Another factor that may affect the isolation rate, is the presence of other pathogens that overgrow the initially poor

growing Pneumovirus. It is possible that the NDV positive and IBV positive samples could have contained the TRTV-like virus but was not detected because it was masked by the more prolific growing viruses. Also, the symptoms associated with SHS could have been caused by these pathogens in association with adverse environmental factors and immunosuppressive agents. Seven NDV and three IBV isolates were made from the 37 samples processed. Also, a novel H. paragallinarum, which appears to be capable of causing SHS type symptoms in birds, was isolated from four samples. E. coli, isolated from most samples, appears to be a secondary pathogen introduced after injury by the bird scratching its head due to the head swelling and irritation caused by viral pathogens.

The sample material was initially inoculated via the a/s of SPF eggs and not directly into the y/s. This was to reduce the risk of bacterial contamination. The y/s is rich in nutrients thereby facilitating the proliferation of many bacteria. The samples were not filtered through a 0.22μ filter since this would have further reduced the low level of virus present in the sample. It was therefore necessary to inoculate the sample into a system that would survive low levels of bacterial contamination.

Although there was no embryo pathology when isolates 4916/91, 652/93 and 711/93 were inoculated via the a/s route, the virus grew in this system. This was evident when the 1st a/s material and the 3rd a/s material were inoculated into the y/s and CEF respectively. Both materials caused pathology in these systems,

although the 3rd a/s material had to be first adapted to CEF's. The three isolates grew well in the y/s, producing a titre of $10^{4.6}$/ml for isolate 4916/91, $10^{6.8}$/ml for 652/93 and $10^{4.2}$/ml for 711/93. Isolate 652/93 was the most prolific growing of the three isolates, producing the highest titres in all growth systems.

There appear to be differences between isolate 652/93 and the other two isolates as shown by their varying abilities to grow in various growth systems. It is therefore likely that several strains of the virus exist and isolate 652/93 could represent one strain whilst isolates 4916/91 and 711/93 could represent another strain. Cook et al. (1993) reported differences between the UK isolates and those from other countries, but the South African isolates were closely related to the UK isolates.

Although some researchers found that TRTV grew to high titres when chicken TOC's were used (Cook and Ellis, 1990), this study showed that the titre of the three isolates in this system was dependent upon the strain of the virus. Isolate 4916/91 and 711/93, which have similar characteristics, grew poorly in TOC's, but isolate 652/93 grew to a high titre in this system. However, despite the better growth of isolate 652/93 compared to isolates 4916/91 and 711/93, the titre in TOC's was among the lowest. It appears that these strains of avian Pneumovirus differ from those of other countries which grow to very high titres in TOC's. There may be a geographical difference in strains of the virus, which is consistent with the findings of Cook and Ellis (1990).

The type of pathology caused by the three isolates in all the growth systems, is consistent with that of other workers (Gough et al., 1988b; Naylor and Jones, 1993; Picault et al., 1987). The level of CPE produced in CEK monolayers could not be increased in subsequent passages. However, unlike CEF and Vero cell monolayers, the isolates produced pathology in the CEK cell monolayer without trypsin treatment. It appears that the isolates require less adaptation for chicken epithelial cells, than for chicken fibroblast cells or cells of mammalian origin. It was not possible to trypsinise the CEK monolayers because the CEK cells in a monolayer cannot withstand the harsh trypsin treatment. According to Dr Jane Cook (Intervet UK, 1994 pers. comm.), several researchers have found that TRTV could be adapted to grow in various cell lines sometimes with trypsin treatment and sometimes without. It is possible that the trypsin cleaves surface glycoproteins that are necessary for infection, thereby facilitating infection, as is the case with NDV (Beard and Hanson, 1984).

Despite the ease of adaptation of the isolates to CEK monolayers compared to CEF and Vero cell monolayers, the isolates, when finally adapted for growth in CEF and Vero cells, grew to higher titres than in the CEK monolayers. Of all the cell culture systems employed, the isolates grew best in the CEF and Vero cell monolayers. However, it is preferable to use Vero cell monolayers since it is easier to generate them for inoculation than it is for any of the avian cell types.

The results suggest that the best route for the isolation of avian Pneumoviruses is the y/s route. Subsequent propagation can be performed via the y/s, CEF and Vero cell routes.

CHAPTER 3

PHYSICAL AND CHEMICAL CHARACTERISATION OF THREE TRTV-LIKE ISOLATES

3.1. Introduction

There are several direct and indirect methods for the diagnosis of virus disease. The direct methods use the clinical specimen directly to detect the presence of virus or to demonstrate specific changes caused by the virus. However, it is essential that the results of the direct methods be confirmed by the indirect methods since there may be false positives and negatives and the identity of the virus may not be established (Versteeg, 1985).

Most methods are based on the fact that many viruses cause characteristic changes in the cells of the inoculated or infected host. These cause the production of infectious virus particles in host cells, secretions and excretions. The host usually responds to the infection by the production of antibodies specific for the virus (Hawkes, 1979).

The present criteria for the classification of animal viruses are based on the physical and chemical characteristics of the virus. After the initial isolation and subsequent propagation of the virus, it is essential to determine the type of virus isolated. Since there are seven families of deoxyribonucleic acid (DNA)

containing animal viruses and 16 families of ribonucleic acid (RNA) containing animal viruses, it is important to determine to which of the two groups the virus belongs (Lukert, 1989). Other tests to determine whether the virus possesses an envelope, its susceptibility to adversely high temperatures and its ability to agglutinate red blood cells (RBC's), are also important characteristics for classification.

Microscopic examination of stained cell cultures for inclusion bodies and other pathological changes, is a rapid method of obtaining an indication of the group of virus involved. Serology is also of importance, especially in retrospectively defining the incidence of disease with the virus isolate obtained. One could also inoculate the purified virus into SPF animals and monitor the animal serologically for substantial increases in antibody titres. This would provide circumstantial evidence as to the identity of the virus (Hawkes, 1979). Serological assays commonly used, include enzyme linked immunosorbent assays (ELISA), serum neutralisation (SN), haemagglutination (HA) and haemagglutination inhibition (HI).

Characterisation of chicken TRTV-like isolates 4916/91, 652/93 and 711/93 using some of the above techniques, were attempted, and the results are presented here. Relatively new technological advances for viral diagnosis based on molecular biology, will be discussed in other Chapters.

3.2. Materials and Methods

3.2.1. Haemagglutination

All eggs inoculated with samples 4916/91, 652/93 and 711/93, were tested for haemagglutinating activity (HA) at the end of each passage using the method described by Villegas (1987).

The test was performed by removing a small volume of clarified allantoic fluid with a sterile disposable Pasteur pipette and placing approximately two drops on a white tile. An equal volume of a 10% suspension of RBC's, collected from the brachial wing vein of SPF chickens into Alsever's solution (Appendix, recipe 1) and washed three times with phosphate buffered saline (PBS pH 7.0), was added to the allantoic fluid. The plate was gently swirled by hand for 1 min to mix the allantoic fluid and RBC's. A positive control consisting of NDV-containing allantoic fluid 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 RBC's which appear "sandy".

3.2.2. Heat stability

The 4th y/s A/F of the three isolates, 4916/91, 652/93 and 711/93, were serially diluted in ten fold dilutions from 10^{-3} to 10^{-7} and separately inoculated into the y/s of 6-day-old SPF eggs. The A/F from the lowest dilution with pathological changes were harvested, diluted to 10^{-3}, and re-inoculated into the y/s

of SPF eggs. This purified 6th y/s A/F was used in further tests and as seed material for vaccine production.

Approximately 1.5 ml of the 6th y/s allantoic fluid of each of the three isolates, 4916/91, 652/93 and 711/93, was placed into two separate 2 ml thin-walled glass screw-top vials and one vial of each placed in a water bath at a temperature of 56°C for 1 h. The remaining vials were kept at 4°C until required. After 1 hr, the heat treated vials were immediately transferred to a beaker containing ice. Serial ten-fold dilutions of the heat treated and 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 inoculated into the y/s of 6-day-old SPF eggs. Five eggs were used for each dilution. The eggs were incubated at 37°C for seven days or until mortality of the embryos.

After seven days, the eggs were opened, examined for pathological effects and the titre of the heat treated and non-heat treated samples determined as described earlier. A heat labile and a heat resistant control sample comprising the H_{120} IB vaccine virus (IBV) (TAD Pharmazeutisches) and a chicken embryo lethal orphan (CELO) virus (Rainbow Farms Laboratory) respectively, were included in the test and treated identically to the test samples.

The titres of the purified 6th y/s allantoic fluids of isolates 4916/91, 652/93 and 711/93, freshly harvested, at 4°C for 24 and 72 h, at -20°C for 3 and 28 days, and at -70°C and in liquid

nitrogen for 6 months, were determined by exposure of the isolates to these temperatures for the stipulated time. The samples were inoculated into eggs as described earlier, and the titres calculated.

3.2.3. Chloroform sensitivity

The protocol for this test was similar to that of Villegas (1987).

Two milliliters of the 6th y/s allantoic fluid of isolates 4916/91, 652/93 and 711/93 were separately aliquoted into two sterile Bijou bottles each and 0.2 ml of chloroform added to one set of the duplicates. The untreated samples were kept at 4°C until required. The chloroform treated samples were vortexed for 10 min, with the bottles kept in an ice bath between mixes. The treated samples were centrifuged at approximately 1500 g for 30 min in a Hereaus Hettich Universal II centrifuge. Without disturbing the precipitate, the upper clear layer was transferred into a sterile Bijou bottle with a sterile Pasteur pipette. The bottle was left opened in a laminar flow hood for 10 min to allow traces of chloroform to evaporate. Serial 10^{-1} and 10^{-2} dilutions of the treated and untreated samples were made in nutrient broth and separately inoculated into the y/s of SPF eggs as described earlier. A chloroform-sensitive and a resistant control sample, comprising the TAD H_{120} IBV and CELO virus respectively, were used as controls and treated identically to the test samples.

3.2.4. Nucleic acid determination

Nucleic acid determination was performed according to the method described by Lukert (1989) and Villegas (1987). Preformed CEF monolayers in 50 cm^3 tissue culture flasks (Greiner Labortechnik) were separately inoculated with the 6th y/s A/F of isolates 4916/91, 652/93 and 711/93. After adaptation of the isolates to CEF cells, CEF monolayers were separately inoculated with the 5th CEF passage of each isolate at dilutions of 10^{-3} to 10^{-6} and also with reference strains of the CELO virus and with the 10^{-6} to 10^{-9} dilutions of the Hitchner B_1 strain of NDV (TAD Pharmazeutisches). After an incubation time of 45 min at 37°C, the inoculum was discarded and maintenance medium containing 50μg/ml of 5-iodo-2'-deoxyuridine (IDU) (Sigma Chemicals) was added. These virus samples were also inoculated onto CEF's using regular maintenance medium without IDU. Five replicates per dilution were used for each sample.

The titre of each isolate, in the presence and absence of IDU, was calculated according to the modified method of Spearman-Karber (Villegas and Purchase, 1989).

3.2.5. Serum neutralisation

Antisera to TRTV, 4916/91, 652/93, 711/93 and IBV were produced in approximately 6-week-old SPF white leghorn chickens by separately inoculating each of five birds with 0.5 ml of TRTV (Pittman-Moore vaccine virus), the 6th y/s A/F of each of the

three isolates, and with IBV vaccine virus (TAD Pharmazeutisches) respectively, directly into the infraorbital sinus. The birds were bled pre-inoculation and two weeks post-inoculation via the brachial wing vein. If the ELISA antibody titre against TRTV for the TRTV, 4916/91, 652/93, and 711/93 isolate inoculated birds, and IBV for the IBV inoculated birds, shifted from a negative value pre-inoculation to a highly positive value (≥ ELISA kit positive control) 4 wks post-inoculation, the birds were sacrificed and the serum collected from the blood.

The sera were clarified by centrifugation at 2000 g in a Hearaeus Hettich Universal II centrifuge and heated at 56°C for 1 h. The inactivated sera were filtered through a 0.22μ Sterivex filter (Millipore). These sera were tested by D.N Bhoora (Rainbow Farms Laboratory), using haemagglutination inhibition for antibodies to NDV, by ELISA for antibodies to IBV, reovirus, infectious bursal disease virus (IBDV) (Delta Bioproducts), TRTV (Pathasure), and by the rapid serum plate agglutination test (RSPA) for antibodies to Mycoplasma gallisepticum (MG) and Mycoplasma synovia (MS). The serum was only used for the serum neutralisation test when found to be monospecific for TRTV.

The alpha neutralisation procedure was used (Beard, 1989). This procedure involved the use of serial dilutions of virus mixed with a standard dilution of serum. The TRTV monospecific serum was diluted 1:10 in nutrient broth. The 6th y/s A/F of the three isolates were separately used as antigen for the neutralisation tests. Serial dilutions of the antigens (10^{-1} to 10^{-7}) were made in

nutrient broth and 0.7 ml of the 10^{-4} to 10^{-7} dilutions separately placed into sterile vials containing 0.7 ml nutrient broth. To another set of four vials was separately added 0.7 ml of the 10^{-2} to 10^{-5} dilution of antigen and 0.7 ml of the diluted TRTV serum.

These viruses and virus-serum complexes were allowed to react at room temperature for approximately 45 min and 0.2 ml inoculated into each of five SPF eggs per dilution. A negative and a positive control, comprising the TAD H_{120} IBV and the Pittman-Moore TRT vaccine virus respectively, were included in the test and treated as described above. The eggs were opened 7 days post-inoculation and mortality and pathology recorded. The titre of the viruses and their neutralisation indices were calculated. The indices were calculated by subtracting the titre of the serum-virus complex from the titre of the respective virus.

A cross-neutralisation test was also performed using the Pittman-Moore TRT vaccine virus and the monospecific antisera of the three isolates. The test was performed as described above.

3.2.6. Enzyme linked immunosorbent assay

Approximately 0.5 ml of the 6th y/s A/F of each of the three isolates, 4916/91, 652/93 and 711/93, were separately inoculated intra-sinusly into each of three white leghorn SPF chickens housed in isolators. Positive and negative controls consisted of SPF birds inoculated with the Pittman-Moore TRT vaccine virus and the TAD H_{120} IBV respectively.

The birds were bled before inoculation and 14 days post-inoculation from the brachial wing vein. The serum was collected from the blood and analysed by ELISA, using the avian rhinotracheitis ELISA kit (Pathasure). The method was performed according to the kit specifications described below.

Using a Beckman Accu-Prep 221 system, 50 µl of the kit positive and negative control sera were separately pipetted into two wells each of the 96 well TRTV-coated microwell assay plate. The test serum samples were first diluted in a sterile microwell plate by pipetting 5 µl of each serum sample into a well (three serum samples per isolate), using a clean pipette tip for each sample, and then adding 200 µl of the kit sample diluent into each of these wells. The contents of each well were mixed by aspirating and discharging several times with a 50 µl 8-channel Titertek pipette. After the pre-dilution, 50 µl of the test samples were transferred with the 8-channel Titertek pipette into the TRTV-coated ELISA plate wells. The plate was covered with a clean lid and allowed to incubate for 30 min at room temperature.

After the incubation, the contents of the plate were discarded and the plate blotted with absorbent paper towel. The wells were washed five times with distilled water using an EL403H microplate Autowasher (Bio Tek Instruments). After the final wash, the plate was blotted onto paper towel and 50 µl of conjugate reagent added to each well. The plate was covered with a lid and allowed to incubate for 30 min at room temperature. The contents were discarded and the plate blotted with paper towel. The wells were

washed five times and blotted as described earlier. After this step, 100 μl of the kit substrate reagent was quickly added to each well and the plate covered and allowed to incubate for 10 min at room temperature.

After the 10 min incubation, 100 μl of the kit stopping reagent was added to each well with an 8-channel Titertek micropipette. The contents of the wells were then agitated with the aid of a micro-orbital shaker (Bellco Biotechnology).

An EL308 microplate reader (Bio Tek Instruments), was blanked on air and the absorbance of each well measured at 450nm. The results were calculated by determining the sample (S) to mean positive control (P) ratio multiplied by 100 ie. S/P X 100

According to the kit specifications, the test was valid only if the mean positive to negative control ratio was above 6. Samples were deemed positive for TRTV antibodies if the S/P ratio X 100 was greater than 30. Samples with a figure less than 30 were diagnosed as negative for antibodies against TRTV.

3.2.7. Haematoxylin and eosin staining of monolayers

The 4th Vero cell passage of each of the three isolates was serially diluted from 10^{-4} to 10^{-7} and separately inoculated onto preformed Vero cell monolayers. The lowest dilution with CPE 4 days pi, was diluted to 10^{-3} and re-inoculated onto Vero cell monolayers. This purified 6th Vero cell adapted passage of each

of the three isolates (4916/91, 652/93 and 711/93), was used for the inoculation of Vero cell monolayers for the haematoxylin and eosin staining test. The inoculated and uninoculated control monolayers were subsequently stained by the haematoxylin and eosin (H and E) staining method. Monolayers were grown on sterile coverslips placed in disposable 60mm tissue culture dishes (Greiner Labortechnik).

The coverslips with the inoculated monolayers were stained two days after inoculation. The H and E staining method used was similar to that of Villegas (1987). The coverslips were washed with PBS (pH 7.0) and the cells fixed with Bouins fixative for 1 h. The coverslips were rinsed in 100% ethanol and transferred to 70% ethanol for 5 min, washed under slowly running tap water for 5 min and stained with Harris' haematoxylin containing 4% glacial acetic acid for 5-10 min. After haematoxylin staining, the coverslips were briefly washed under tap water and differentiated with two dips in acid alcohol (0.5% HCl in 70% alcohol). To stop differentiation, they were immediately washed under running tap water for 20 min.

The coverslips were counter-stained with a water soluble eosin-phloxine solution (1:1) for 30 sec and the cells on the coverslips dehydrated by dipping once each in a graded alcohol series (70%, 96%, 100%). The coverslips were placed in 100% alcohol for a further 30 sec 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 a Zeiss

Axioskop light microscope for inclusion bodies and other cytopathic effects.

3.3. Results

3.3.1. Haemagglutination

All passages of the three isolates, isolates 4916/91, 652/93 and 711/93, were found to be free of haemagglutinating activity.

3.3.2. Heat stability

All three isolates, as well as the H_{120} IBV, were completely inactivated by exposure to 56°C for 1h. However, the CELO virus titre was virtually unaltered by the heating. Table 3.1 shows the results obtained with the test and control samples.

The titres of the isolates after exposure to various temperatures for varying periods of time, are shown in Table 3.2. There was no effect on the titres of the isolates after storage at -70°C and liquid nitrogen for 6 months. There was a drop in the titre of each isolate when stored at -20°C for 28 days but little effect on the titres when the isolates were stored at this temperature for 3 days. There was a slight drop in the titres of the isolates when stored at 4°C for 24 h, but the titres dropped more when stored at this temperature for 72 h, especially isolates 4916/91 and 652/93.

Table 3.1. Titres of the controls and three TRTV-like isolates exposed to 56°C for 1h.

SAMPLE	TREATMENT	TITRE (EID_{50}/ml)
4916/91	control	$10^{4.6}$
4916/91	56°C 1 h	0
652/93	control	$10^{6.8}$
652/93	56°C 1 h	0
711/93	control	$10^{4.2}$
711/93	56°C 1h	0
H_{120} IBV	control	$10^{6.2}$
H_{120} IBV	56°C 1h	0
CELO virus	control	$10^{6.6}$
CELO virus	56°C 1h	$10^{6.2}$

Table 3.2. Titre of TRTV-like isolates 4916/91, 652/93 and 711/93 after various exposure times to different temperatures.

TEMPERATURE	TIME	4916/91*	652/93*	711/93*
4°C	24 h	$10^{4.2}$	$10^{6.6}$	$10^{4.0}$
4°C	72 h	$10^{2.6}$	$10^{4.8}$	$10^{3.8}$
-20°C	3 days	$10^{4.4}$	$10^{6.6}$	$10^{4.2}$
-20°C	28 days	$10^{3.8}$	$10^{5.6}$	$10^{3.6}$
-70°C	6 months	$10^{4.6}$	$10^{6.8}$	$10^{4.2}$
Liq. N_2	6 months	$10^{4.6}$	$10^{6.8}$	$10^{4.2}$
control	fresh	$10^{4.6}$	$10^{6.8}$	$10^{4.2}$

* EID_{50}/ml of respective isolate

3.3.3. Chloroform sensitivity

The chloroform treated samples of all three isolates failed to cause any infection when the 10^{-1} and 10^{-2} dilutions were inoculated into SPF eggs. However, there was 100% mortality when the 10^{-1} and 10^{-2} dilutions of the untreated test samples were inoculated into SPF eggs. The chloroform sensitive H_{120} IBV also failed to cause effects in SPF eggs when treated with chloroform. The chloroform resistant CELO virus was not affected by chloroform treatment and caused 100% mortality in SPF eggs at the 10^{-1} and 10^{-2} dilution when inoculated with the treated and untreated samples.

3.3.4. Nucleic acid determination

There was no difference in the titre of the three isolates (4916/91, 652/93 and 711/93) as well as NDV, in the presence or absence of IDU. However, the titre of the positive control sample, CELO virus, dropped from $10^{7.2}$ $TCID_{50}$/ml to $10^{3.6}$ $TCID_{50}$/ml. The results are shown in Table 3.3.

Table 3.3. Results of the nucleic acid determination test of three TRTV-like isolates

SAMPLE	TREATMENT	TITRE ($TCID_{50}$/ml)
4916/91	untreated	$10^{4.2}$
4916/91	IDU treated	$10^{4.2}$
652/93	untreated	$10^{6.2}$
652/93	IDU treated	$10^{6.2}$
711/93	untreated	$10^{4.8}$
711/93	IDU treated	$10^{4.8}$
NDV	untreated	$10^{9.2}$
NDV	IDU treated	$10^{9.0}$
CELO virus	untreated	$10^{7.2}$
CELO virus	IDU treated	$10^{3.6}$

3.3.5. Serum neutralisation

The titres of the isolates in the absence of TRTV specific antibodies and when reacted with TRTV specific antiserum for 45 min, are shown in Table 3.4 below.

Table 3.4. Results of the serum neutralisation tests of three TRTV-like isolates and controls

ISOLATE	VIRUS TITRE (EID_{50}/ml)	NEUTRALISATION TITRE (EID_{50}/ml)	NEUTRALISATION INDEX
4916/91	$10^{4.6}$	$10^{0.9}$	3.7
652/93	$10^{6.8}$	$10^{1.1}$	5.7
711/93	$10^{4.2}$	$10^{0.9}$	3.3
H_{120} IBV	$10^{6.2}$	$>10^{4.5}$	<1.7
TRTV	$10^{6.6}$	$10^{1.3}$	5.3

Table 3.5. Results of the cross-neutralisation test using the TRTV and monospecific antisera to isolates 4916/91, 652/93 and 711/93

SERA	NEUTRALISATION TITRE ($EID_{50/ml}$)	NEUTRALISATION INDEX
TRTV	$10^{1.3}$	5.3
4916/91	$10^{2.5}$	4.1
652/93	$10^{1.5}$	5.1
711/93	$10^{2.5}$	4.1
IBV	$10^{4.9}$	1.7
TRTV TITRE (EID_{50}) = $10^{6.6}$/ml		

The three isolates, 4916/91, 652/93 and 711/93, were all neutralised to some extent by the TRTV specific antiserum and the TRTV was also strongly neutralised by the 4916/91, 652/93 and 711/93 antisera (Table 3.5). The 652/93 antiserum neutralised the TRTV more strongly than the other two isolates. The positive controls were strongly neutralised whereas the negative IBV control antigen and antiserum was not neutralised by the TRTV specific antisera or virus respectively.

3.3.6. Enzyme linked immunosorbent assay

All the birds were found to be negative for antibodies against TRTV before inoculation. However, serum samples from birds inoculated with the three isolates and the Pittman-Moore TRTV, were found to have very high levels of antibodies against TRTV. Serum from birds inoculated with the TAD IBV, remained negative 2 wks post-inoculation. The results are shown in Table 3.6 below.

The optical density (OD) and S/P ratio X 100 of the kits positive and negative controls were 671, 100 and 105, 16 respectively. The positive : negative control ratio was 6.4. A value below six, invalidates the results.

Table 3.6. TRTV ELISA results with isolates 4916/91, 652/93 and 711/93

SAMPLE	PRE-INOCULATION		POST-INOCULATION	
	OD	S/P X 100	OD	S/P X 100
4916/91	127	19	684	102
652/93	138	21	731	109
711/93	121	18	611	91
TRTV	134	20	644	96
IBV	138	21	168	25

3.3.7. Haematoxylin and eosin staining of monolayers

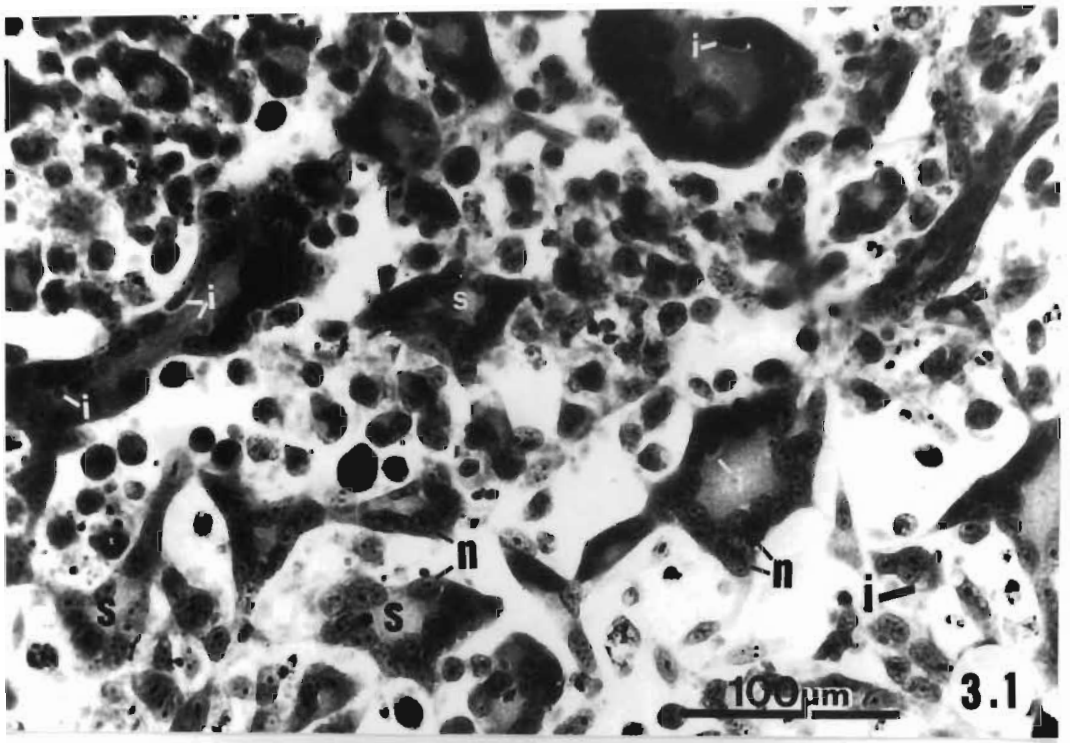
All the inoculated monolayers displayed a similar type of CPE visualised by the H and E staining. Apart from the occurrence of syncytia, there were eosinophilic inclusions in the cytoplasm of the infected cells (Plate 3.1). The uninoculated control monolayer showed no signs of CPE (Plate 3.2).

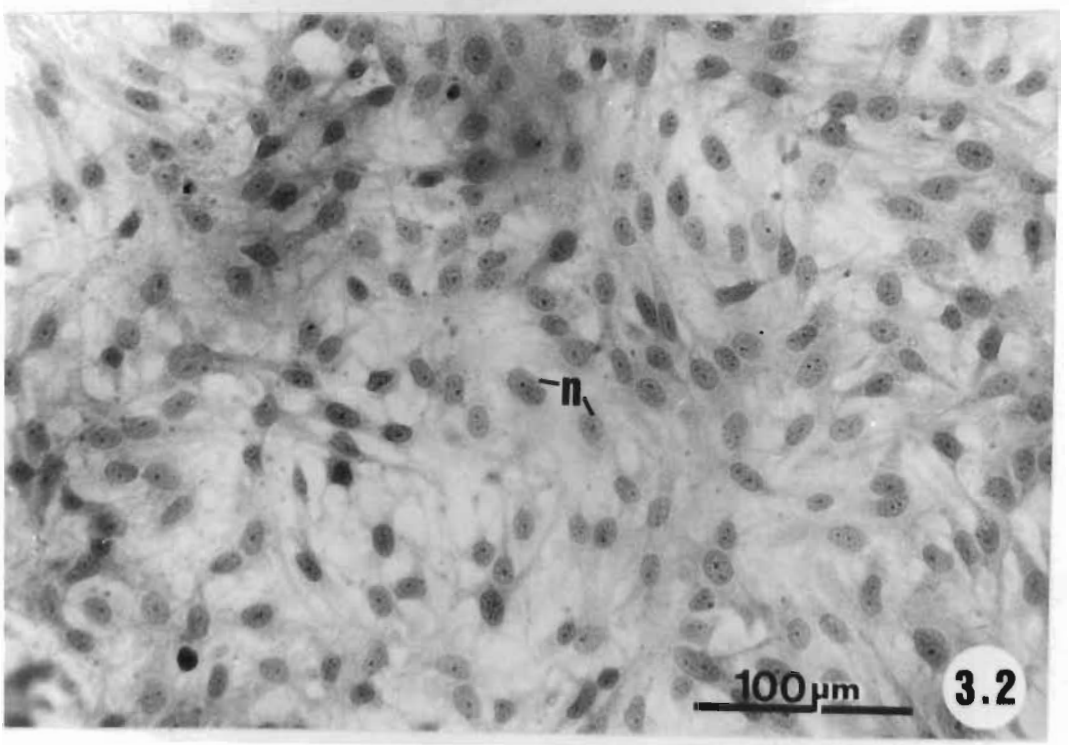
3.4. Discussion

The results of the physico-chemical tests were similar for the three isolates (4916/91, 652/93 and 711/93), indicative of the

Plate 3.1. Vero cell monolayer stained with haematoxylin and eosin. The syncytia (s) and inclusions (i) are clearly visible. Numerous nuclei (n) can be observed within a syncytium.

Plate 3.2. Healthy uninoculated Vero cell monolayer. Note the absence of syncytia and inclusions. (n = nucleus)


i
i
i
s
s
s
n
n
i
100µm
3.1


n
100µm
3.2

possible relatedness between them.

The HA tests with the isolates were negative, suggesting that the isolates do not contain haemagglutinin in their membrane. A negative HA test excludes viruses that do possess haemagglutinin eg. NDV and avian influenza (Alexander, 1989).

The isolates were found to be heat labile since they were inactivated when exposed to a temperature of 56°C for 1 h. Most viruses that possess an outer membrane around the nucleocapsid are inactivated by prolonged exposure to this temperature which denatures the membrane. This also indicates that the virus requires the membrane for infection. Further evidence for the presence of a membrane around the isolates, is the fact that they were completely inactivated after treatment with chloroform. Chloroform is a lipid solvent that destroys lipoprotein membranes. The sensitivity of a virus to lipid solvents therefore indicates whether it has a lipoprotein envelope or not (Lukert, 1989).

The isolates were found to be stable for a long period of time at temperatures of about -70°C or lower. The titre of the isolates did not drop when stored at -70°C or in liquid nitrogen (N_2). However, when stored at 4°C for longer than 24 h, there was a drop in the titre of the isolates. Prolonged exposure to a temperature of -20°C also caused a drop in the titre of the isolates. These results suggest that the isolates are relatively fragile and if storage for a period exceeding 3 days is required,

then they should be stored at a temperature of -70°C or lower.

The titre of the isolates was unaltered by IDU treatment, indicative of the fact that the isolates contain a genome of RNA (Wyeth and Alexander, 1989). IDU is a thymidine analog and an inhibitor of DNA replication (Lukert, 1989). The DNA-containing CELO virus positive control was significantly reduced in titre by IDU treatment whilst the RNA-containing NDV negative control was virtually unaltered by IDU treatment.

All three isolates were significantly neutralised by TRTV specific antisera and the TRTV was significantly neutralised by antisera against each of the three isolates, especially 652/93, which suggests a close relationship between the isolates and with TRTV. Neutralisation tests with specific antisera are usually accurate and useful in identifying unknown viruses and differentiating between serotypes of a virus (Lukert, 1989). The reason for the lower neutralisation indices of isolate 4916/91 and 711/93 compared to that of 652/93, may be due to the lower titres of the former and because they are probably not as immunogenic as isolate 652/93.

The results of the ELISA tests indicate a high degree of serological relatedness between the three isolates and TRTV. Since the commercial TRTV ELISA kit was able to detect antibodies against the three isolates, it was not necessary to develop specific ELISA kits for each of the three isolates. The ELISA S/P ratios of isolates 4916/91 and 652/93 were higher than that of

the positive control. The difference in ELISA results amongst the three isolates could be due to a difference in the ability of the isolates to replicate, as well as the amount of virus initially present in the inoculum. Isolate 652/93 which grows prolifically and to a significantly higher titre than that of the other two isolates, also produced the highest antibody response against TRTV in SPF birds.

All three isolates caused CPE comprising giant cells or syncytia and the production of eosinophilic cytoplasmic inclusions. This type of CPE is a characteristic of the Pneumoviruses eg. respiratory syncytial virus (RSV) and TRTV (Buys et al., 1989a,b; Naylor and Jones, 1993; Parrott, Kim, Brandt, Beem, Richardson, Gerin, and Chanock, 1979).

The results suggest that the three isolates, although possessing different abilities to grow in laboratory host systems, are remarkably similar and possibly strains of the same serotype. Furthermore, the serological evidence indicates that the isolates are similar to TRTV. It is therefore suggested that the three isolates are enveloped RNA viruses and possibly strains of the same serotype as the Pittman-Moore vaccine TRTV. This theory must be substantiated by electron microscopy and molecular biological techniques such as SDS-PAGE, western blotting, the polymerase chain reaction (PCR) and DNA probing which are discussed in later Chapters.

CHAPTER 4

ELECTRON MICROSCOPY OF CHICKEN TRTV-LIKE VIRUS AND TRTV-LIKE VIRUS-INFECTED TISSUES

4.1. Introduction

The electron microscope (EM) is one of the most useful single tools available to virologists, but it is also an extremely expensive instrument which requires qualified and experienced technicians for its efficient operation and maintenance (Versteeg, 1985). When available, the EM can be of immense diagnostic value and may be used as the first step towards virus diagnosis (Hill, 1984). Since several new human and animal pathogens were recognised for the first time by this technique, electron microscopy has been used as a routine screening for viruses, especially when less intensive methods were not within reach (Versteeg, 1985).

The diagnosis made by electron microscopy is morphological. However, since viruses from a group have basically the same morphology, they cannot be typed with the EM. With known viruses, the EM can be used to examine detailed virus substructure, to study various aspects of virus-cell interactions, and to examine the localisation of virus development in the host.

Buys *et al*. (1989a) and Giraud *et al*. (1987), using the EM, observed accumulations of virus nucleoprotein on the plasma

membrane of TRTV-infected Vero cells. The accumulations later budded through the plasma membrane. Apart from these studies, there are no records of other electron microscopical work on TRTV-infected tissues. No electron microscopy has been performed on the localisation and virus development in TOC's. However, there are numerous reports on TRTV and chicken isolates of a TRTV-like virus particle morphology.

Results of electron microscopy on isolate 4916/91, 652/93 and 711/93 infective allantoic fluid to determine virus morphology, and on infected TOC's and Vero cells to study virus development, are presented in this Chapter.

4.2. Materials and Method

4.2.1. Treatment of infective allantoic fluid for the EM

The allantoic fluid from embryos separately inoculated via the y/s route with the 6th y/s passage of isolates 4916/91, 652/93 and 711/93, were harvested 4 days post-inoculation. The allantoic fluid was then treated for electron microscopy according to the method of Gough and Collins (1989).

The allantoic fluid was centrifuged at approximately 3000 g for 10 min in a Hereaus Hettich Universal II centrifuge to remove red blood cells and debris. The fluid was then centrifuged at 70000g for 60 min in a Sorvall RC28S centrifuge using a S-20 rotor. The pellet was resuspended in 0.5 ml of sterile deionised water and placed on a 15 ml step gradient comprising 12.5 ml 30%(w/w)

sucrose and 2.5 ml 55% (w/w) sucrose. After centrifugation at 56500g for 60 min in a Sorvall RC28S centrifuge using a S-20 rotor, the band at the interface was removed with a syringe fitted with a 21 gauge hypodermic needle which was bent horizontally at the end. This fraction was diluted with PBS (pH 7.0) and pelleted at 70000g for 60 min. The resultant pellet was resuspended in 0.5 ml 0.01M Tris-HCl buffer (pH 7.0). Healthy allantoic fluid from uninoculated SPF eggs of the same age as that of the test samples, was treated in the same manner as the test samples. However, there was no band at the interface of the gradients of the healthy control sample. Therefore, the fluid at the general location of the band was removed and further processed.

One drop of the purified suspension was placed on formvar coated copper grids, negatively stained for 2 min with 2% phosphotungstic acid (PTA, pH 6.0), and viewed with a Jeol 100CX transmission electron microscope (TEM).

4.2.2. Preparation of infected Vero cells and TOC's for EM

TOCs with good cilial activity were prepared from 19-21 day old SPF chicken embryos and separately inoculated with the 5th y/s material of isolates 4916/91, 652/93 and 711/93. A few TOC's were also inoculated with healthy allantoic fluid. After 2 days, the TOC's were placed in 6% glutaraldehyde in 0.05M sodium cacodylate buffer for fixation until ready for subsequent processing.

Confluent layers of healthy Vero cells were separately inoculated with healthy allantoic fluid and with the 7th Vero cell passaged material of isolates 4916/91, 652/93 and 711/93. The medium above the monolayers was discarded 2 days pi and replaced with a small volume of 6% glutaraldehyde in 0.05M sodium cacodylate buffer which completely covered the monolayer. The monolayers were stored at 4°C in this fixative until ready for further processing.

The glutaraldehyde fixed samples were washed twice for 30 min each in 0.05M sodium cacodylate buffer, post-fixed in 2% osmium tetroxide for 2 h followed by two 30-min washes in buffer. The samples were then block-stained in 2% uranyl acetate for 30 min followed by two 30-min washes with double distilled water.

The samples were dehydrated in a graded ethanol series ranging from 10-100% in increasing steps of 10%. The samples were submersed in each ethanol concentration for 10 min and the 100% dehydration step was repeated three times. The samples were then washed in two 30-min changes of propylene oxide and embedded in Spurr's resin according to the following embedding formulation recommended by Spurr (1969):

ERL 4206 .....10g

DER 736 .....6g

NSA (nonenyl succinic anhydride) 26g

S-1 (dimethylaminoethanol) 0.4g

The samples were submersed in increasing resin concentrations as

shown below:

25% Spurrs : 75% absolute alcohol .....2 h

50% Spurrs : 50% absolute alcohol .....2 h

75% Spurrs : 25% absolute alcohol .....2 h

100% Spurrs .....overnight

The samples in 100% Spurrs resin were poured into labelled embedding dishes and polymerised in an oven at 70°C for 16 h. The samples were cut out of the mould and mounted on labelled perspex stubs. The blocks were trimmed with a glass knife on a Reichert ultramicrotome. Ultrathin sections of the specimens were cut and three sections picked up on each 200 mesh copper grid, stained with 2% uranyl acetate for 10 min followed by lead citrate for a further 10 min, and viewed with a Jeol 100CX transmission electron microscope.

4.3. Results

Since the EM results obtained with isolates 4916/91, 652/93 and 711/93 were similar, only the results obtained with isolate 652/93, are presented.

4.3.1. EM of infective allantoic fluid

Electron microscopy of 6th y/s passage allantoic fluid of all isolates, revealed the presence of highly pleomorphic virus-like particles (VLP's) approximately 100-300 nm in diameter with a fringe of spikes approximately 12 nm in length (Plate 4.1).

However, although there were often numerous VLP's present in a field, the spikes were not always clearly visible (Plate 4.2). In some preparations of allantoic fluid, structures resembling helical nucleocapsids with the typical "herringbone" type structure of approximately 15 nm in diameter, was observed extruding from the VLP's (Plates 4.3 and 4.4). A coiled nucleocapsid-like structure was also observed within a VLP in Plate 4.2. No VLP's or nucleocapsids were observed in preparations made from healthy allantoic fluid.

4.3.2. EM of sections of infected TOC's and Vero cells

Various developing forms of VLP's were observed in sections of TOC's. Plate 4.5 shows the presence of developing VLP's in a vesicle. Electron dense accumulations of viral nucleoprotein-like material were observed close to the cell membrane (Plates 4.6, 4.7 and 4.8). Plate 4.8 shows a nucleocapsid-like structure with electron dense material located beneath the cell membrane which appears to be altered. Fine structures resembling spikes were vaguely discernable on the outer surface of the plasma membrane as shown in Plates 4.7 and 4.8. The development of electron dense filamentous and pleomorphic nucleoprotein-like material were also observed in a vesicle-like structure (Plate 4.9). All such accumulations were located in the cytoplasm of the tracheal epithelial cells.

The development of VLP's in Vero cells was found to be similar to that in TOC's. Numerous developing nucleocapsid-like

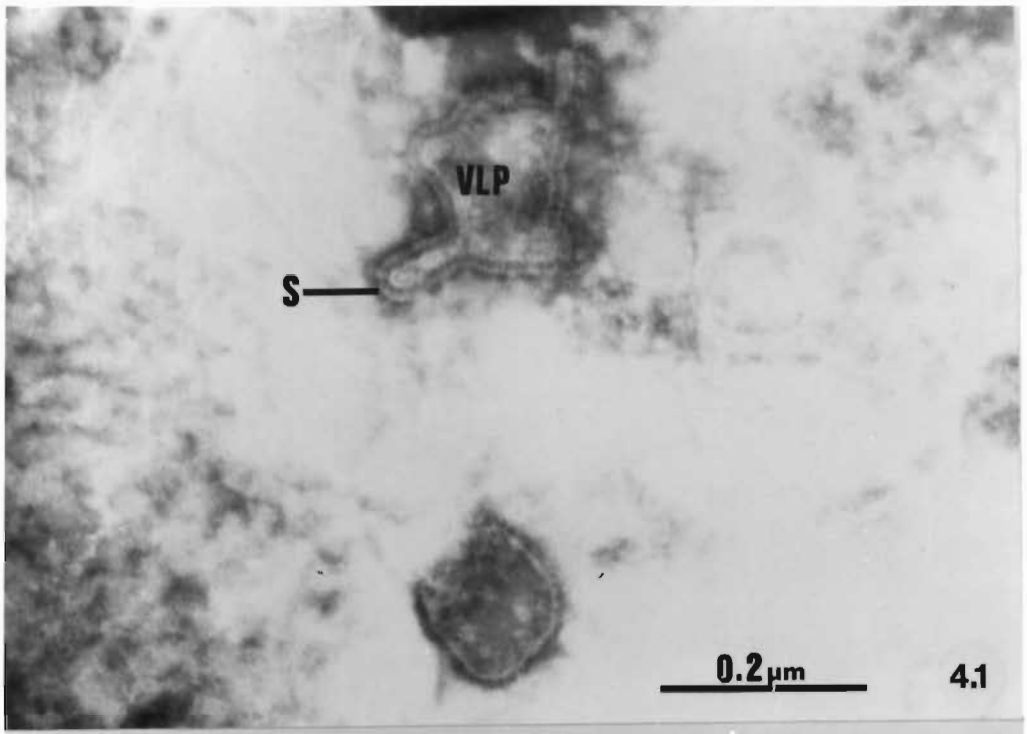
structures were observed in vesicular-like structures in the cytoplasm of Vero cells (Plates 4.10-4.13). Electron dense nucleoprotein accumulations, similar to that of infected TOC's, were observed in vesicle-like structures (Plate 4.14 and 4.15). Plate 4.15 shows a well defined nucleoprotein-like accumulation in the process of differentiation. Electron dense accumulations close to the plasma membrane, in the process of budding, are shown in Plate 4.16. Each accumulation of nucleoprotein may form a separate VLP. Four such accumulations are depicted in Plate 4.16. There were no accumulations of nucleoprotein and a marked lack of vesicles in healthy TOC and Vero cell sections. Vesicles that were observed in healthy material were always devoid of any distinctive material.

4.4. Discussion

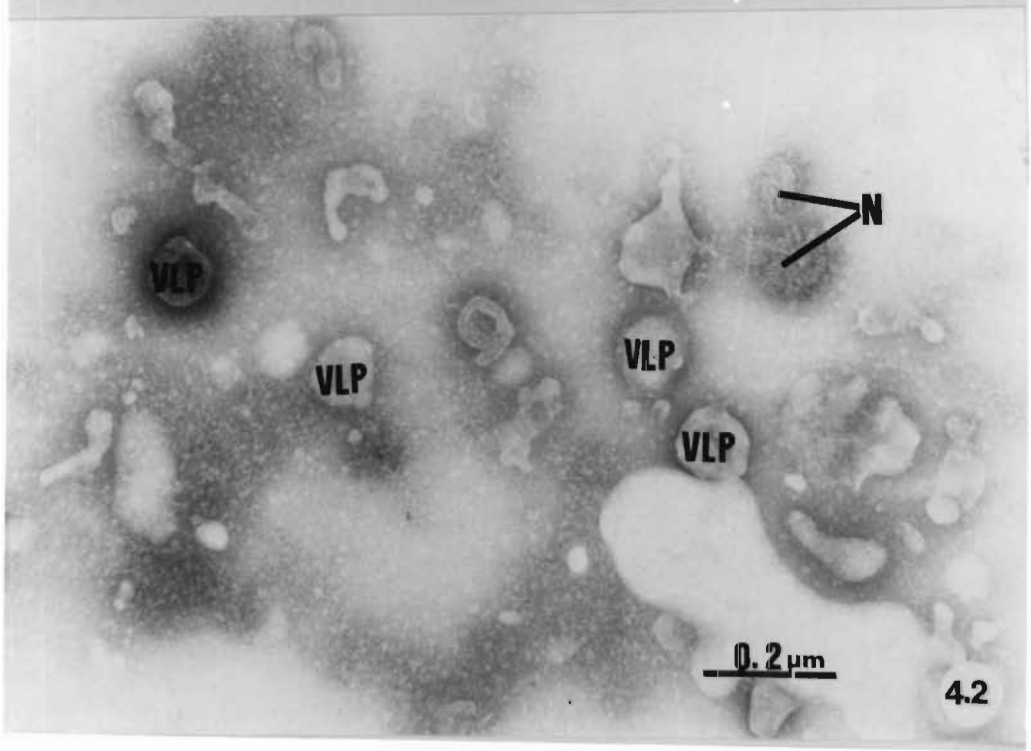
The EM results on infective allantoic fluid were consistent with the findings of other workers for TRTV (Buys et al., 1989a,b; Collins and Gough, 1988; Gough and Collins, 1989; Picault et al., 1987). They found the agents associated with TRT and SHS to be highly pleomorphic virus particles with diameters ranging from 40-800 nm possessing a fringe of approximately 13nm long spikes. The diameter of the internal nucleocapsid was approximately 14 nm and had a "herringbone" type appearance. The VLP'S observed in EM preparations of infective allantoic fluid in this study, were approximately 100-300 nm in diameter with the spikes approximately 12 nm in length. The helical nucleocapsid was approximately 15 nm in diameter. There were also a few VLP's

Plate 4.1. Pleomorphic virus-like particles (VLP) with a fringe of spikes (S)

Plate 4.2. Numerous pleomorphic VLP's. The fringe of spikes is not well defined. The coiled nucleocapsid-like structure (N) is vaguely visible inside a VLP.

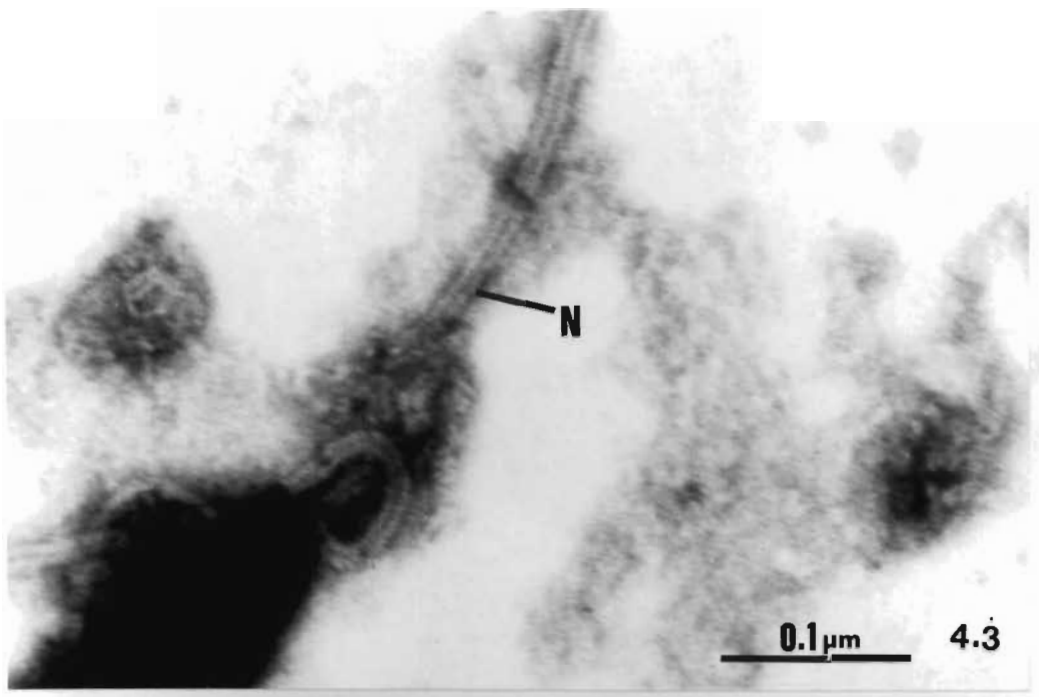


4.1

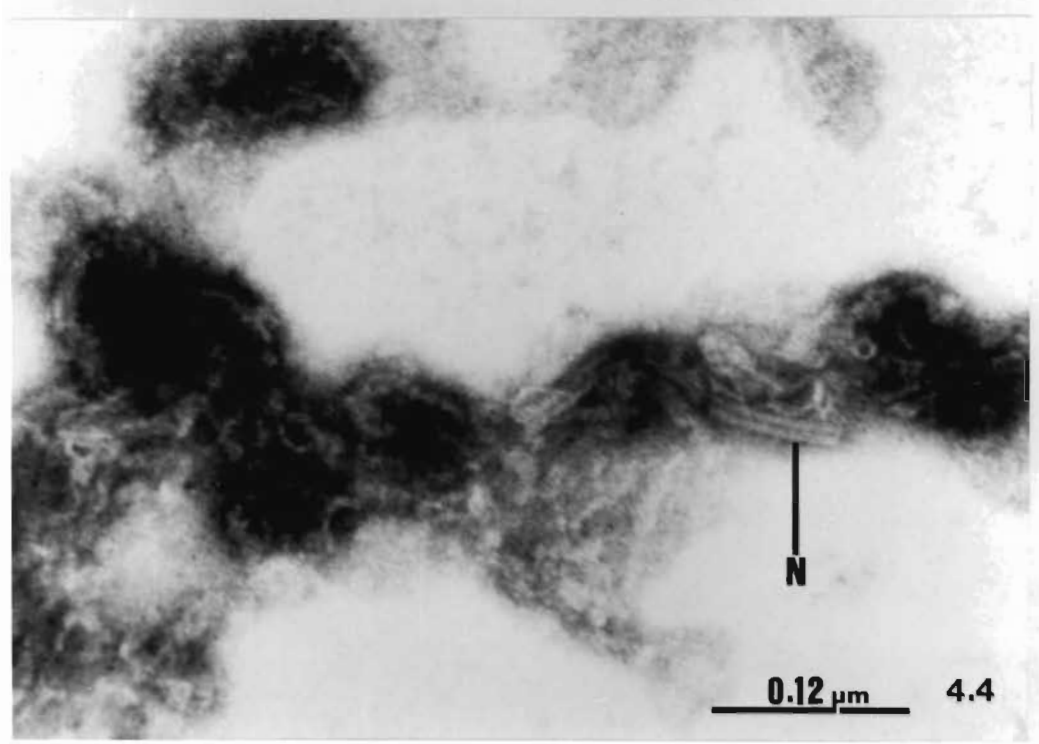


4.2

Plate 4.3 and 4.4. Helical nucleocapsid (N) approximately 15 nm in diameter from disrupted VLPs.



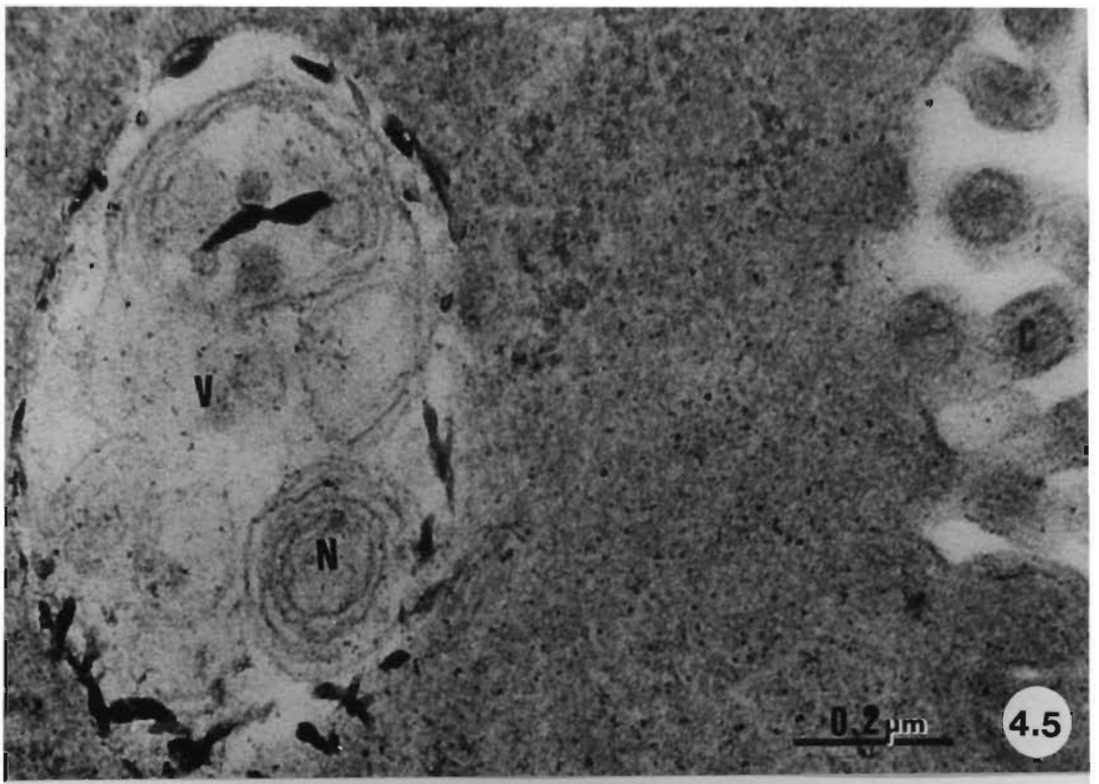
4.3



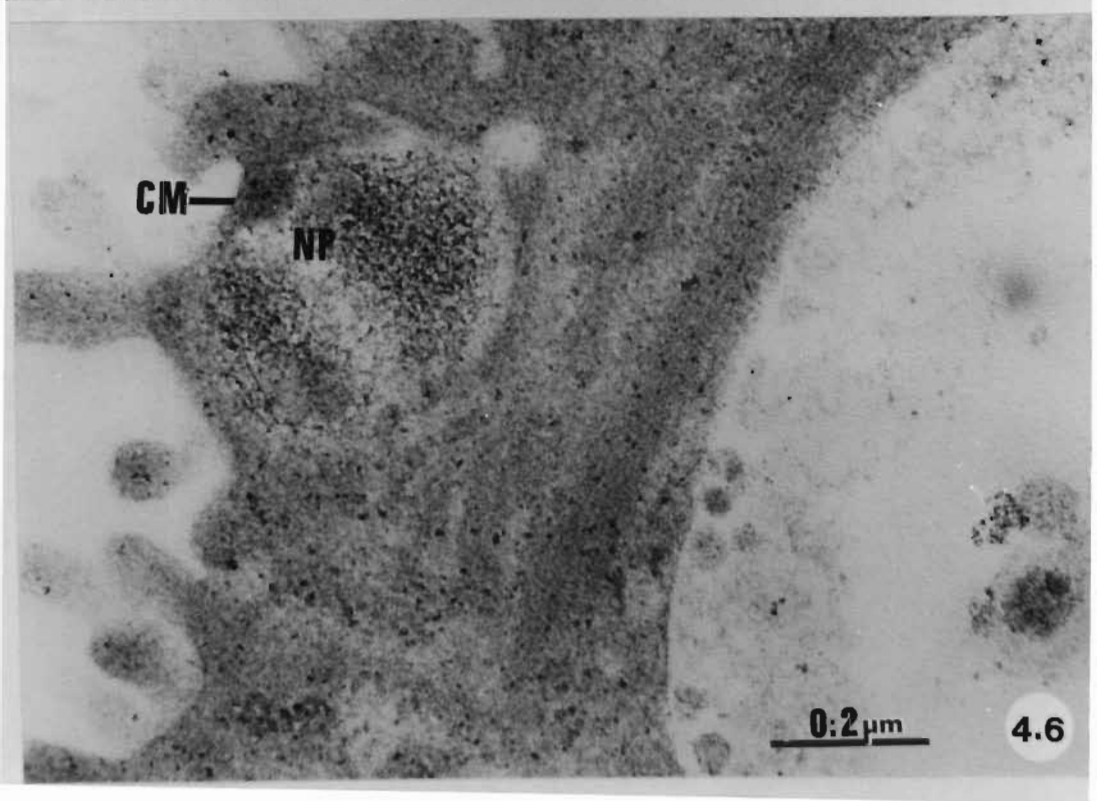
4.4

Plate 4.5. Possible nucleocapsids (N) of developing VLPs in a vesicle (V) of a tracheal epithelial cell. The cilia (C) are visible.

Plate 4.6. Viral nucleoprotein-like material (NP) close to the cell membrane (CM) of a tracheal epithelial cell.

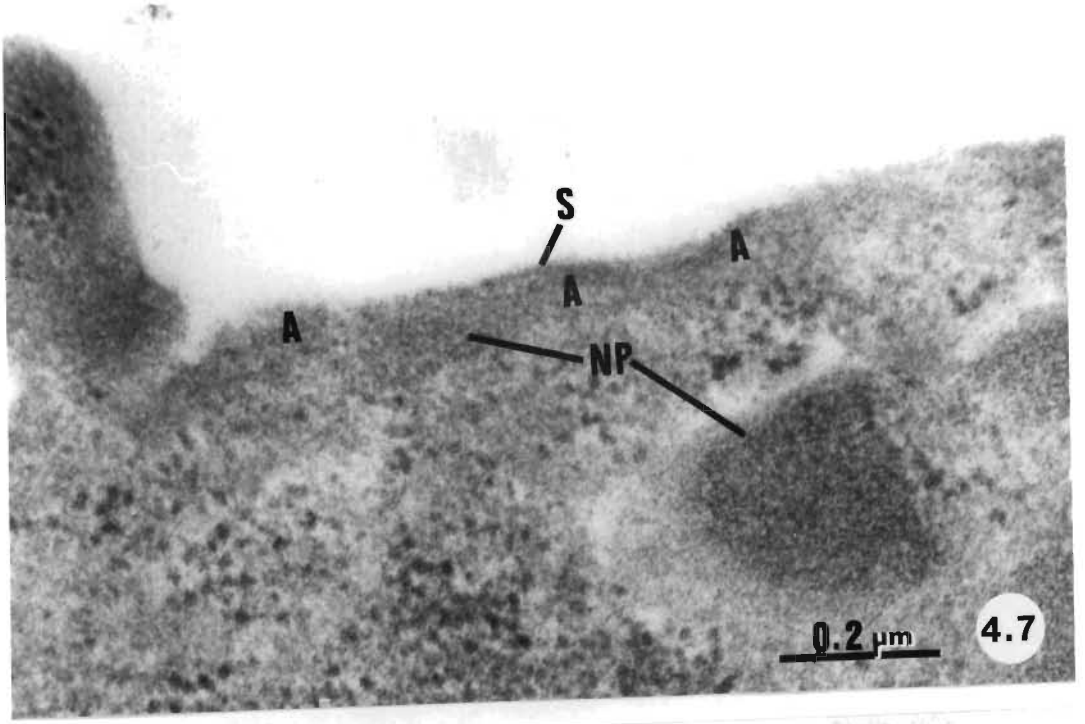


4.5



4.6

Plate 4.7 and 4.8. Electron dense nucleoprotein-like accumulations (NP) just beneath the cell membrane of a tracheal epithelial cell that appears to be altered (A) in areas. Structures resembling spikes (S) are vaguely discernable in some areas of the outer surface of the cell membrane (r = ribosome).


S
A
A
A
NP
0.2 µm
4.7

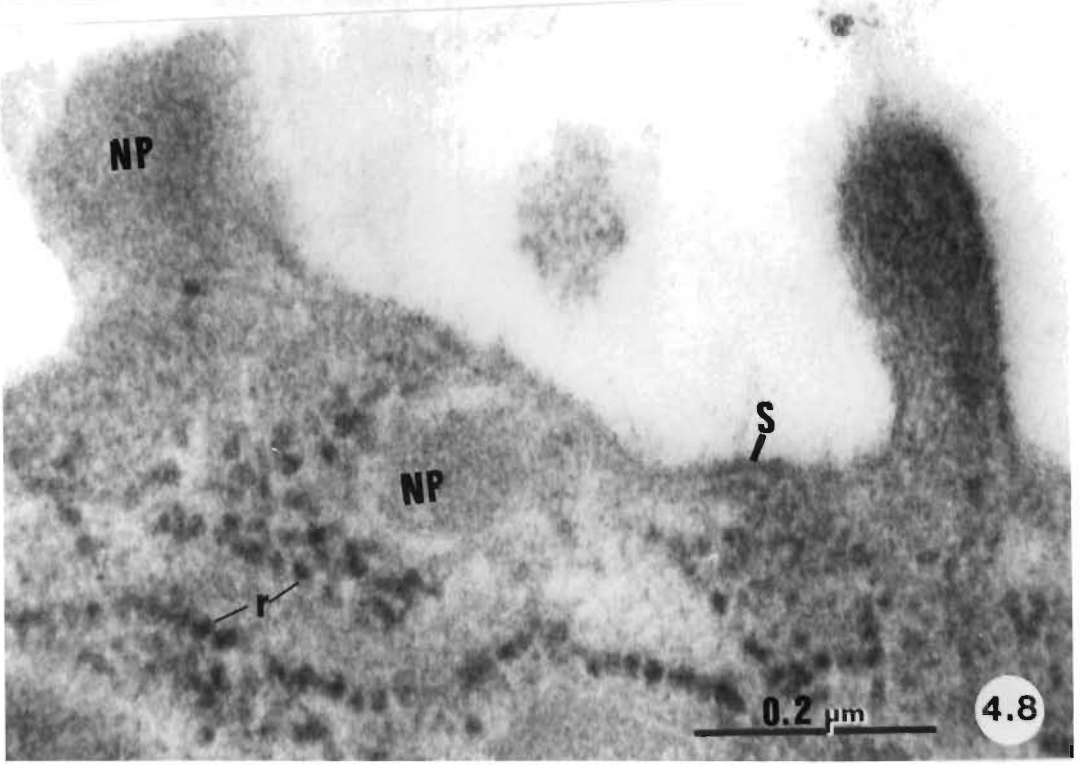
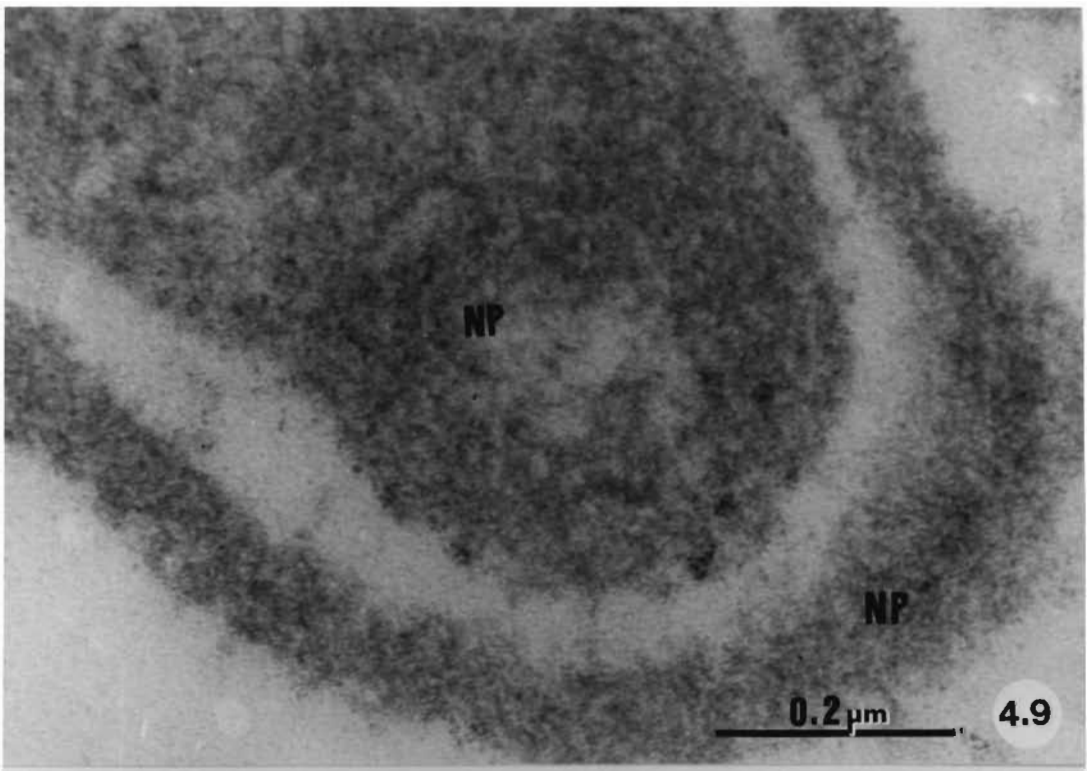
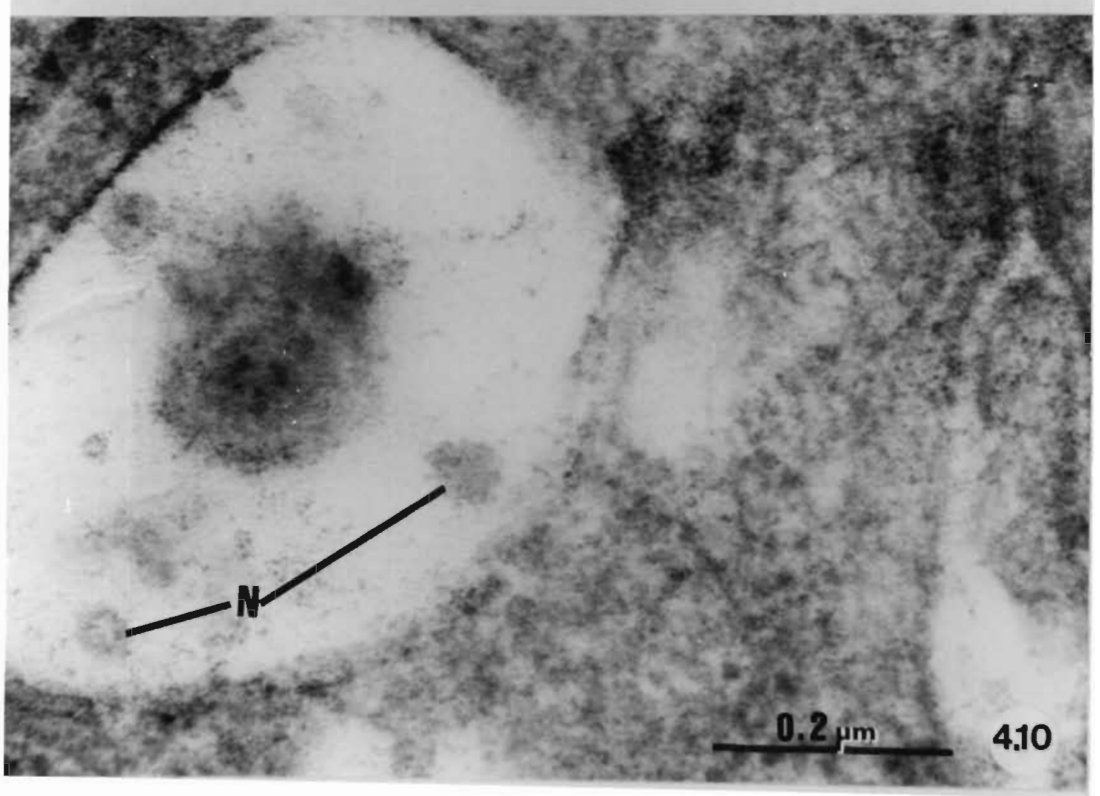

NP
S
NP
r
0.2 µm
4.8

Plate 4.9. Electron-dense viral nucleoprotein-like material (NP) in a vesicle of a tracheal epithelial cell.

Plate 4.10. Possible viral nucleocapsid (N) in the process of development in a vesicle of a Vero cell.

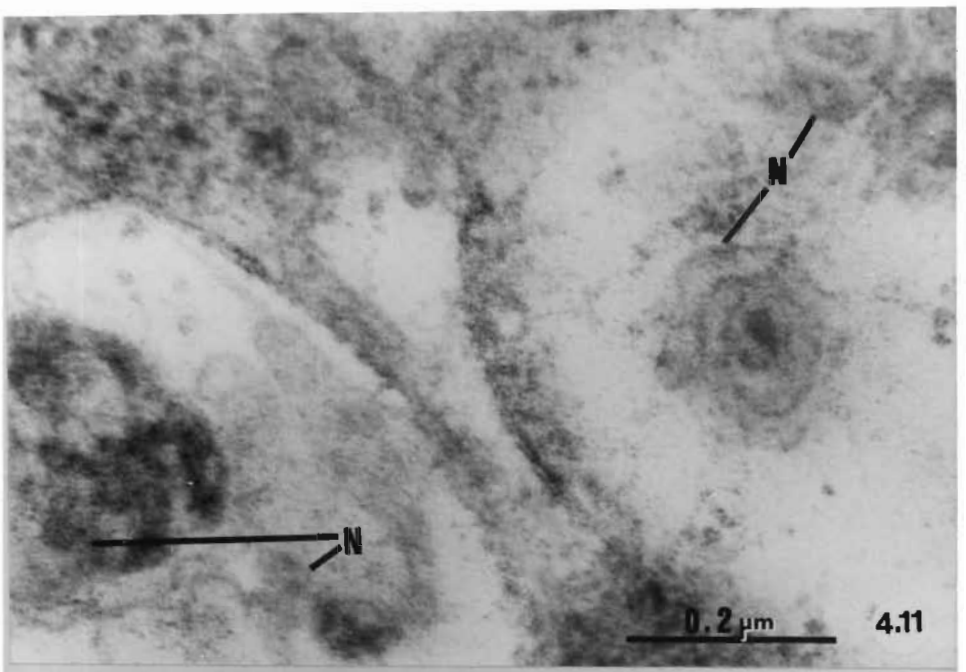


4.9

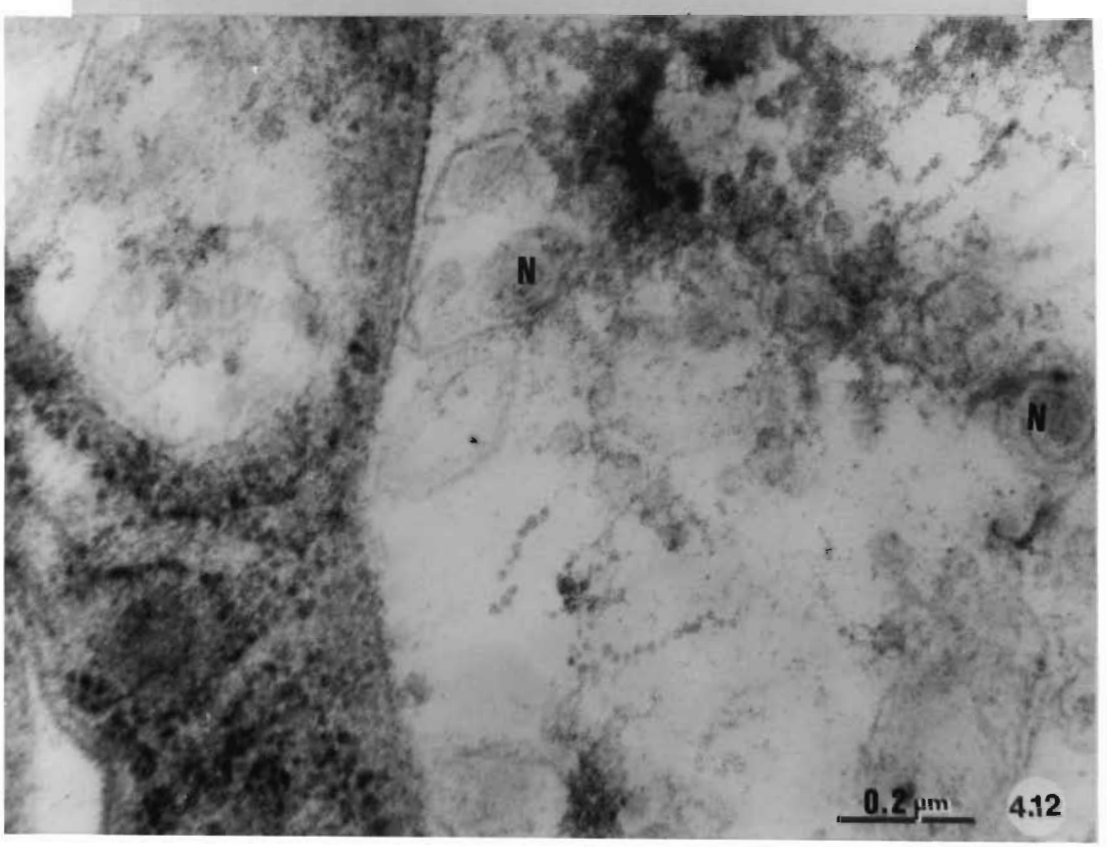


4.10

Plate 4.11 and 4.12. Developing viral nucleocapsid-like (N) structures in vesicles of Vero cells.



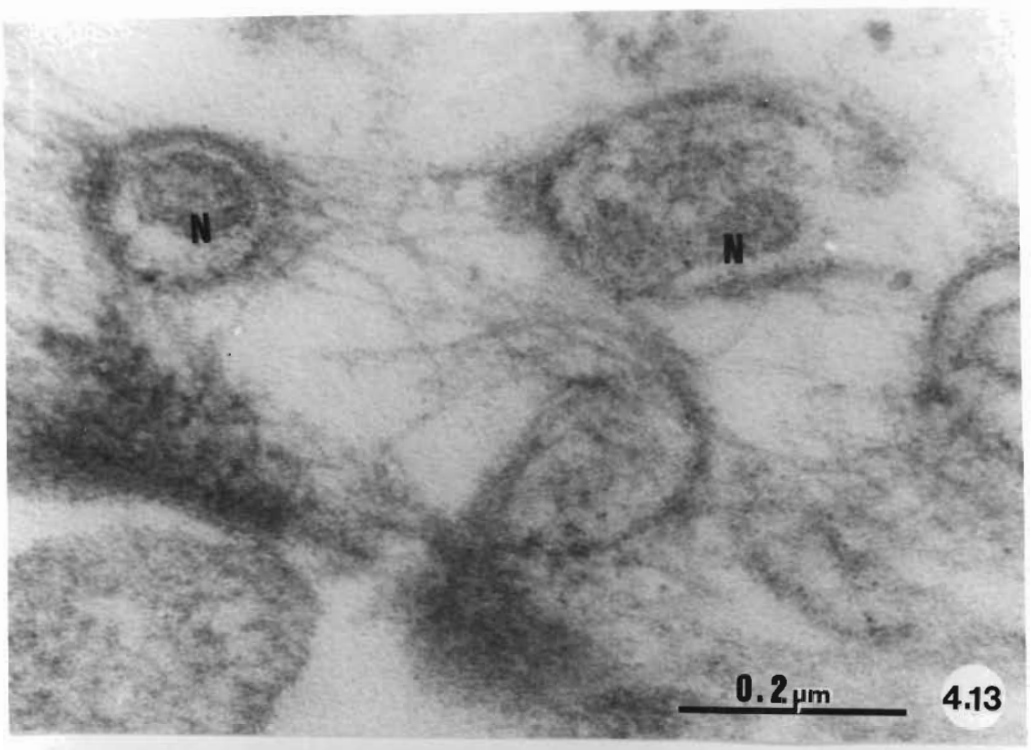
4.11



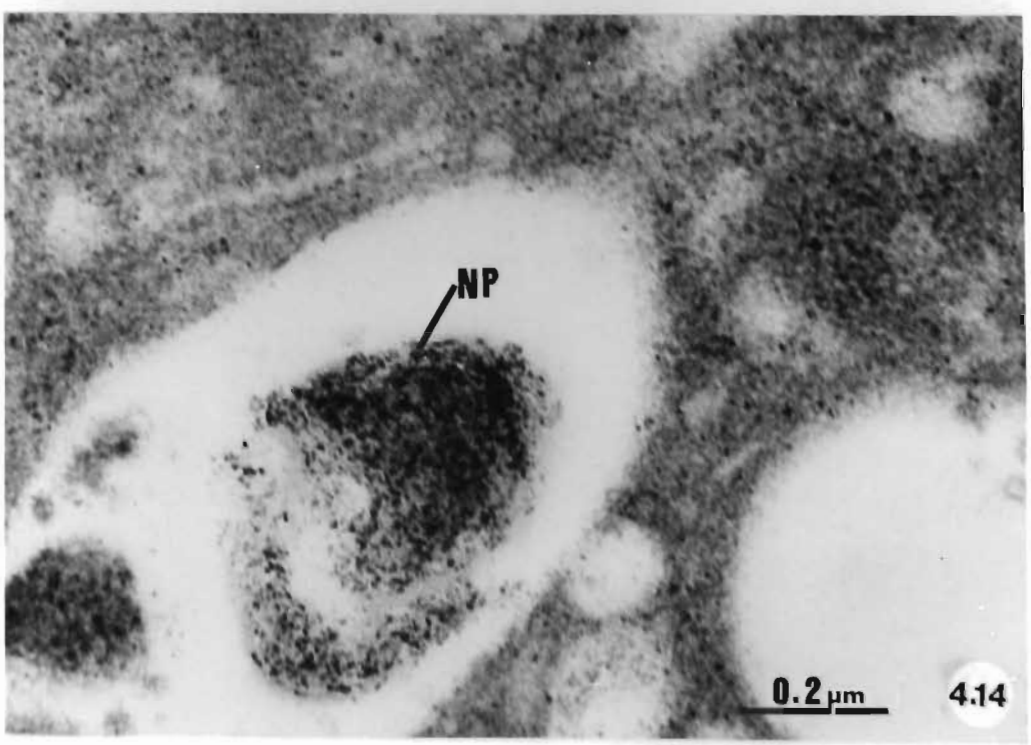
4.12

Plate 4.13. Possible viral nucleocapsids (N) in the process of development in a vesicle of a Vero cell.

Plate 4.14. Electron-dense nucleoprotein-like accumulations (NP) in vesicles of a Vero cell.



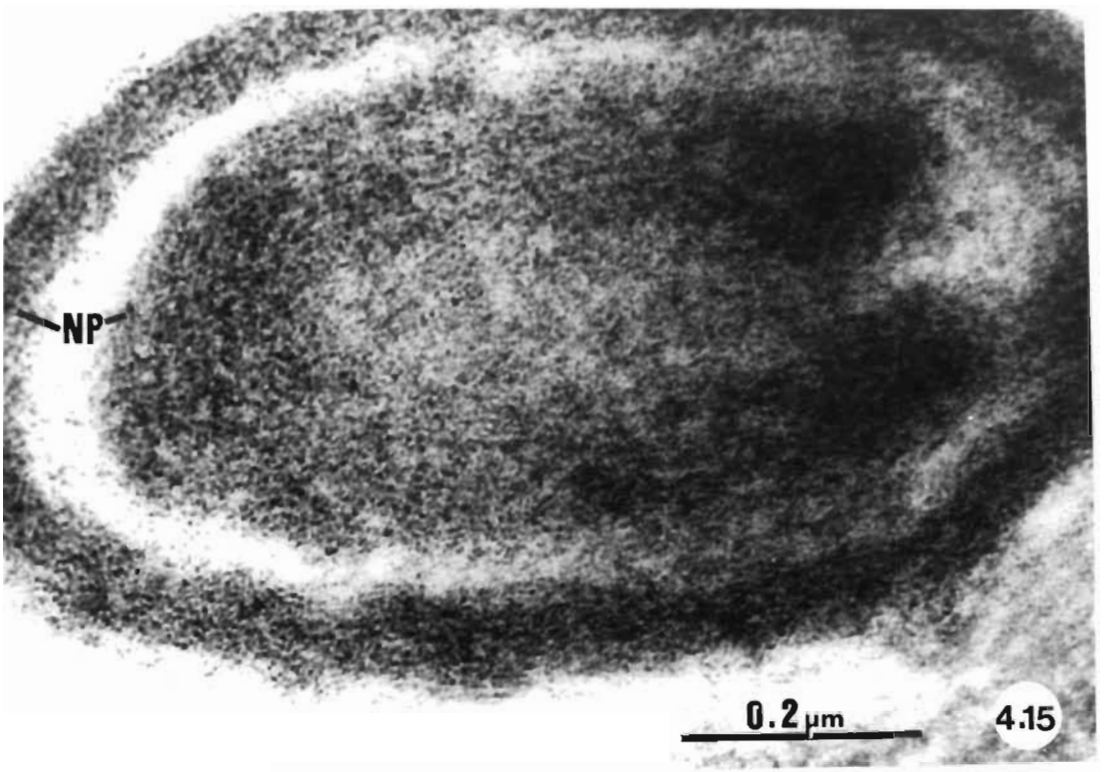
4.13



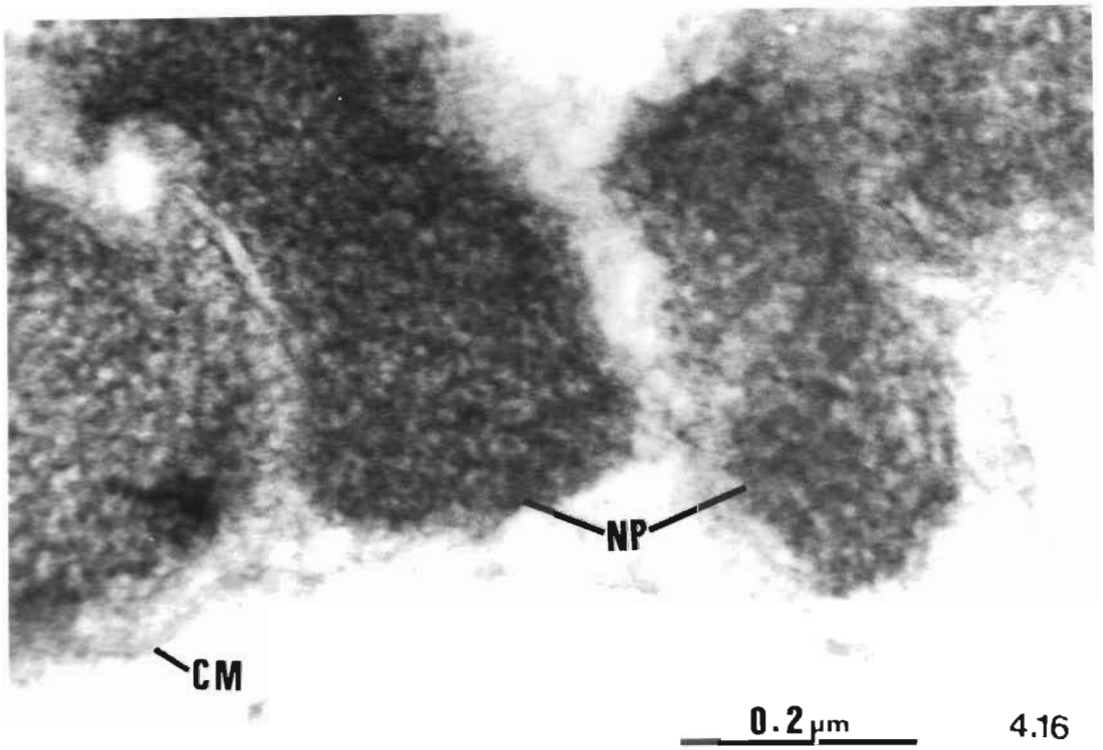
4.14

Plate 4.15. Accumulations of nucleoprotein-like material (NP) in the process of differentiation in a vesicle of a Vero cell.

Plate 4.16. Electron-dense accumulations of viral nucleoprotein-like material (NP) in the process of budding from the cell membrane (CM) of a Vero cell. Each accumulation probably results in a VLP.



4.15



4.16

smaller than 100 nm, depicted in Plate 4.2, which were fragmented particles caused during preparation for EM.

The results obtained in TOC and Vero cells were identical, suggesting that the process of VLP development and maturation is similar in both systems. It appears that virus-like nucleoprotein may accumulate in vesicles where virus assembly may occur and these vesicles move toward the plasma membrane where maturation and budding of the VLP's occur. Also, accumulations of nucleoprotein-like material occurred close to the cell plasma membrane and appeared to form a possible nucleocapsid as described by Fraser and Martin (1978) for measles virus. Virus-like particle specified spikes are then probably inserted into the plasma membrane (Plate 4.8). The mature VLP then buds from the cell plasma membrane which contains spikes, incorporating this cell membrane as its own. Plate 4.8 shows a possible VLP in the process of budding from a tracheal epithelial cell. These findings are similar to those of Buys et al. (1989a) and Giraud et al. (1987) for TRTV in Vero cells. These results are also a characteristic of another Pneumovirus, RSV, which has highly pleomorphic virions ranging in diameters between 90-860 nm with an internal nucleocapsid 14 nm in diameter (Parrott et al., 1979).

The smaller diameter of the nucleocapsid differentiates these isolates from those of NDV, a Paramyxovirus, which has a wider helical nucleoprotein component 18 nm in diameter (Chanock, 1979). The influenza viruses are also pleomorphic, but are

generally spherical with an approximate diameter of 100 nm, surface spikes 8-10 nm long, and a nucleoprotein component approximately 9 nm in diameter (Dowdle, Kendal, and Noble, 1979). The results obtained here are similar to features associated with RSV and TRTV both of which are Pneumoviruses. The EM differences between TRTV and isolates 4916/91, 652/93 and 711/93, are indistinguishable. It is therefore probable that isolates 4916/91, 652/93 and 711/93 are similar to RSV and TRTV and are likely members of the genus Pneumovirus of the Paramyxoviridae family.

CHAPTER 5

SDS-PAGE AND WESTERN BLOTTING OF THREE TRTV-LIKE ISOLATES

5.1. Introduction

Advances in molecular biology and immunology have provided pathologists with powerful tools to enhance morphologic and clinical laboratory diagnoses. The application of this technology to pathology represents the transfer of recent basic research tools into the laboratory (Fenoglio-Preiser and Willman, 1987).

Electrophoresis in polyacrylamide gels in the presence of the anionic detergent sodium dodecyl sulphate (SDS), has proven to be a useful tool for the separation of proteins and the determination of their molecular weights (Sigma Chemical Company, 1988). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) can therefore greatly 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 dissociate 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 gel 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 complexes 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 SDS-polyacrylamide gels depends 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 (R_m) with known protein markers. A linear relationship is obtained if the logarithms of the molecular weights of the protein markers are plotted against their respective electrophoretic mobility (Sigma Chemical Company, 1988).

Many researchers have performed SDS-PAGE on TRTV (Cavanagh and Barrett, 1988; Collins and Gough, 1988; Gough and Collins, 1989; Ling and Pringle, 1988) and although their results are largely similar, there are some differences which have been explained as being caused by differences in the SDS-PAGE protocols used and not to strain differences.

Western blotting involves the electrophoretic transfer of proteins from the gel to a solid support such as nitrocellulose paper. This paper is then probed with antibodies that react specifically with antigenic epitopes displayed by the target

protein. Western blotting can therefore be used to identify specific proteins in complex mixtures of proteins and to indicate immunogenic relatedness between different strains of a virus (Sambrook et al., 1989).

This Chapter describes the SDS-PAGE and western blotting results obtained with three TRTV-like isolates, 4916/91, 652/93 and 711/93.

5.2. Materials and Method

5.2.1. Preparation of samples for SDS-PAGE

The 5th y/s allantoic fluid of isolates 4916/91, 652/93 and 711/93 were each harvested from SPF eggs and centrifuged in a Heraeus Hettich Universal II centrifuge at approximately 3000 g for 10 min. The supernatant of each sample was recentrifuged using a Sorvall S-20 rotor at 70000 g for 1 h in a Sorvall RC28S centrifuge. The resulting pellet was resuspended in 1 ml of sterile distilled water and layered onto a 15 ml 30-55% (12.5 ml and 2.5 ml respectively) stepped sucrose gradient. The sucrose gradients were centrifuged at 56500 g for 1 h in the Sorvall S-20 rotor. The band at the interface of the gradient was removed, diluted with distilled water to 15 ml, and centrifuged as before at 56500 g for 1 h. The resulting pellet was resuspended in 0.5 ml of sterile distilled water and used as sample for SDS-PAGE and subsequent western blots. Allantoic fluid from healthy 13-day-old embryonated SPF eggs was harvested and treated identically to that of the three isolates.

Just prior to SDS-PAGE, 50 μl of each purified sample was added to an equal volume of SDS gel loading buffer (Appendix, recipe 2) and heated to 100°C for 3 min.

5.2.2. SDS-PAGE

The method used for SDS-PAGE was according to that of Sambrook et al. (1989). A 12% running gel and a 5% stacking gel were cast according to the formulation in Table 5.1.

Table 5.1. The formulation for the preparation of the stacking and running SDS-polyacrylamide gels.

INGREDIENT	STACKING GEL (ml)	RUNNING 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% (w/v) acrylamide and 1% (w/v) N,N'-methylenebisacrylamide in deionised water.
* N,N,N',N'-tetramethylethylenediamine.

The glass plates for gel casting were assembled and the freshly prepared running gel mixture introduced between them with the aid of a syringe and 21 gauge needle to approximately 2 cm from the top of the plates. With the aid of a Pasteur pipette, isobutanol was overlayed 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 washed with distilled water. The water was drained and the stacking gel mixture poured on top of the running gel. Immediately thereafter, a clean teflon comb with 10 "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 the electrophoresis apparatus and Tris-glycine electrophoresis buffer (Appendix, recipe 3) added to the top and bottom buffer reservoirs.

Approximately 50 μl of each sample for electrophoresis was separately loaded into the bottom of the well with the aid of a micropipette. Well 1 was loaded with MW-SDS-70 (Sigma) molecular weight markers, well 2 with healthy control material and well 3,4 and 5 with 4916/91, 652/93 and 711/93 material respectively. SDS-gel loading buffer was loaded into unused wells. Two such gels were loaded identically and run together in the same apparatus. The electrophoresis apparatus was connected to a Consort E702 power pack and run at 180 volts for approximately 2 h or until the bromophenol blue dye front reached the bottom of the running gel.

One of the two 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 glacial acetic acid. The solution was filtered through Whatman No. 1 filter paper and the

gel immersed in this solution with gentle agitation on a micro-orbital shaker for 24 h 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% acetic acid on a micro-orbital shaker. The destain solution was regularly changed at 2 h intervals. After 8 h 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 weight of the protein markers (lane 1) on the y-axis and their R_m valus on the x-axis. The molecular weights of all other protein bands (lanes 2-5) were determined from this standard graph.

5.2.3. Western blotting

The unstained SDS polyacrylamide gel was used for western blotting according to the method of Sambrook et al. (1989). A piece of Hybond-C nitrocellulose paper (Amersham), corresponding to the size of the gel, was cut. The blotting apparatus, manufactured and supplied by South African Scientific Products, was assembled. The nitrocellulose paper was placed against one side of the gel and the gel and nitrocellulose paper were sandwiched between two Whatman 3M filter papers cut to the size of the gel. A porous pad of sponge was placed on either side of

the sandwich. A gauze support was clipped together on either side of the sandwich with teflon clips to hold the sandwich in place.

The sandwich was placed in the electrotransfer unit so that the gel faced the negative electrode and the nitrocellulose paper the positive electrode. Refrigerated transfer buffer (Appendix, recipe 4) was added to the buffer chamber to a level just below the electrodes. A current of 200 mA was applied to the sandwich for 16 hrs with a Consort E702 power pack. The sandwich was removed and the nitrocellulose paper carefully removed and transferred to a clean 3M Whatman filter paper. The nitrocellulose paper was allowed to dry at room temperature for 1 h.

The nitrocellulose paper was submerged in a tray of deionised water for 5 min and transferred to a working solution of Ponceau S stain for 10 min with gentle agitation on a micro-orbital shaker. The Ponceau S stock solution was prepared as follows:

Ponceau S		2 g
Trichloroacetic acid		30 g
Sulfosalicylic acid		30 g
Distilled Water	to	100 ml

The working solution of Ponceau S was prepared by diluting the stock tenfold with distilled water. The Ponceau S stained nitrocellulose paper was washed several times in distilled water. The stained protein bands of the molecular weight markers were marked with waterproof ink. The nitrocellulose paper was then

placed in a container and covered with a blocking solution, consisting of a solution of 5% non-fat dried milk powder in 1M PBS (pH 7.0), for 1 h at room temperature on a micro-orbital shaker.

The nitrocellulose paper was washed twice with 1M PBS (pH 7.0) for 5 min each. The primary antibody solution, prepared by adding 100 μl of high titre TRTV monospecific antisera. (refer Chapter 3) to 10 ml 1M PBS (pH7.0), was incubated with the blot for 24 h at 37°C. The blot was washed with PBS (4 X 5 min), incubated (2 h, RT) with 10 ml of anti-chicken horseradish peroxidase enzyme conjugate (Sigma) (1:2000 dilution in PBS, pH 7.0) and again washed (4 X 5 min) with PBS.

Just prior to developing the blot, 0.1 ml of a 4-chloro-1-naphtol (Promega) stock solution was added to 10 ml of 50 mM Tris buffer (pH 7.6). The solution was filtered through Whatman 3M filter paper. Ten μl of a 30% hydrogen peroxide solution was added and the blot was incubated (30 min, RT), with gentle agitation on a micro-orbital shaker. The reaction was stopped by decanting the substrate solution and rinsing the blot with the PBS solution for 10 min.

The TRTV-specific antigenic proteins of the three TRTV-like isolates were identified on the blot and their molecular weights determined from the standard graph.

5.3. Results

5.3.1. SDS-PAGE analysis of the proteins of the three TRTV-like isolates

Plate 5.1 shows the result obtained after CBB staining of the SDS-PAGE gel. The molecular weights of the protein bands in the control (lane 2) and test samples (lane 3-5) were determined from the standard graph (Fig. 5.1). Table 5.2 depicts the molecular weights of the various bands obtained from each sample.

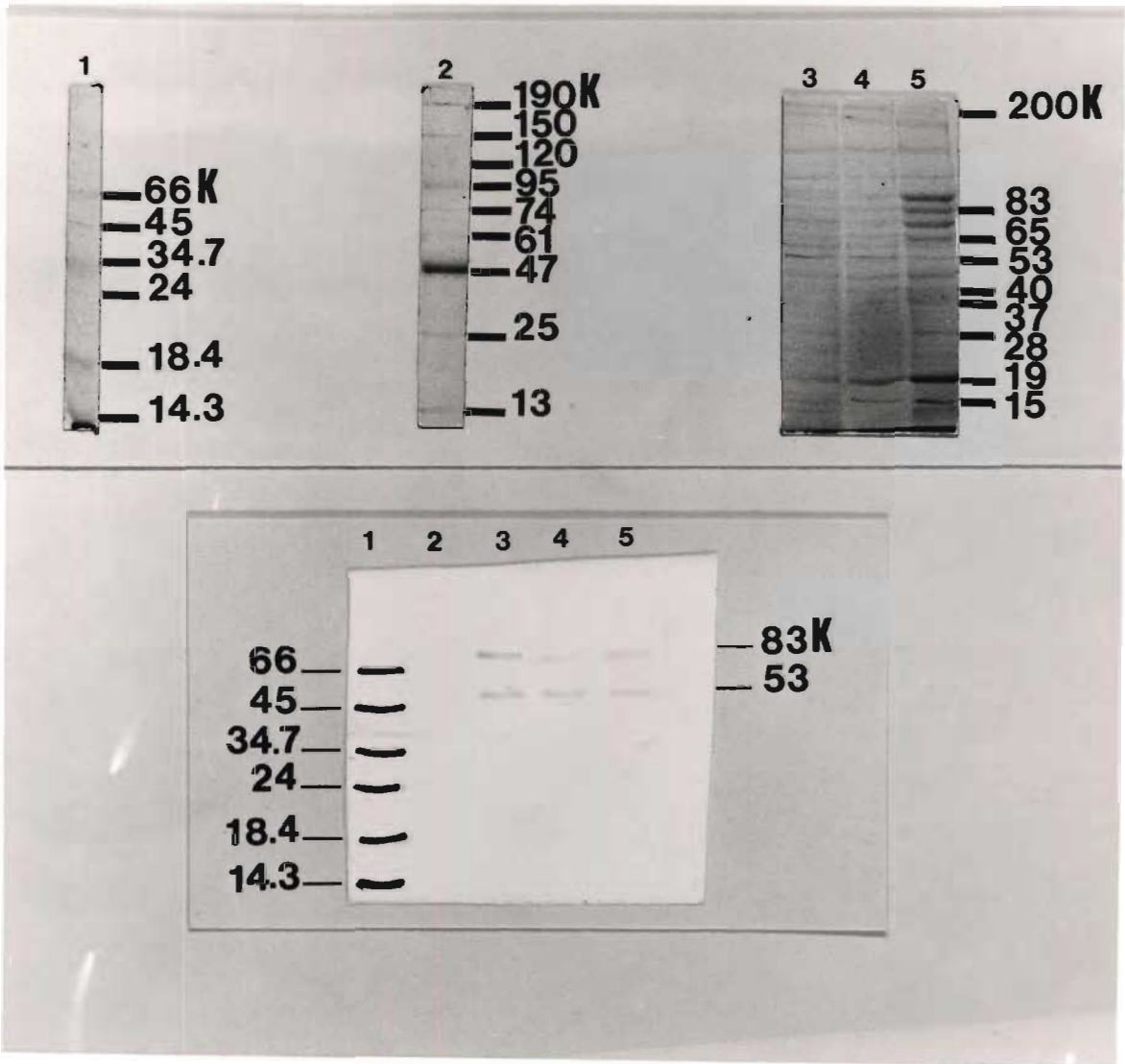
Since the results obtained with all three TRTV-like isolates were identical, only those of 4916/91 are shown in Table 5.2.

5.3.2. Western blot analysis of the immunogenic proteins of the three TRTV-like isolates using TRTV-monospecific antisera

Two bands of polypeptides of molecular weights approximately 83 kDa and 53 kDa (Plate 5.2) were observed in the developed blot of samples 4916/91, 652/93 and 711/93. No bands were observed in the blot of the healthy control material.

Plate 5.1. SDS-PAGE results obtained with isolates 4916/91, 652/93 and 711/93. Lane 1 = molecular weight markers; Lane 2 = healthy allantoic fluid; Lanes 3, 4 and 5 were loaded with 4916/91, 652/93 and 711/93 infected allantoic fluid respectively.

Plate 5.2. Western blot with isolates 4916/91, 652/93 and 711/93 using TRTV-specific antisera. Samples in each lane are as for Plate 5.1.


1
66K
45
34.7
24
18.4
14.3
2
190K
150
120
95
74
61
47
25
13
3
4
5
200K
83
65
53
40
37
28
19
15
1
2
3
4
5
66
45
34.7
24
18.4
14.3
83K
53

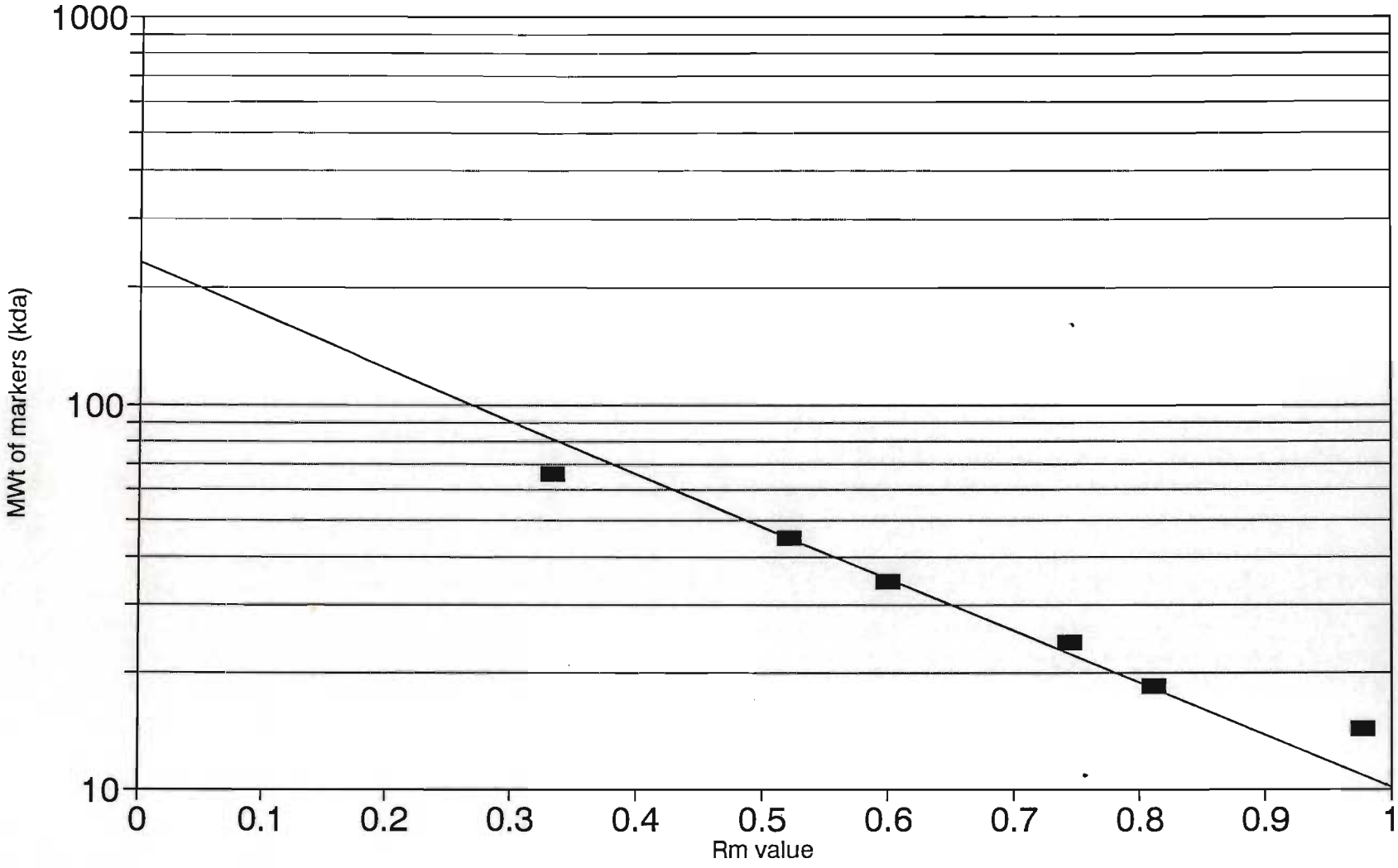


Fig. 5.1. Molecular weight of markers against their respective Rm values

Table 5.2. The R_m values and resultant molecular weights (M_r) of the protein bands of the 4916/91 isolate obtained using the standard graph

CONTROL		4916/91	
R_m	M_r(kDa)	R_m	M_r(kDa)
-	-	0.03	200
0.06	190	0.06	190
0.16	150	0.16	150
0.23	120	0.23	120
0.29	95	0.29	95
-	-	0.32	83
0.37	74	0.37	74
-	-	0.4	65
0.42	61	0.42	61
-	-	0.466	53
0.51	47	0.51	47
-	-	0.56	40
-	-	0.6	37
-	-	0.69	28
0.72	25	0.72	25
-	-	0.82	19
-	-	0.89	15
0.93	13	0.93	13

5.4. Discussion

According to Naylor and Jones (1993) several researchers have reported on the polypeptide composition of TRTV based on SDS-PAGE results. Most have reported detecting seven viral polypeptides of molecular weights 200 kDa, 82-84 kDa, 68 kDa, 38-43 kDa, 35-40 kDa, 30-35 kDa and 19-22 kDa. The 68 kDa polypeptide could dissociate into a 53-54 kDa and a 14-15 kDa

polypeptide (Cavanagh and Barrett, 1988). Cavanagh and Barrett (1988) found the 82-84 kDa and the 53-54 kDa polypeptides to be glycosylated. According to them, three polypeptides distinguish TRTV from Paramyxoviruses and Morbilliviruses and group it with the Pneumovirus. The nucleocapsid protein (38-43 kDa) and the P-protein (35-40 kDa) of TRTV were found to be much smaller in size whilst the 22 kDa protein was not found in the other two genera.

The results obtained with the three TRTV-like isolates (4916/91, 652/93 and 711/93), were very similar to those obtained by Cavanagh and Barrett (1988), Collins and Gough (1988), Gough and Collins (1989) and Ling and Pringle (1988). Nine viral polypeptides were detected in this study with molecular weights corresponding to those obtained for TRTV.

The 83 kDa band detected corresponds to the 82-84 kDa band of TRTV, the 53 kDa and 15 kDa bands correspond to the dissociated 68 kDa band of TRTV which results in two bands of 53-54 kDa and 14-15 kDa. The 40 kDa, 37 kDa, 28 kDa and 19 kDa proteins are similar to the 38-43 kDa, 35-40 kDa, 30-35 kDa and 19-22 kDa TRTV polypeptides respectively.

The slight differences in molecular weights between a few of the TRTV polypeptides and those of the three TRTV-like isolates are probably due to differences in the gel running conditions and the plotting of the standard curve.

The western blot analysis of the SDS-PAGE gel, revealed the presence of two stained bands of molecular weights 83 kDa and 53 kDa. Protein bands will only develop on a western blot if the primary antibody used in the analysis contains sufficient antibodies to react with the antigen on the blotted nitrocellulose paper. Therefore, with the use of specific antisera, one could use the western blot to determine relatedness between isolates. Since there were stained bands on the western blot of the three TRTV-like isolates and none on that of the control sample, it would appear that there is a close relatedness between TRTV and the three isolates. It is also likely that the TRTV-like isolates are strains of TRTV. The SDS-PAGE and western blot analysis of 4916/91, 652/93 and 711/93 support this suggestion. However, in order to firmly establish the relatedness between TRTV and the three TRTV-like isolates, it is essential that a DNA probe against TRTV be developed and tested against these isolates. A positive result would provide conclusive evidence of the close relatedness between TRTV and the TRTV-like isolates.

CHAPTER 6

THE CONSTRUCTION AND USE OF A TRTV DNA PROBE AGAINST THREE TRTV-LIKE ISOLATES

6.1. Introduction

The ability to achieve a rapid, accurate diagnosis of a pathogen is fundamental to maintaining good programmes to monitor health and disease in poultry flocks (Murtaugh, 1993). Current methods of laboratory diagnosis of infectious diseases are based on isolation of the agent, or serological methods. Clinical signs alone cannot be used to diagnose the causal organism since many can produce similar diseases (Andreasen, Jackwood and Hilt, 1991). Often, many passages of a virus in laboratory host systems are required before it becomes adapted for growth in these systems. This often makes the isolation of viruses a lengthy process. The process is labour-intensive and expensive, especially considering the fact that two to three passages are required before the isolation attempt can be aborted as being negative for virus (Kwon, Jackwood, Brown and Hilt, 1993). Alternative diagnostic methods such as detection of increasing antibody titres and fluorescent antibody tests, also have disadvantages such as non-specificity, insensitivity or delayed diagnosis. A sensitive, specific and rapid means of diagnosis is desirable (Andreasen *et al.*, 1991).

Recent advances in molecular biology and recombinant DNA

technology have provided new opportunities for improving the effectiveness and productivity of disease diagnosis. This provides the ability to identify disease agents rapidly and accurately by their molecular structure rather than by indirect measurements based on what they do in cell culture, animals or embryonated eggs (Murtaugh, 1993).

The polymerase chain reaction (PCR) is an in vitro method for the enzymatic synthesis of selected pieces of DNA or RNA sequences, specific for each type of organism, using two oligonucleotide primers that hybridise to opposite strands and flank the region of interest in the target DNA (Saiki, 1989). In PCR, DNA polymerase is incubated together with primers, deoxynucleotide triphosphates (dNTP) and the sample DNA. If DNA from the target species is present, the bases in the primers will pair with the complementary bases in the sample DNA. To perform PCR on RNA, it has to be initially transcribed into DNA using a reverse transcriptase enzyme. This DNA (cDNA) is then used as the target DNA in the PCR reaction. The enzyme machinery will then extend the chain by adding complementary bases (Murtaugh, 1993).

PCR is a three-step procedure of template DNA denaturation to separate the two strands of DNA, primer annealing to the template and the extension of the annealed primers by the DNA polymerase. This constitutes one cycle of PCR. Because the primer extension products synthesised in one cycle can serve as a template in the next, the number of target DNA copies approximately doubles at every cycle. Thus, 20 cycles of PCR yields about a million-fold

amplification (Jackwood, Kwon and Hilt, 1992).

The selection of times, temperatures and number of cycles for PCR depends on a number of factors (Taylor, 1991). Incubation times are normally kept as short as possible to reduce the overall cycling times and to minimise the risk of non-specific amplification. The number of cycles also depends on the abundance of target DNA and the efficiency of the PCR (Saiki, 1989). The annealing temperature chosen for a PCR depends on the length and composition of the primers (Prof. E. Rybicki, University of Cape Town, 1993 pers. comm.).

Extraction of RNA from material can be performed by the guanidinium thiocyanate extraction procedure that inactivates RNases and the RNA is then purified by organic extraction and ethanol precipitation (Sambrook et al., 1989), or by the use of Proteinase K and SDS for tissue disruption, followed by organic extraction of the RNA (Jackwood et al., 1992). After transcription of the RNA and PCR, the PCR product is purified and then labelled with a molecule that can be easily detected (Kwon et al., 1993). The specific base sequence of this labelled probe enables it to bind to a specific complementary region of a microorganism's nucleic acid. This makes the test specific for the target organism (Andreasen et al., 1991).

The use of PCR and specific oligonucleotide probes in routine microbiological screening, is an area of great potential and has been used to detect many infectious diseases (Peake, 1989).

Jing, Cook, Brown, Shaw and Cavanagh (1993) detected TRTV in turkeys using the PCR. Turkeys were inoculated with virulent strains of TRTV and tracheal swabs were taken from the inoculated birds every 2-3 days pi and RNA extracted from them. A reverse transcription (RT) reaction was performed on the RNA using a primer, designated PCRF.1st, complementary to the 3' end of the fusion protein mRNA. This cDNA was then used in a PCR with an upstream primer designated F.GRH to generate a 541 base pair (bp) product. To further enhance the result and to exclude non-specific reactions, a hemi-nested PCR was performed with PCRF.1st and another primer designated AN17, using the product of the 1st PCR as the sample for this second PCR. This yielded a 175 bp product.

This Chapter describes the synthesis of a TRTV and 652/93 isolate probe using the primers described by Jing et al. (1993). The 652/93 probe will be evaluated as a routine diagnostic tool for virus detection. The TRTV probe was used to confirm the identity of three TRTV-like isolates and the 652/93 probe to detect the presence of the 652/93 isolate at regular intervals pi in tracheal swabs of birds inoculated with this isolate.

6.2. Materials and methods

6.2.1. Extraction of RNA from allantoic fluid

Allantoic fluid from SPF eggs separately inoculated with isolates 4916/91, 652/93, 711/93, Pittman-Moore TRT vaccine virus, IBV (TAD) and healthy A/F, were separately harvested 6 days pi and

the procedure of Jackwood et al. (1992) for the extraction of RNA from IBV-infected A/F, was used for the extraction of RNA from the allantoic fluid of the six samples.

To 438 µl of A/F in a 1.5 ml sterile microfuge tube, was added 50 µl of 20% SDS and 12.5 µl of a 20 mg/ml Proteinase K solution (Sigma). The mixture was incubated at 50°C for 5 min and cooled for 20 min at 20°C. An equal volume of electrophoresis grade buffered phenol (10mM Tris pH 7.5, 1mM EDTA) (Sigma) was added to the solution and thoroughly mixed. This mixture was centrifuged for 2 min at 12500 g in a microfuge (Hawksley) and the aqueous phase retained. This procedure was repeated twice. Thereafter, an equal volume of a chloroform : isoamyl alcohol mixture (24:1) was added to the aqueous phase and treated as for the phenol extraction. This step was repeated once.

Precipitation of RNA was performed by adding 0.1 volume (vol) of 3M sodium acetate (pH 5.0) and 2.5 vol cold 95% ethanol and incubating at -20°C for 24 h. The mixture was then centrifuged at 12500 g for 30 min in a microfuge. The supernatant was discarded and the often invisible pellet resuspended in 6 µl of diethyl pyrocarbonate (DEPC)-treated sterile water. The RNA was then transcribed into cDNA by reverse transcriptase.

6.2.2. Primer synthesis

Primers used in this study were synthesised by Oligo Express (UK) according to the sequence of Jing et al. (1993) for TRTV. The

primers were complementary to the PCRF.1st, F.GRH and AN17 of the fusion protein (F) mRNA of TRTV. The sequences of the primers are shown below:

PCRF.1st:	5'-AAATAACTTAACTGACATAAGCCAT-3'	....TRT1
F.GRH:	5'-AAAGTCAGCACAGGTAGACAC-3'	....TRT2
AN17:	5'-GATGCCAAGAGCAAGGCTGG-3'	....TRT3

Oligonucleotide primer TRT1 was complementary to nucleotides 1612 to 1636 at the 3' end of the F mRNA, whilst oligonucleotide TRT2 corresponded to nucleotides 1096 to 1116 of the F mRNA. Oligonucleotide TRT3 corresponded to nucleotides 1462 to 1481 of the F mRNA (Jing et al., 1993).

6.2.3. Reverse transcription of RNA

Reverse transcription (RT) was performed according to the method of Sambrook et al. (1989) using the avian myeloblastosis virus (AMV) reverse transcriptase (Boehringer). The RT reaction mixture was prepared according to the method shown in Table 6.1.

Table 6.1. Ingredients used in the RT reaction

INGREDIENT	VOLUME
5 X AMV-RT incubation buffer (Boehringer)	4μl
Sterile DEPC-treated water	6μl
RNAsin (40 units/ml) (Pharmacia)	2μl
Primer TRT1 (320 μg/ml)	2μl
dNTP mix (20mM) (Boehringer)	1μl
AMV-RT (40 units/μl) (Boehringer)	1μl
Sample RNA	4μl
Total reaction volume	20μl

The above mixture was incubated at 25°C for 10 min and then at 42°C for 60 min in a thermal cycler (Hybaid).

6.2.4. The polymerase chain reaction (PCR)

The product of the RT reaction of samples 4916/91, 652/93, 711/93, TRTV, IBV and healthy material produced by AMV-RT, was used as template in the PCR reaction as described by Jing et al. (1993). The PCR was performed in the same tube as the RT reaction. The reaction mixture used is shown in Table 6.2 with the reagents added to the tube in the order prescribed in the table. The mixture was incubated in a thermal cycler (Hybaid) at 94°C for 1 min, then at 52°C for 2 min followed by 72°C for 3 min. This constituted one PCR cycle. Thirty five such cycles were performed.

Table 6.2. Quantity of ingredients used for the PCR

INGREDIENTS	VOLUME
Sterile DEPC-treated water	48 µl
dNTP mix (20mM) (Boehringer)	2 µl
Primer TRT1 (320 µg/ml)	2 µl
Primer TRT2 (400 µg/ml)	2 µl
10 X PCR buffer (Appendix 5)	4 µl
Taq DNA polymerase (2 units) (AB)	2 µl
Sterile mineral oil overlay	30 µl
Template DNA (RT tube contents)	20 µl
Total reaction volume	80 µl

A second hemi-nested PCR was performed using 4 µl of the product of the 1st PCR as template with primers TRT1 and TRT3. The reaction conditions were similar to the 1st PCR conditions described above, except that the volume of sterile water was increased to 64 µl to compensate for the reduced volume of template DNA (4 µl), and the TRT2 primer was substituted with primer TRT3. The thermocycler settings and the number of PCR cycles were as for the 1st PCR.

6.2.5. Agarose gel electrophoresis

The PCR products were resolved by electrophoresis in 2% agarose gels using a Mini-Horizontal Electrophoresis Unit (Fisher Biotech) connected to a Consort E702 power pack. The gel was prepared by melting 0.8g agarose in 40 ml of Tris-acetate-EDTA (TAE) buffer (pH 8.0) (Appendix, recipe 6). The gel mixture was cooled to approximately 50°C and 2 µl of a 10 mg/ml ethidium

bromide solution added to the mixture. The gel mixture was poured into the gel casting tray with the "comb" in place.

After solidification of the gel, the comb was carefully removed and the gel transferred to the electrophoresis apparatus. TAE electrophoresis buffer (pH 8.0) was poured into the electrophoresis apparatus buffer chamber to a level of approximately 2 cm above the gel surface.

1 μl of molecular markers consisting of ϕX174 DNA HAE III fragments (Promega), were mixed with 13 μl sterile water and 3μl agarose gel loading buffer (Appendix, recipe 7), and loaded into well No. 1 of the gel. Fifteen μl of each PCR sample was mixed with 3 μl of agarose gel loading buffer and individually loaded into separate wells of the gel. Well 2 was loaded with 4916/91 PCR product, well 3 with 652/93 PCR product, well 4 with 711/93 PCR product, well 5 with TRTV PCR product, well 6 with IBV PCR product and well 7 with PCR product obtained with healthy A/F. All agarose gels were loaded in this sequence.

Electrophoresis was performed at 100 mA for 1 h. After electrophoresis, the gel was viewed with a Fotodyne UV Transilluminator and photographed with a FCR 10 Polaroid Camera using Polaroid 667 instant pack film.

6.2.6. Purification of 175 bp DNA fragment

The Magic PCR Prep DNA Purification kit (Promega) was used to

clean-up the 175 bp TRTV and isolate 652/93 DNA fragments produced by the hemi-nested PCR with primers TRT1 and TRT3.

The oil overlay used for the PCR (Table 6.2) was removed from the 175 bp PCR product and 100μl of the kit buffer placed in a sterile 1.5 ml microfuge tube. Sixty μl of PCR sample were also placed in this tube and briefly mixed by vortexing. One ml of well mixed kit resin was dispensed in the tube and briefly vortexed 3 times over a period of 1 min. Thereafter, the plunger was removed from a sterile 3 ml syringe and a kit microcolumn fitted onto the tip of the syringe. The resin-sample mixture was poured into the syringe. The syringe plunger was replaced and the resin slowly forced into the microcolumn. The fluid discharged from the column was discarded.

The microcolumn was detached from the syringe, the plunger removed and the microcolumn reattached to the syringe. Two ml of an 80% propan-2-ol solution was introduced into the syringe and the plunger replaced. The liquid was gently forced through the column. The column was removed from the syringe and placed in a new sterile 1.5 ml microcentrifuge tube. This was centrifuged in a Hawksley microcentrifuge for 20 sec at 12500 g. The column was placed in another sterile 1.5 ml microcentrifuge tube and 50 μl of sterile distilled water added to it. This was allowed to stand at room temperature for 1 min followed by centrifugation at 12500 g for 20 sec in a microfuge. The fluid discharged into the microcentrifuge tube contained the clean 175 bp DNA fragment which was stored at 4°C until ready for labelling with

digoxygenin (DIG) (Boehringer).

6.2.7. Labelling of 175 bp DNA fragment with DIG

The DIG Labelling and Detection Kit from Boehringer was used for the labelling of the 175 bp DNA fragments of TRTV and the 652/93 isolate.

The protocol used was as described in the Boehringer Kit insert. The DNA fragment was denatured at 95°C for 10 min in a thermal cycler (Hybaid) and immediately chilled in an ice-bath thereafter. The following ingredients were then mixed together in a sterile 1.5 ml microcentrifuge tube:

5 µl denatured 175 bp DNA fragment
2 µl dNTP labelling mix (Kit reagent)
2 µl hexanucleotide mix (Kit reagent)
10 µl sterile distilled water
1 µl Klenow enzyme (Kit reagent)

The mixture was briefly centrifuged in a microfuge and incubated at 37°C for 2 h. The reaction was stopped by the addition of 2 µl 0.2 M EDTA (pH 8.0). The labelled 175 bp probe was precipitated with 2.5 µl 4 M lithium chloride (LiCl) and 75 µl chilled 95% ethanol. The solution was thoroughly mixed and allowed to stand at -70°C for 30 min. The mixture was centrifuged at 12500 g for 10 min and the pellet washed with chilled 95% ethanol. The resuspended mixture was again centrifuged at 12500g

for 10 min. The supernatant was discarded and the excess ethanol allowed to evaporate. The dried pellet was dissolved in 50 µl Tris-EDTA (TE) buffer (1M Tris-HCl, pH 8.0; 0.1M EDTA) (Sigma) and stored at 4°C until required for use.

6.2.8. Evaluation of DIG labelled probe

Three groups of 10 4-week-old SPF birds were housed in separate isolators and one group inoculated via the eyedrop method with 0.2 ml of the 5th y/s passaged 652/93 isolate containing $10^{6.2}$ EID_{50}/ml. Another group was inoculated with $10^{3.4}$ EID_{50}/ml of IBV (TAD) and the third group was the uninoculated control group. Tracheal swabs were taken from these birds daily up to 21 days pi. RNA was extracted from these swabs according to the method of Sambrook et al. (1989).

Swabs were shaken in 0.5 ml guanidinium isothiocyanate (GITC) extraction solution (Appendix, recipe 8) in a 1.5 ml microfuge tube. The tubes were microfuged at 12500 g for 30 min. The supernatant was discarded and the pellet resuspended in 0.5 ml extraction buffer containing 1 µl of a 20 µg/µl glycogen solution. To this solution was added 50 µl of a 2 M sodium acetate (pH 4.1) solution, 0.5 ml 0.1M citrate buffer (pH 4.3) saturated phenol (Sigma), and 0.1 ml of a 49:1 mixture of chloroform : isoamyl alcohol. The mixture was vortexed and microfuged at 12500 g for 5 min. The aqueous phase was recovered and mixed with 2.5 vols of cold 95% ethanol. This solution was stored at -20°C overnight and microfuged at 12500 g for 30 min.

The pellet was resuspended in 10 µl TE buffer (pH 8.0). The RT reaction and PCR with primers TRT1 and TRT2 were performed on these samples as previously described. The PCR products were probed with the 175 bp 652/93 isolate DIG-labelled probe.

The RT-PCR products of 4916/91, 652/93, 711/93, TRTV, IBV, and healthy material from A/F-extracted RNA, were also probed with the 652/93-isolate and TRTV DIG-labelled probes..

6.2.9. Blotting procedure for DIG-labelled probe

The Boehringer blotting and probing procedure, as described in the DIG DNA Labelling and Detection Kit insert, was used.

One µl of each PCR sample product was dot blotted onto nylon membrane (Amersham), baked at 80°C for 30 min and UV cross-linked for 30 secs on a UV transilluminator (Fotodyne). Thereafter, the blot was placed in hybridising buffer (Appendix, recipe 9) for 1 h at 68°C. The labelled probe (652/93 or TRTV) was denatured at 95°C for 10 min and diluted to 5 µl/ml of hybridising buffer. The blot was incubated with the diluted probe for 2 h at 68°C and washed twice for 5 min each at 68°C with 2 X SSC buffer (pH 7.0) (Appendix, recipe 10). Two 15 min washes were performed with 0.1 X SSC buffer followed by one 1 min wash with buffer 1 (Appendix, recipe 12) and one 30 min wash with buffer 2 (Appendix, recipe 13). These washes were performed at 68°C.

The Boehringer DIG DNA Labelling and Detection Kit anti-DIG

reagent was diluted 1:5000 in buffer 2 and the blot incubated with it for 30 min at 37°C followed by two 15 min washes with buffer 1. The blot was then washed for 2 min with buffer 3 (Appendix, recipe 14).

Just prior to use, colour-substrate solution was prepared by mixing 22.5 µl nitroblue tetrazolium (NBT) (Boehringer) with 17 µl 5-bromo-4-chloro-3-indolyl phosphate (Boehringer) and 5 ml buffer 3. The blot was incubated with this substrate at 37°C for 2 h. The reaction was stopped by washing the blot with TE buffer (pH 8.0) for 5 min.

Each blot contained negative and positive control samples. Negative control samples consisted of PCR product of IBV and healthy A/F material whilst the positive control sample consisted of the 652/93 or TRTV 175 bp DNA fragment, complementary to the probe being used.

6.3. Results

6.3.1. Agarose gel electrophoresis of PCR products

The results of the agarose gel electrophoresis of the PCR products using primers TRT1 and TRT2, are shown in Plate 6.1. The size of the DNA bands obtained in the test sample lanes were determined from the standard graph (Fig. 6.1). Table 6.3 depicts the size of the ϕX174 DNA marker fragments and their R_m values as well as the R_m values and sizes of the fragments obtained with 652/93. Since 4916/91, 652/93, 711/93 and TRTV all yielded

identical results, only those for 652/93 are shown in Table 6.3. A band of approximately 541 bp in size was visualised in the sample lanes containing 4916/91, 652/93, 711/93 and TRTV PCR product. No such band was detected in the IBV and healthy material PCR product lanes.

Agarose gel electrophoresis results of PCR product obtained with primers TRT1 and TRT3, are shown in Plate 6.2. A band of approximately 175 bp in length was detected in the same lanes as for the 541 bp bands reported above. No bands were detected in the IBV and healthy material PCR product lanes.

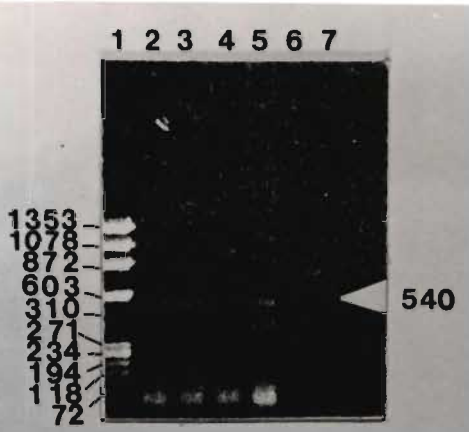
Table 6.3. The R_m values and sizes obtained after electrophoresis of TRTV and TRTV-like isolate PCR products in a 2% agarose gel using primers TRT1 + TRT2 and TRT1 and TRT3 with ϕX174 DNA fragments as markers.

ϕX174 DNA MARKERS		652/93 PCR PRODUCT	
SIZE(bp)	R_m	R_m	SIZE(bp)
1353	0.50	0.90	175
1078	0.55	0.68	540
872	0.59		
603	0.66		
310	0.79		
271	0.81		
234	0.84		
194	0.88		
118	0.96		
72	1.00		

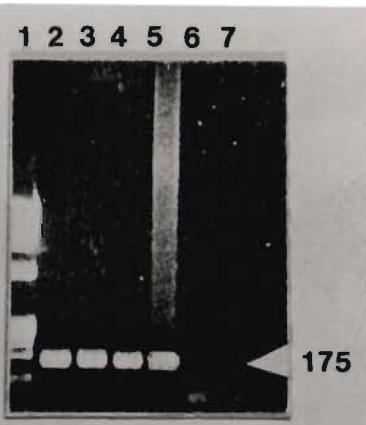
Plate 6.1. PCR product of TRTV and TRTV-like isolates using primers TRT1 and TRT2 electrophoresed in 2% agarose gel. Lane 1 = ϕX174 DNA markers; lane 2 = Isolate 4916/91 PCR product; lane 3 = Isolate 652/93 PCR product; lane 4 = Isolate 711/93 PCR product; lane 5 = TRTV PCR product; lane 6 = IBV PCR product; lane 7 = healthy A/F PCR product.

Plate 6.2. PCR product of TRTV and TRTV-like isolates using primers TRT1 and TRT3 electrophoresed in 2% agarose gel. Lanes loaded as for Plate 6.1.

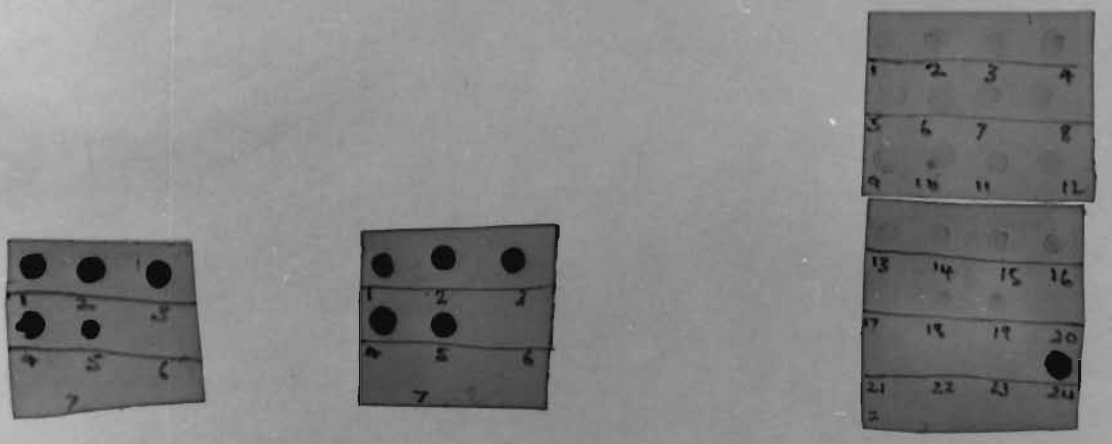
Plate 6.3. DNA probe blots of PCR product using the 652/93 probe (6.3a) and the TRTV probe (6.3b). Dot 1 = 4916/91; 2 = 652/93; 3 = 711/93; 4 = TRTV; 5 = positive control; 6 = IBV; 7 = healthy uninoculated A/F. 6.3(c) is the DNA probe blot using the 652/93 probe on swabs taken from 652/93-inoculated birds from day 1 to day 21 after inoculation. Dot 22 = swabs from IBV-inoculated birds; 23 = healthy uninoculated birds; 24 = positive control DNA.


1 2 3 4 5 6 7
1353
1078
872
603
310
271
234
194
118
72
540

6.1


1 2 3 4 5 6 7
175

6.2


a
b
c

6.3

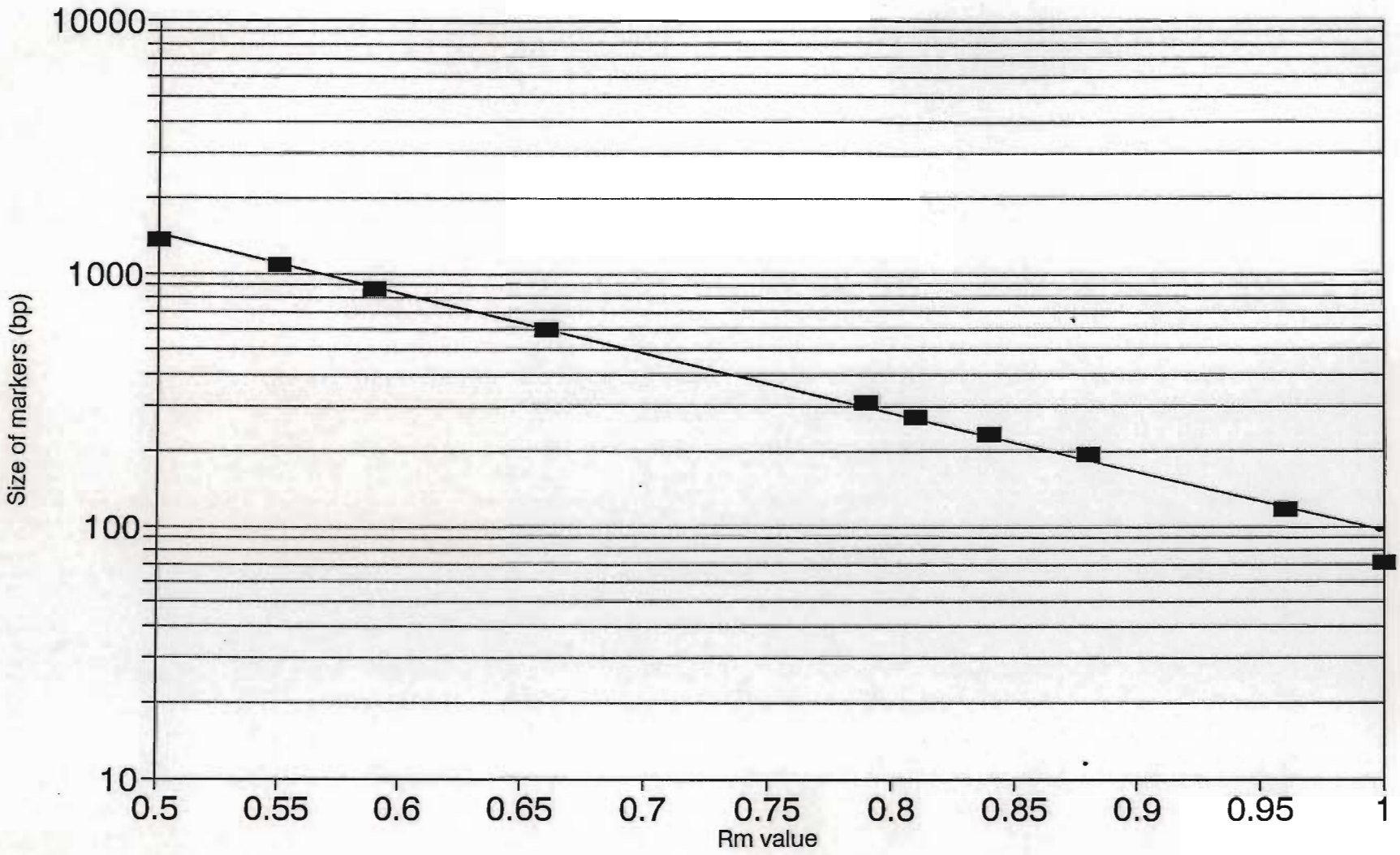


Fig. 6.1. Size of markers against their respective Rm values

6.3.2. DNA-probe blots

The results of the probing of PCR samples with the 175 bp DIG-labelled TRTV and 652/93 isolate probes, are shown in Plates 6.3a, b and c.

The 4916/91, 652/93, 711/93 and TRTV PCR products from A/F, yielded positive probe results with both the TRTV and 652/93 isolate probes (Plates 6.3a and 6.3b respectively). The PCR products of RNA extracted from IBV infected A/F and healthy A/F, did not hybridise to either probe. Positive 652/93 isolate probe results were obtained for the PCR products of tracheal swab samples up to 19 days pi as shown in Plate 6.3c. The PCR product of swabs taken from healthy birds and birds inoculated with IBV, did not hybridise with the 652/93 isolate probe and therefore yielded negative probe results.

6.4. Discussion

Every organism has a unique genetic composition which distinguishes it from other organisms. The principles of nucleic acid probes are based on this phenomenon. Numerous nucleic acid probes are currently in use for the diagnosis of infectious organisms and genetic disorders (Roberts, Montandon, Green and Bentley, 1991).

According to Jing et al. (1993), the 541 bp band obtained with primers TRT1 and TRT2 are specific for TRTV and can be used for

the diagnosis of TRTV. Using the RT-PCR, the 541 bp band was detected by agarose gel electrophoresis using A/F separately infected with isolates 4916/91, 652/93, 711/93 and TRTV. This band was not detected with healthy A/F or IBV infected A/F, thereby demonstrating the specificity of the primers for TRTV or TRTV-like isolates. However, non-specific reactions have been reported to occur in some PCR's (Kwok and Higuchi, 1989). Therefore, to further validate the results, the hemi-nested PCR with primers TRT1 and TRT3 was performed using the product of the 1st PCR as sample template. This generated a 175 bp product specific for TRTV. The 175 bp fragment occurs inside the 541 bp fragment thereby eliminating the risk of non-specific priming. Hence, the presence of a 175 bp band after PCR with primer sets TRT1 and TRT2 followed by TRT1 and TRT3, is indicative of the presence of TRTV (Jing et al., 1993).

Although the 175 bp band visualised in the agarose gels is specific for TRTV, to show that it is of the same sequence as TRTV, a DNA probe was developed by labelling the 175 bp fragment produced from 652/93 isolate and TRTV extracted RNA, with DIG. If a sample DNA contained the same sequence of nucleotide bases as the specific 175 bp fragment, then the 175 bp labelled probe will hybridise to this fragment producing a colour reaction when the probe-blot is developed.

A/F samples of isolates 4916/91, 652/93, 711/93 and TRTV hybridised to the TRTV probe and yielded positive results. TRTV-infected A/F was used as the positive control and IBV-infected

A/F as the negative control. These results confirm the identity of 4916/91, 652/93 and 711/93 as chicken isolates of TRTV. The term avian rhinotracheitis virus (ARTV) is therefore used to distinguish the chicken isolates from the turkey isolates of rhinotracheitis virus.

The obvious benefit of an infectious disease nucleic acid probe is to detect the presence of a virus in the field early in the disease outbreak, thereby enabling the early treatment of the disease. The 652/93 isolate probe was therefore evaluated in a controlled laboratory study by infecting birds with the chicken isolate of TRTV (652/93 isolate), and tracheal swabbing these birds daily for up to 21 days pi. The probe was able to detect the virus for 19 days pi, suggesting that the virus persists in the bird, although at low levels, for 19 days after infection. The reason for the inability to isolate virus from birds beyond 6 days pi, could be due to the low titres at which the virus persists in the birds. These results are consistent with the findings of Jing et al. (1993) who were able to detect TRTV in turkeys for 17-19 days pi.

These results are encouraging in that the probe could be used for the field detection of ARTV infection. This has far reaching implications with regard to disease diagnosis. Conventional virus isolations for the diagnosis of ARTV are time consuming and difficult. A successful ARTV isolation from field material would take a minimum of 2 weeks to grow the virus and a further 2 weeks for identification purposes. The result of the virus isolation

attempt would therefore only be available a month after the sample was taken. The cost of the isolation and subsequent tests are enormous since SPF eggs with a cost of approximately R8.00 per egg, are used for virus isolation attempts. Also, the virus can only be isolated from fresh field material for a maximum of 7 days pi (Cook et al., 1991; Naylor, Al-Ankari, Al-Afaleq, Bradbury and Jones, 1992).

The detection of ARTV from tracheal swabs by RT-PCR followed by the DNA probe hybridisation assay, would take only 2 days or a maximum of 3 days if the hemi-nested PCR was performed. The RT-PCR-probe assay can detect minute quantities of ARTV for a long period of time (19 days) without the need for other confirmatory tests to identify the pathogen, since the RT-PCR-probe assay is specific (Jing et al., 1993). The advantages of the RT-PCR-probe system over the conventional methods of isolation, are therefore obvious.

The results presented in this Chapter confirm that the three isolates, 4916/91, 652/93 and 711/93, are chicken strains of TRTV. However, the probe could not distinguish between the chicken and turkey strains of the virus due to their close relatedness and the fact that the TRTV primers used were derived from sequences of the nucleic acid common to TRTV and ARTV. Sequencing of the ARTV genome is required to determine differences, if any, between the TRTV and ARTV genomes.

CHAPTER 7

PRODUCTION OF A VACCINE AGAINST A TRTV-LIKE VIRUS

7.1. Introduction

Disease of animals occurs when the animal's body defences are impaired and the severity of the disease is determined by the degree of impairment. Diseases caused by infectious agents may be complex and dependent upon the characteristics of the host and infectious agent. Whether an infectious agent is capable of causing disease, depends on a number of factors such as the virulence of the agent, its route of entry into the host, environmental factors, genetic susceptibility and immunological status of the host, and the presence of other infectious agents.

Some organisms exist as strains of different virulence. Velogenic strains of an organism may overcome the hosts' natural resistance and cause severe disease and mortality. Mesogenic strains may cause moderate disease with little mortality whilst lentogenic strains normally cause very slight or no reaction in healthy hosts. The lentogenic strains may be used as potential vaccines. However, some strains may not cause disease on their own but predispose the host to infection by other infectious agents which may then cause disease. Also, adverse environmental conditions and stress of birds, may result in strains that are normally considered nonpathogenic, causing disease (Zander, 1984).

Vaccination of flocks of commercially reared birds, is very useful in the control of most viral disease outbreaks. Theoretically, the ideal viral vaccines are those manufactured with live avirulent strains since they normally stimulate the immunising mechanisms in a similar way to the natural infection, although less intensively, and also because live vaccines are easily mass applied thus making them less labour intensive than vaccination with inactivated vaccines (Zanella and Marchi, 1982). Also, the age of application of live vaccines could be easily maneuvered to accommodate changes in vaccination programmes, whereas inactivated vaccines, for practical reasons, would have to be applied at 1 day of age.

The method of live vaccine application is very important. Vaccination of day-old chicks can be performed by the "eye-drop" method where a drop of reconstituted vaccine is introduced onto the eye, or by spray vaccinating where the vaccine is reconstituted in a volume of deionised water and sprayed over the chicks. Birds older than one day of age, can be spray vaccinated or the vaccine may be administered via the drinking water. However, according to Dr Philip Box (Pittman-Moore Ltd UK, 1992 pers. comm.), avian Pneumoviruses should not be administered via the drinking water since these viruses are rapidly inactivated in chlorinated water. Different vaccines work well at different ages. The time and frequency of application depend on conditions prevalent in the area.

Several commercial vaccine manufacturers have marketed attenuated

TRTV vaccines for use in turkeys and chickens. However, all these vaccines comprise viruses isolated from turkeys. None of the vaccines consist of a virus isolated from chickens. The method of large scale production of the vaccine differs from one manufacturer to the next. Some use chicken eggs as the medium for vaccine production (Box, 1989), whilst others use Vero cells (Ivaz Vaccine Manufacturers Italy, 1993 pers. comm.). Williams et al. (1991a; b) produced a TRTV vaccine in Vero cells which provided protection to susceptible turkey poults from challenge with a virulent virus, whilst Cook and Ellis (1990), used alternate passaging between TOC's and chicken eggs to sufficiently attenuate a TRTV for use as a potential vaccine.

Vaccines against any disease must be pure, effective, stable, economically applicable and must not constitute a danger to the health of the vaccinates (Lensing, 1973). The vaccine must therefore be subjected to extensive bacteriological and mycological tests and must contain no microorganism other than the vaccine microorganism. The vaccine has to contain a suitable number of infective virus particles capable of causing a seroconversion in birds and offering protection against virulent virus. Also, there should be a demonstrable benefit in vaccinates compared to non-vaccinated birds.

In this Chapter, the selection of an isolate, derived from chickens, for use as a vaccine and further testing in laboratory and field trials to evaluate its efficacy, are described. The stability of the attenuation and the purity of the vaccine was

also tested.

7.2. Materials and Methods

7.2.1. Selection of an isolate for use in a vaccine

The purified 6th y/s A/F of each of the three isolates, 4916/91, 652/93 and 711/93, were separately inoculated in SPF chick TOC's and at the first sign of ciliostasis, the fluid was harvested and inoculated into the y/s of 6-day-old embryonated SPF chicken eggs. After 6 days, the A/F was harvested and re-inoculated into TOC's. This alternate passaging between TOC's and eggs was performed for 10 passages. This is similar to the method of Cook and Ellis (1990) where they used TOC-a/s passaging. This procedure of TOC-a/s passaging was also performed.

Approximately 0.5 ml of the 10th TOC-y/s passaged A/F, yolk, and homogenised CAMs, the 10th TOC-a/s passaged A/F, the 5th Vero cell passaged material, and the 5th CEF passaged fluid of all three isolates, were separately inoculated intrasinusly or by the eyedrop method, in 4-week-old SPF birds housed in isolators. The birds were bled before inoculation and 14 days post-inoculation from the brachial wing vein. The sera were tested for antibodies against TRTV with the Pathasure avian rhinotracheitis ELISA kit.

The EID_{50} or $TCID_{50}$ of the isolates in each system, except the 10th TOC-y/s passaged material, was not determined as this was done previously and discussed in Chapter 3.

7.2.2. Stability of the attenuation

The 11th TOC-y/s passaged isolate of 652/93, was given an additional passage via the y/s route and then a total of 0.5 ml of this A/F was inoculated intrasinusly, orally, and via the eye drop method, into a total of 10 day-old SPF chicks which were then housed in an isolator. Six days post-inoculation, the trachea and turbinates were harvested from the 10 chicks, homogenised in 6 ml of PBS, centrifuged at 3000 g in a Heraeus Hettich Universal II centrifuge to sediment the debris, and the supernatant was inoculated into a further 10 day-old SPF chickens. This procedure was repeated for five passages. After the final passage, 50% of the birds were kept for 12 days post-inoculation. Birds in each passage were observed daily for symptoms of infection.

After the 5th passage, the trachea and turbinates were removed from 50% of the birds, processed as described earlier, and the supernatant used for virus isolation attempts as described in Chapter 2. Upon isolation of virus, a serum neutralisation test was performed on the isolate using monospecific antisera against the 652/93 isolate. The entire procedure was repeated with 4-week-old SPF chickens.

7.2.3. Vaccine production

For various reasons discussed later in this Chapter, isolate 652/93 was selected as the candidate for use as a vaccine. The

10th TOC-y/s passaged material was used as the master seed for vaccine production. The working seed for vaccine production consisted of an additional passage of the master seed. Six-day-old embryonated SPF chicken eggs were inoculated via the y/s with 0.2 ml of a 10^{-2} dilution of the working seed. The eggs were chilled at 4°C for 24 hrs when the first signs of embryo mortality of a proportion of the embryos (15-20%) were observed.

The A/F, yolk fluid, and CAM were then harvested. The A/F and yolk were combined and clarified by centrifugation at 6000 g for 15 min in a Sorval GSA rotor using a Sorval RC28S centrifuge. The supernatant was decanted through sterile muslin cloth into a sterile collection vessel kept on ice. The CAMs were homogenised in a half volume of PBS (pH7.2) and centrifuged at 6000 g as before. The supernatant was filtered through sterile muslin cloth into the vessel containing the clarified allantoic and yolk fluids. Two ml of a penicillin / streptomycin solution containing 25000 units/ml and 25000 ug/ml respectively (Bio Whittaker), were added to each litre of supernatant. This material constituted the vaccine which was dispensed into 5 ml cryogenic vials and stored at -70°C until released for use.

A vial of the freshly dispensed vaccine as well as a vial of vaccine stored at -70°C for 7 days, were titrated in the y/s of 6-day-old embryonated SPF eggs as previously described.

7.2.4. Sterility tests

Sterility tests were performed by inoculating thioglycollate and Freys broth with 0.5 ml of the vaccine and the vaccine was streaked onto blood agar, nutrient agar and sabaroud agar. The Freys broth was incubated at 37°C for 10 days whilst the remaining agars and broth were incubated at 37°C for 48 h and then visually inspected for growth of microorganisms.

7.2.5. Purity test

Monospecific antiserum against the 652/93 isolate was reacted with an equal volume of vaccine in a serum neutralisation test as described in Chapter 3. The test was carried out in the y/s of 6-day-old embryonated SPF chicken eggs.

7.2.6. Safety

Each of 10 1-day-old and 10 4-week-old SPF chickens, housed separately in isolators, were inoculated intranasally and by the ocular route with a total of 0.5 ml of vaccine. The birds were observed daily for 28 days for mortality, and symptoms of infection such as rhinitis, sinusitis, swollen heads, and snicking.

7.2.7. Serological tests for extraneous viruses

Ten 4-week-old SPF chickens, housed in an isolator, were

inoculated intrasinusly, intramuscularly and by the ocular route with a total 1 ml of vaccine. This inoculation was repeated at 10 days of age. The birds were kept in the isolators for a period of 4 wks post-inoculation. Serum was drawn from these birds before inoculation and at 4 wks post-inoculation for the detection of antibodies against IBV, NDV, IBDV, reovirus, avian encephalomyelitis virus (AEV), M. gallisepticum (MG), M. synovia (MS) and TRTV as shown in Table 7.1. The table also indicates the test and the manufacturer of the kit used for the detection.

Table 7.1. Tests employed to detect antibodies against various diseases in SPF chickens inoculated with the 652/93 isolate vaccine.

ORGANISM	TEST EMPLOYED	SOURCE
IBV	ELISA	Delta Biologicals
IBDV	ELISA	Delta Biologicals
Reovirus	ELISA	Delta Biologicals
AEV	ELISA	Idexx
NDV	HI	Rainbow Isolate
MG	RSPA*	Intervet
MS	RSPA*	Intervet
TRTV	ELISA	Pathasure

*Rapid Serum Plate Agglutination

7.2.8. Evaluation of the efficacy of the vaccine in laboratory trials

Ten day-old SPF chicks were each inoculated by the eye drop method with a bird dose of vaccine ($10^{3.6}$ EID_{50}/bird). A further 10 14-day-old SPF chicks were inoculated intranasally with 0.2 ml of the 3rd y/s A/F of isolate 652/93. Another group of 10 day-old

SPF chicks were inoculated via the eye drop method with a bird dose of vaccine and challenged 14 days later by intranasal instillation of 0.2 ml of the 3rd y/s A/F of isolate 652/93. The fourth group of day-old SPF chicks were inoculated via the eye drop method with 0.2 ml of PBS only. All groups of birds were housed under similar conditions in separate isolators for the duration of the experiment. The chicks were observed daily for symptoms of infection or vaccine reaction.

Twenty day-old SPF chicks, housed in an isolator, were each vaccinated with one bird dose of vaccine via the eye drop method. After 14 days, 10 birds were removed from the isolator and placed in another isolator. The remaining 10 birds in the first isolator were re-vaccinated with a bird dose of vaccine via the eye drop method and maintained in the isolator for a further 14 days. Another group of 10 SPF birds, maintained in an isolator, were vaccinated only at 14 days of age with a bird dose of the vaccine. Ten day-old SPF chicks were maintained in an isolator for 28 days without any vaccination. All groups of birds were bled at 14 and 28 days of age. Serum for determination of the TRTV antibody status at day-old was obtained by sacrificing 10 day-old SPF chicks. TRTV ELISA, using the Pathasure avian rhinotracheitis kit, was performed on all the serum samples.

7.2.9. Evaluation of the efficacy of the vaccine in field trials

A trial was conducted at a broiler site using the 652/93 vaccine.

The site consisted of four subsites designated A, B, C, and D. Each subsite, except for A which had six houses, possessed four houses. Chicks to be placed at subsite A were vaccinated at one day of age by the eye drop method. Chicks placed at subsite B were vaccinated at one day of age by the eye drop method and at 14 days of age by the spray method. Chicks placed at subsite C were vaccinated only at 14 days of age whilst chicks at subsite D were not vaccinated with the 652/93 vaccine. All day-old vaccinations were performed at the hatchery. All other vaccinations were identical for each group. Thirty thousand birds were placed in each house.

Twenty-five chicks from each subsite were sacrificed at day-old for serum and twenty-five birds were bled fortnightly from the brachial wing vein thereafter. The sera were tested for the presence of antibodies against NDV by the HI test, M. gallisepticum and M. synovia by the RSPA test, and IBV and TRTV by the Delta ELISA and the Pathasure ELISA systems respectively. A proportion of the sick and dead birds from each subsite were examined by a Rainbow Farms Veterinarian (Dr. M.J Versfeld). Samples for virus isolation attempts and bacteriology were taken when necessary. The trial was terminated when the birds were 35 days of age since a large proportion of the birds were slaughtered as grillers at this age. The serology, mortality and mass of the birds in each group were compared to the unvaccinated control group.

7.3. Results

7.3.1. Selection of an isolate for vaccine production

The titres of the 10th TOC-y/s passaged A/F of the three isolates are shown in Table 7.2. The 652/93 isolate 10th TOC-y/s passaged A/F had the highest EID_{50}/ml. The SPF birds inoculated intrasinusly with the 10th TOC-y/s passaged 652/93 isolate A/F, produced the highest level of seroconversion against TRTV. The similiarly passaged 4916/91 and 711/93 isolates, also caused seroconversion in SPF birds but at a lower level than that of the 652/93 isolate. The Vero cell passaged material of all three isolates caused a much reduced level of seroconversion than the TOC-y/s passaged material whilst the CEF material produced no seroconversion. The TOC-a/s passaged material of all the three isolates inoculated intrasinusly, produced only slight seroconversion in SPF birds with the highest level produced by the 652/93 isolate. The results of the intrasinus inoculation of SPF birds with the various materials are depicted in Table 7.3.

Only the TOC-y/s passaged 652/93 isolate caused a good seroconversion in SPF chicks when inoculated via the eye drop method. All the other passaged material caused negligible or no seroconversion in SPF birds inoculated via the eye drop method. The results are depicted in Table 7.3. The yolk material and homogenised CAMs of the TOC-y/s passaged material of all three isolates caused a good seroconversion in SPF birds when inoculated intrasinusly. However, this seroconversion was drastically reduced when the same samples were inoculated via the

eye drop method. Nonetheless, the 652/93 material consistently produced higher titres of antibodies against TRTV, than any of the other isolates. The results are presented in Table 7.4.

Table 7.2. The titres of the 10th TOC-y/s passaged A/F of the three TRTV-like isolates

ISOLATE	TITRE (EID_{50}/ml)
4916/91	$10^{4.6}$
652/93	$10^{6.6}$
711/93	$10^{4.2}$

Table 7.3. Seroconversion results obtained before and after intrasinus and eye drop inoculation of SPF chickens with various passages of the three TRTV-like isolates

ISOLATE	INTRASINUS		EYE DROP	
	PRE*	POST*	PRE*	POST*
4916/91 10th TOC-y/s	21	92	22	36
4916/91 10th TOC-a/s	17	42	24	30
4916/91 5th CEF	20	23	21	20
4916/91 5th Vero	20	42	15	28
652/93 10th TOC-y/s	22	104	24	69
652/93 10th TOC-a/s	18	66	24	38
652/93 5th CEF	25	26	18	25
652/93 5th Vero	24	78	20	31
711/93 10th TOC-y/s	22	90	18	37
711/93 10th TOC-a/s	21	39	22	32
711/93 5th CEF	20	25	20	18
711/93 5th Vero	18	52	26	29

*= ELISA index value pre-inoculation or post-inoculation

Table 7.4. Seroconversion results obtained before and after intrasinus and eyedrop inoculation of SPF chickens with the CAMs and yolk of the three TRTV-like isolates

ISOLATE	INTRASINUS		EYE DROP	
	PRE*	POST*	PRE*	POST*
4916/91 yolk	24	78	21	32
4916/91 CAM	16	90	23	33
652/93 yolk	19	90	24	39
652/93 CAM	18	96	18	48
711/93 yolk	22	72	26	29
711/93 CAM	24	82	19	31

*= ELISA index value pre-inoculation or post-inoculation

7.3.2. Stability of the attenuation

There were no signs of infection in both groups of chickens, the group inoculated at day-old and that inoculated at 4 wks of age, in all the 5 bird-bird passages. Virus was isolated from both groups of chickens after the 5th passage and they were both neutralised with the 652/93 isolate monospecific antisera by the serum neutralisation test.

7.3.3. Vaccine production

Eggs inoculated with the working seed were chilled 6 days post-inoculation when the first signs of embryo mortality occurred. Approximately 20% of the embryos were dead at this stage with the remaining embryos showing signs of stunting. Approximately 25 ml of a combination of A/F, yolk and homogenised CAMs was obtained

from each SPF egg inoculated with the 652/93 vaccine isolate. The titre of the final blend of vaccine before and after freezing at -70°C, was $10^{6.6}$ EID_{50}/ml and $10^{6.5}$ EID_{50}/ml respectively.

7.3.4. Sterility tests

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

7.3.5. Purity tests

The vaccine virus was neutralised by monospecific antisera against the 652/93 isolate. The neutralisation index was 5.7.

7.3.6. Safety

None of the birds in the two groups, the day-old inoculated and 4-week-old inoculated groups, showed any signs of infection. The birds appeared healthy throughout the duration of the experiment.

7.3.7. Serological tests for extraneous viruses

Except for TRTV, the SPF birds inoculated with the vaccine were serologically negative for the presence of antibodies against the various organisms tested. There was a dramatic increase in antibodies against TRTV in the inoculated birds. Table 7.5 shows the differrence in antibody titres before and after inoculation with the vaccine.

Table 7.5. Antibody levels of SPF birds against various diseases before and after vaccination with the 652/93 vaccine

ORGANISM	ANTIBODY TITRES*	
	PRE-INOCULATION	POST-INOCULATION
IBV	80	82
IBDV	72	68
Reovirus	72	70
AEV	14	16
NDV	0	0
MG	-ve	-ve
MS	-ve	-ve
TRTV	20	109

* = TRTV ELISA index values

7.3.8. Evaluation of the efficacy of the vaccine in laboratory trials

The day-old chicks vaccinated with one bird dose of vaccine, showed no symptoms of infection or any vaccine reaction for the entire duration of the trial. However, the group of chicks inoculated intranasally with the 3rd y/s A/F of isolate 652/93 caused a slight sinusitis 3 days post-inoculation in 40% of the chicks. This symptom lasted only for 1 day and the chicks appeared to have fully recovered by the following day.

Neither the group of chicks vaccinated at day-old and then challenged at 14 days of age with the 3rd y/s A/F of isolate 652/93, nor the control group inoculated with PBS, showed any symptoms of disease for the duration of the experiment.

The serological results of the 20 day-old SPF chicks vaccinated at day-old, at day-old plus 14 days, and only at 14 days of age are depicted in Table 7.6. The chicks seroconverted from a negative TRTV ELISA index value at day-old, to a value indicating a large amount of TRTV antibodies at 14 days of age. The level of antibodies increased to a higher value by 28 days of age. The group vaccinated only at day-old had a slightly lower TRTV ELISA index value at 28 days of age. The group vaccinated only at 14 days of age had a similar ELISA index value as the group vaccinated only at day-old. The control group of birds showed virtually no shift in their day-old, 14 day- and 28 day-TRTV antibody titres

Table 7.6. TRTV ELISA results of chicks vaccinated with the 652/93 vaccine at day-old, day-old and 14 days, and at 14 days only

VACCINATION AGE	TRTV ELISA INDEX VALUE		
	DAY-OLD	14 DAYS	28 DAYS
day-old	18	36	44
day-old + 14 days	18	36	48
14 days	18	20	42
non-vaccinated	18	21	20

7.3.9. Evaluation of the efficacy of the vaccine in field trials

The average mass of the birds from each subsite at various ages, are shown in Table 7.7 below. The average mass of the birds at 35 days of age at all the vaccinated sites was higher than that of the unvaccinated control site. Also, the mass of the birds at all sites was higher than the Rainbow Farms inhouse standard mass

of birds at that age. No statistical analysis was performed on these figures since due to the lack of manpower, only one house on each subsite was weighed. The serology results of the birds in each subsite are shown in Table 7.8. Subsite B had an elevated NDV HI titre at 35 days whilst the IBV ELISA titre of subsite C at this age was higher than that of the other subsites. The TRTV ELISA titres of all subsites, except C which was lower, were similar. All subsites were positive for antibodies against M. gallisepticum, but negative for antibodies against M. synovia.

Table 7.9 shows the daily mortality of birds in each house at the various subsites. The average mortality at subsites A (6.09%) and B (6.28%) was lower than that at C (7.37%) and D (7.26%). However, using the "Minitab Statistical Software" package, a oneway analysis of variance was performed ("t" test). It was found that at the 95% confidence limit, there were no significant differences between the treatments. Fig. 7.1 graphically depicts the average daily mortality of subsites A, B, C and D.

Table 7.7. Average mass of birds at various ages from subsites A, B, C and D

AGE (DAYS)	STANDARD AND AVERAGE MASS AT SUBSITES (g)				
	STD	A	B	C	D
7	221	236	233	235	233
14	335	358	357	359	352
17	449	479	481	486	479
21	610	662	672	674	648
24	751	808	812	820.	795 .
31	1095	1169	1173	1173	1148
35	1300	1377	1384	1386	136

Table 7.8. Serology results of vaccinated and unvaccinated birds from subsites A, B, C and D at fortnightly intervals

SITE	TRT ELISA				IBV ELISA				NDV HI			
	0	14	28	35	0	14	28	35	0	14	28	35
A	65	33	37	65	118	54	80	100	5	2	2	2
B	59	32	38	65	120	60	90	80	6	2	3	6
C	56	28	26	37	118	72	102	140	5	3	3	2
D	62	28	30	35	110	56	80	77	5	2	3	3

The Veterinarian did not take samples for viral or bacterial isolation attempts from any of the subsites, as he felt that it was not justified. Also, he did not note any major disease problems during post-mortem examination of dead and live birds from each subsite. The cause of death was non-specific.

Table 7.9.: Daily and average mortality of birds at subsite A, B, C and D

	SITE A MORTALITY							SITE B MORTALITY					SITE C MORTALITY					SITE D MORTALITY				
AGE (days)	HSE 1	HSE 2	HSE 3	HSE 4	HSE 5	HSE 6	AVG	HSE 1	HSE 2	HSE 3	HSE 4	AVG	HSE 1	HSE 2	HSE 3	HSE 4	AVG	HSE 1	HSE 2	HSE 3	HSE 4	AVG
1	36	24	11	17	25	25	23	34	32	28	10	26	17	15	107	15	39	22	43	30	30	31
2	32	29	42	34	30	41	35	35	30	53	30	37	50	45	85	20	50	22	30	47	48	37
3	33	30	51	22	25	46	35	35	38	25	60	40	38	18	80	23	40	39	45	49	19	38
4	20	15	48	50	25	38	33	40	30	56	63	47	30	22	96	16	41	18	53	25	37	33
5	26	27	55	43	43	39	39	42	35	60	65	51	35	26	97	20	45	34	51	46	27	40
6	16	28	60	31	30	45	35	40	32	55	62	47	30	32	65	26	38	31	32	21	14	25
7	19	20	32	34	30	46	30	40	33	43	56	43	26	26	70	3	31	17	36	31	14	25
8	18	25	40	29	22	42	29	22	20	30	45	29	19	22	29	36	27	46	33	26	19	32
9	18	22	32	30	32	49	31	36	21	32	41	33	11	17	14	12	14	35	33	32	14	29
10	26	32	55	19	30	41	34	22	26	21	24	24	14	27	30	16	22	32	26	39	50	37
11	39	23	47	20	25	28	30	24	21	21	20	22	30	20	47	15	28	39	36	40	40	39
12	17	30	32	36	35	54	34	22	23	22	17	21	23	18	37	15	23	40	36	23	24	31
13	26	60	28	36	23	43	36	24	16	18	21	20	20	16	29	17	21	46	55	21	31	51
14	21	35	23	43	28	36	31	32	14	20	21	22	66	25	126	59	69	97	84	95	23	75
15	29	45	30	65	37	48	46	58	97	77	76	77	15	16	22	18	18	44	24	26	20	29
16	28	81	43	30	36	46	44	24	16	19	20	20	13	14	20	15	16	33	20	21	24	25
17	20	34	30	22	27	30	27	19	16	17	20	18	23	31	26	15	24	103	44	22	28	49
18	17	26	20	51	49	55	36	56	13	24	16	27	23	30	33	17	26	67	39	43	23	43
19	28	43	43	47	60	57	46	37	20	50	26	33	41	25	36	16	30	48	30	30	25	33
20	37	66	42	39	38	68	48	86	28	66	28	52	35	36	40	18	32	50	31	33	37	38
21	23	53	34	26	32	50	37	30	20	40	25	29	65	67	34	39	51	77	50	42	34	51
22	23	40	33	37	58	57	41	47	32	59	40	45	78	105	67	40	73	44	43	46	23	39
23	43	46	36	24	59	90	50	37	23	43	42	36	42	173	58	36	77	69	86	55	47	64
24	57	46	18	21	53	61	43	30	30	49	33	36	68	162	51	48	82	74	97	96	52	80
25	102	39	36	23	62	95	60	20	24	83	35	41	50	178	50	58	87	92	114	73	31	78
26	147	42	55	22	50	71	65	23	30	129	91	68	53	212	66	53	96	122	102	63	104	98
27	138	41	53	27	74	97	72	25	28	73	106	58	65	185	65	66	95	156	142	84	57	110
28	119	25	33	39	73	107	66	28	40	266	125	115	47	188	62	63	90	105	150	55	56	92
29	213	40	47	39	63	99	84	34	39	231	130	109	46	137	83	64	83	140	100	75	67	96
30	72	49	60	42	110	122	76	56	60	208	140	116	51	167	73	65	89	132	74	51	125	96
31	85	54	43	37	120	110	75	78	65	168	106	104	60	126	131	80	99	166	96	118	67	112
32	34	74	71	113	172	117	97	130	78	158	122	122	50	120	150	74	99	120	90	145	166	130
33	90	100	104	84	200	110	115	149	124	103	110	122	117	115	266	125	161	85	92	239	149	141
34	60	180	90	204	182	133	142	140	113	91	86	108	167	120	300	120	177	75	60	212	156	126
35	50	128	166	81	88	121	106	143	77	69	65	89	258	100	355	175	222	100	40	233	147	130
SUM	1762	1652	1643	1541	2046	2317	1827	1698	1350	2509	1977	1884	1776	2636	2922	1508	2211	2420	2119	2289	1878	2177
% MORT	5.87	5.51	5.48	5.14	6.82	7.72	* 6.09	5.66	4.50	6.36	6.59	* 6.28	5.92	8.79	9.74	5.03	* 7.37	8.07	7.06	7.63	6.26	* 7.26

* not significant at 95% confidence limit

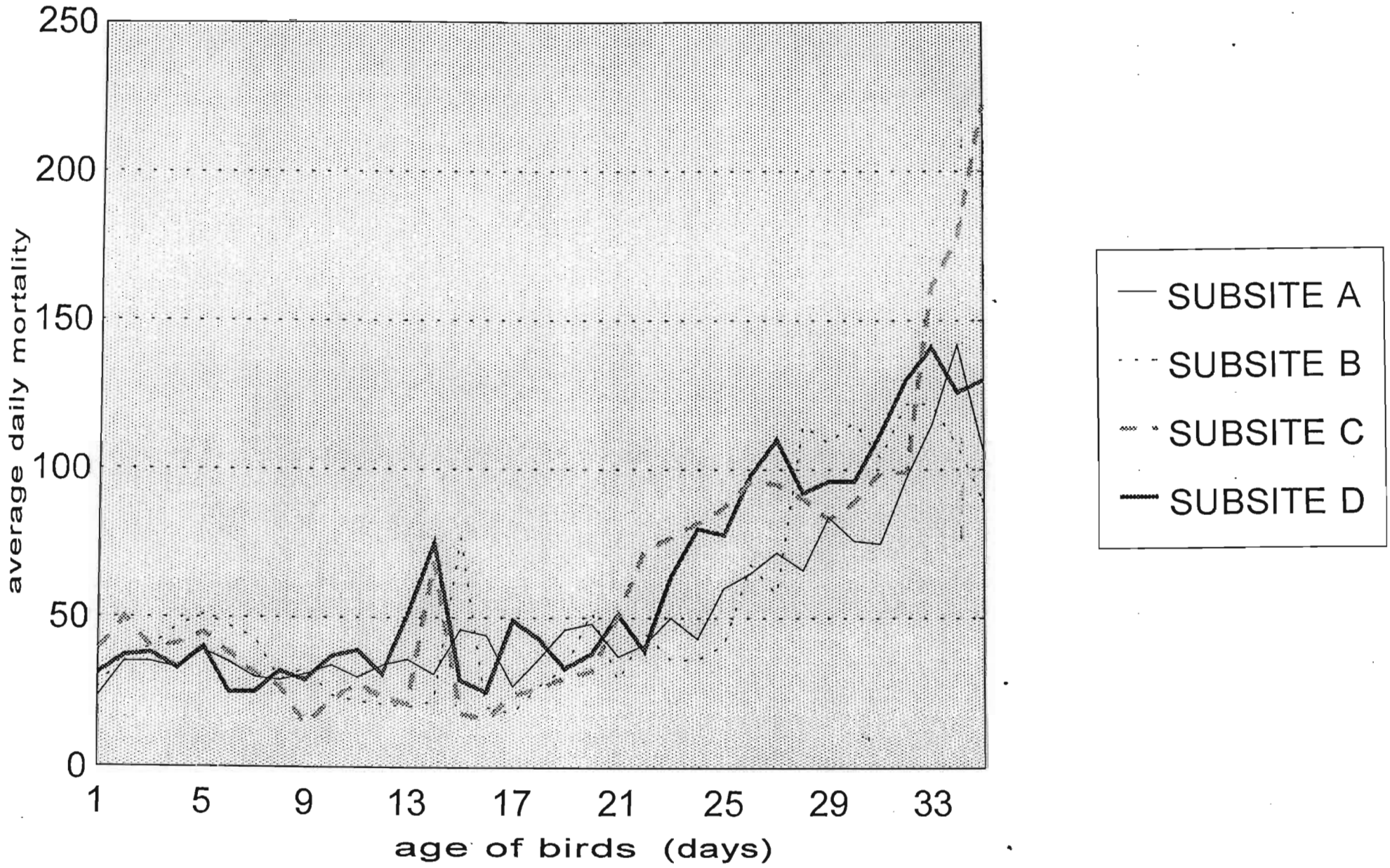


Fig 7.1. Average daily mortality at subsites A, B, C and D

7.4. Discussion

Several groups have licensed live attenuated TRTV vaccines for use against virulent TRTV in turkeys. The vaccines are not indicated for use in chickens. All of these vaccines consist of isolates made from turkeys (Pitman-Moore Ltd, UK; Ivaz Vaccine Manufacturers, Italy; Intervet, Holland). Some groups have used Vero cell propagated virus (Williams et al., 1991a) whilst others employ SPF chicken eggs (Pitman-Moore Ltd, UK). In this study, various routes of propagation of three TRTV-like isolates were tested for their suitability as a live chicken derived vaccine. Alternation between TOC-y/s passaging was attempted as a method to attenuate the isolates without significantly altering them. This TOC-y/s passaging is similar to the method employed by Cook and Ellis (1990) where they used TOC-a/s passaging to attenuate their TRTV isolate without drastically affecting its immunogenicity. According to them, this worked well as a vaccine, but there were always clinical signs of infection following vaccination of turkeys.

From the results, it is evident that the best candidate for a live vaccine was the 10th TOC-y/s passaged 652/93 isolate. This isolate had the highest titre when passaged via this system and also induced the highest antibody response against TRTV in SPF birds. The TOC-a/s passaged A/F did not stimulate the same high level of antibodies against TRTV. This is contrary to the results of Cook and Ellis (1990) who obtained very good seroconversion with their TOC-a/s material. This could be due to slight

differences between the two isolates caused by differences in their isolation and propagation. This view was also expressed by Cook et al. (1993a) to explain differences between isolates from different geographical areas.

The Vero cell passaged material of all three isolates caused a much reduced level of seroconversion against TRTV compared to the TOC-y/s material. This was unexpected since there was a severe CPE in the Vero cell monolayer and other workers obtained good seroconversion with Vero cell propagated isolates (Gaudry, 1991; Williams et al., 1991a;b). The isolates were probably modified to some extent by the Vero cell passaging, resulting in a loss of immunogenicity.

The CEF passaged material of all three isolates failed to cause seroconversion against TRTV in SPF birds. This is consistent with the findings of Williams et al. (1991a). The reason for this phenomenon could be that the infected cell membrane, which is used by the virus to form its outer envelope, was not significantly altered by the viral glycoproteins.

The homogenised CAM's and yolk of the TOC-y/s passaged material also caused good seroconversion when inoculated intrasinusly in SPF birds, but the level of seroconversion was sharply reduced when the same samples were inoculated via the eye drop method. This drop was not unexpected since direct introduction of inoculum into the sinus will have a greater impact than introduction via the eye. However, the 652/93 TOC-y/s passaged

A/F inoculated via the eye drop method, caused a good seroconversion in SPF birds although lower than the intrasinus inoculation. Since the 652/93 isolate TOC-y/s passaged material consistently produced better results than the other isolates, it was chosen as the candidate for the vaccine. The 652/93 isolate 10th TOC-y/s A/F, CAM and yolk were harvested and blended together for use as the vaccine since all three had high titres of virus and were able to induce significant antibody response against TRTV in SPF birds.

Before approval of a vaccine for use, it has to be shown to be free of contaminants, safe and effective. The purity and sterility tests showed that the vaccine was free of any contaminating bacteria, virus, fungi, or mycoplasmas. The vaccine was serologically demonstrated to be negative for antibodies against IBV, IBDV, reovirus, AEV, NDV, M. gallisepticum and M. synovia. The stability of the attenuated virus was demonstrated by bird to bird passaging in day-old as well as 4-week-old SPF chickens. The isolate did not revert to pathogenicity and cause any symptoms of infection in any of the chickens. The virus was also re-isolated from both groups of birds, demonstrating the presence and viability of the virus in the inoculum.

There was a negligible drop in vaccine virus titre after storage at -70°C for 7 days. The vaccine could therefore be stored at this temperature prior to use. Titration results of Chapter 3 show that the virus could be stored for more than 6 months at this temperature without loss of titre. This makes storage of

large amounts of vaccine convenient in the absence of a freeze drier. Freeze drying is also more time consuming, and a small freeze drier cannot handle millions of doses of vaccine. The best option is therefore to store the vaccine at -70°C after manufacture and transport in liquid nitrogen to the hatchery for application.

Based on the titre of the vaccine which was $10^{6.6}EID_{50}$/ml, and since 1 ml of vaccine is normally reconstituted into 30 ml of vaccine diluent which is sufficient to vaccinate 1000 birds, one bird dose of vaccine would therefore contain $10^{3.6}EID_{50}$/ml. However, this dosage must be shown to be effective and able to cause seroconversion and protection in vaccinates. This was demonstrated in laboratory trials where vaccinated birds seroconverted against the TRTV and showed no symptoms of infection when challenged 14 days post-vaccination with the 3rd y/s A/F of isolate 652/93. There was a transient sinusitis in 40% of the unvaccinated chicks challenged with this isolate. The sinusitis was of a very short duration and only in a few chicks. This suggests that in the field situation, the severity of the disease is dependent upon other predisposing factors such as adverse environmental conditions, the presence of other pathogens which may be the primary or secondary organism in a disease outbreak, and poor flock management. Specific pathogen-free chicks vaccinated at day-old and again at 14 days of age, had the highest seroconversion against TRTV, whilst the groups vaccinated at day-old or 14 days, had lower seroconversions than the double vaccination, but similar to each other. The results of the

laboratory trials suggest that two vaccinations at day-old and 14 days of age may offer the best protection against the virulent virus.

The results of vaccination studies undertaken in the laboratory do not always correlate with the results obtained in the field. This was found to be especially true for TRTV by a major vaccine manufacturer in France (Gaudry, 1991).

The broiler vaccination trial conducted with the 652/93 vaccine showed that the best results were achieved with birds vaccinated at day-old or at day-old and again at 14 days of age. The average mortality of birds at the subsite vaccinated at day-old, was 6.09%, whilst that given the double vaccination was 6.28%. However, statistically, these differences were not significant. There was little difference in the percentage mortality between the subsite vaccinated at 14 days of age only and the unvaccinated control subsite. However, there was one house on subsite C (14 day vaccination), which had an extremely high mortality that elevated the average percentage mortality for the subsite. It is extremely difficult to measure small benefits derived from a vaccine in the field, when other predisposing factors are present.

The elevated mortality could have been due to several factors. This particular house had elevated mortality from the day of chick placement which suggests that the chicks placed in this house were of poor quality compared to the other houses. Also,

subsite C had an elevated IBV antibody titre at 35 days of age with elevated mortality occurring between 29-35 days. This suggests that there may have been an IBV infection which could have contributed to the increased mortality. The TRTV and NDV antibody titres were not very high, suggesting the absence of infection with these viruses.

Fig. 7.1 illustrates two major phases of mortality at all the subsites. The first phase occurred between 13-16 days of age and the second phase occurred between 27-35 days of age and was of a longer duration. The first phase may be due to the culling or death of the poor quality chicks that had been placed on the farm. The second phase of mortality may have been due to disease outbreak and the increased competition for food and water between birds, with the sick and weaker birds unable to compete, resulting in an increase in mortality. The disease could have been caused by NDV at subsite B and by IBV at subsite C since there were increased titres of the respective antibodies.

The cause of the second phase of mortality at subsite A and D is not clearly evident. However, the elevated TRTV titre at the unvaccinated subsite D, could suggest that TRTV may have been responsible. However, the TRTV antibody titres were also elevated at subsites A and B. Although subsites A and B were vaccinated with the 652/93 vaccine, there was little serological response following day-old vaccination. It is therefore unlikely that the elevated TRTV antibody titres at A and B were due to the vaccine although the vaccine may have protected the birds from the field

virus and resulted in the higher 35-day TRTV antibody titres at these two vaccinated sites.

Although one cannot determine precisely the nature or circumstances responsible for the high late mortality, it is believed that no single factor is responsible, but a combination of factors including environmental factors, management of the flock and farm, and disease. In field trials, the best method to evaluate the efficacy of the vaccination, is by comparing the performance of vaccinated and unvaccinated birds. This is the only reliable method when serological results cannot be extrapolated to determine vaccination efficacy.

The average mass of 35-day-old birds at subsite C was the highest with the mass at subsite B only 2 grams lower. The mass at subsite A was 9 grams lower than C but the average mass of birds at the unvaccinated control subsite was much lower at 19 grams below that of C. However, the bird mass of all the subsites was above the in-house standard mass of birds aged 35 days. Based on the mortality figures (although not statistically significant) and the average mass of birds at each subsite, it would appear that day-old or day-old plus 14 day vaccination would yield the best broiler performance results.

Serologically, there was hardly any response to the vaccine 14 days after day-old application. This has also been the experience of other workers. Gaudry (1991) found that although there was good seroconversion in birds vaccinated with their TRTV vaccine

in laboratory trials, there was often no seroconversion when the same vaccine was used in field trials and when seroconversion did occur, it was very low. It is possible that other vaccines administered before, after, or together with the 652/93 vaccine, could inhibit the birds response to this vaccine. Also, it is possible that maternal antibodies present in broiler chicks, drastically affect the seroconversion results. However, although the serological response may be inhibited, the vaccinated birds appeared to perform better than the unvaccinated birds.

The localised immunity induced by the vaccine may be more important in the protection against the virulent virus, than circulating immunity. This view was also expressed by Cook et al. (1989b). They found the serological response of turkey poults with maternal antibodies to be poor with many poults showing no detectable serum antibody response. They were, however, protected against challenge with virulent TRTV, indicating that humoral antibody may not be a good indication of protection against infection. They believed that local or cellular immunity was more important in resistance against TRTV infection. It would appear that the isolates used in their vaccine and in the 652/93 vaccine, are capable of significantly inducing the cellular immunity to provide protection against infection with these field isolates.

Based on the results obtained, it is evident that the 652/93 vaccine provides adequate protection against the field virus and results in improved bird performance. A 1% reduction in mortality

results in enormous savings for a large commercial chicken producer. It is therefore essential that effective vaccination against disease and proper management procedures be introduced wherever possible. Vaccination on its own is unlikely to have a major impact in the control of a disease, but implemented together with adequate biosecurity, proper hygiene and management, can contribute to the containment and eventual eradication of most diseases.

GENERAL DISCUSSION

It is now accepted that the virus responsible for TRT is also involved in SHS of chickens. Many researchers have isolated TRTV-like viruses from turkeys and chickens and shown that some of the symptoms of TRT and SHS could be reproduced in turkeys and chickens respectively although the symptoms in chickens were often mild compared to the symptoms produced in turkeys.

Most of the research has concentrated on the turkey disease rather than its chicken counterpart. The main reason for this is the difficulty involved in the isolation of these viruses from chickens and the apparently more severe disease in turkeys. However, although the disease appears to be more severe in turkeys, the chicken disease may be equally, or more devastating since there may be numerous other factors involved in the disease such as complications with other chicken viruses, secondary bacterial infections, high stocking density, and other management factors. Turkeys are not usually stocked at the high stocking density of chickens.

A phenomenon evident in this study was the inability to achieve a successful TRTV-like virus isolation from chickens if the time of sampling and the choice of birds for sampling were incorrect. It was essential that birds with sinusitis in the early stages of the disease be "hand-picked" for sampling. Samples from birds in the more pronounced or latter stages of the disease yielded negative isolation results. This was also reported by other

workers (Naylor and Jones, 1993). This short isolation period is indicative of the short duration of the disease and the time of peak virus concentration in the birds.

After consultation with Dr S.S. Buys (Early Bird), sinus exudate and sinus washings from birds in the early stages of sinusitis were used for isolation. The three ARTV isolates obtained were made from these samples. The use of other material, eg. trachea, lungs and tracheal swabs, failed to yield any ARTV isolates (results not presented). This could be because the virus does not grow to very high titres in these tissues. The failure to isolate the virus from the lungs is not unexpected, since it has been established by many researchers that TRTV and TRTV-like viruses of chickens, cause upper respiratory tract infections. However, the inability to isolate ARTV from the trachea and tracheal swabs, was unexpected since the virus is reported to cause tracheitis and other workers (Picault et al.,1987) have isolated the virus from this tissue. It is possible that the time of sampling may not have been during the period of maximum virus titre in the trachea.

To confirm the identity of the three ARTV isolates and to show their similarity to TRTV, various physicochemical tests were performed. The virus was shown to be enveloped, heat labile, and non-haemagglutinating. Electron microscopy showed it to be pleomorphic with diameters ranging between 100-300nm and the membrane covered with a fringe of approximately 12 nm long spikes. Also, a helical internal nucleocapsid component

approximately 15nm in diameter, was observed. The IUDR test demonstrated that the genome of the isolates consisted of RNA. All these morphological and chemical findings are consistent with characteristics of members of the Pneumovirus genus.

The culture characteristics of the ARTV isolates were similar to those of TRTV. The serological tests also suggest that the isolates are closely related to TRTV. In fact, the isolates could not be distinguished from TRTV serologically. Monoclonal antibody studies may demonstrate serological differences between these isolates and TRTV. This work is currently being performed by Dr J.K.A Cook (Intervet, UK).

The determination of an organism's identity is contained in its genetic material. Unequivocal evidence of an organism's identity can therefore be obtained by analysis or comparison of its genetic material to that of other related organisms. There exists at present only one commercially available avian disease DNA probe. This probe is manufactured by Idexx (USA) for the detection of M. gallisepticum. Jing et al. (1993) used PCR to detect TRTV in samples but their detection was based on the presence of a 541 or 175 bp DNA fragment in agarose gels. Although these bands are specific for TRTV using the TRT1 and TRT2 primers or the TRT1 and TRT3 primers respectively, hybridisation of the 175 bp TRTV-specific DNA probe to this 541 bp band provides absolute proof as to the identity of the isolate. The results of this study show that the three ARTV isolates are chicken strains of TRTV. Further molecular

characterisation of the three isolates involving sequencing of the genomes, is in progress at the laboratory of Dr A.J. Easton (University of Warwick, UK).

Although the DNA probes synthesised for TRTV and ARTV utilised the primers described by Jing et al. (1993), this is the first TRTV or ARTV DNA probe for routine diagnostic use. This enables a rapid, sensitive and accurate diagnosis of a virus that would otherwise take weeks to identify. PCR and probe technology have revolutionised routine virus identification.

The successful control of a disease can be achieved by suitable biosecurity measures, good disinfection, effective vaccination and proper management. There are currently no commercially available vaccines derived from chicken isolates of TRTV (ARTV). Commercial chicken producers wishing to vaccinate their flocks against ARTV, have to use the TRTV vaccine which is available from many commercial vaccine manufacturers. Although this vaccine has been effective for the protection of turkeys against TRT, no benefit could be demonstrated in broiler chickens (D.K. Thomson, Rainbow Farms, 1993 pers. comm.). It was believed that an autogenous chicken-isolate derived vaccine would provide better results. According to the results presented in Chapter 7, there appeared to be a small benefit to chickens vaccinated with this chicken-derived ARTV vaccine. However, the benefit was not statistically significant. It is extremely difficult to measure small benefits afforded by a vaccine in field trials when other environmental and disease factors have a much more pronounced

effect on the vaccinates. The vaccination trials were repeated at the same sites on numerous occasions but the results were severely distorted by the occurrence of virulent NDV which has subsequently become endemic in the Natal and Transvaal regions.

According to Shane (1992, 1993), SHS is a complicated disease with a host of factors contributing to its severity. This has also been observed at Rainbow Farms (Dr A.J. Morley, Rainbow Farms, 1993 pers. comm.). ARTV is believed to be the primary organism responsible for SHS, but due to the damage caused to the sinuses and the upper respiratory tract, other opportunistic pathogens like E. coli, exacerbate the disease. This is possibly the reason why only mild symptoms of the disease could be reproduced under laboratory conditions. It is therefore obvious that to achieve effective control of ARTV in broilers, steps should be taken to maintain a relatively "clean" environment and to institute effective ARTV vaccination at 1 day of age and again at 14 days of age. Strict biosecurity measures should also be instituted. These procedures should result in effective ARTV control.

This study provides unequivocal proof for the presence of strains of TRTV in chickens and possible control measures using autogenous chicken derived TRTV vaccines. This is the first report of the use of a chicken derived TRTV vaccine for the control of SHS. This is also the first report of a TRTV/ARTV DNA probe for routine diagnostic identification of ARTV infection in chickens.

APPENDIX

1). Alsever's solution

20.5 g dextrose

4.2 g sodium chloride

0.55 g citric acid

8.0 g sodium citrate

1000 ml distilled water

2). SDS-gel loading buffer

50 mM Tris.HCl (pH 6.8)

100 mM dithiothreitol

2 % SDS

0.1 % bromophenol blue

10 % glycerol

3). Tris-glycine electrophoresis buffer

25 mM Tris

250 mM glycine (pH 8.3)

0.1 % SDS

4). Transfer buffer

39 mM glycine

48 mM Tris

0.037 % SDS

20 % methanol

5). 10 X PCR buffer

500 mM KCl

200 mM Tris

20 mM $MgCl_2$

0.1 % gelatin

0.1 % triton-X 100

6). Tris-acetate EDTA (TAE) buffer (50 X) (pH 8.0)

242 g Tris

57.1 ml glacial acetic acid

1000 ml distilled water

7). Agarose gel loading buffer

4 g sucrose

10 ml distilled water

0.025 g bromophenol blue

8). Guanidinium isothiocyanate (GITC) extraction buffer

250 g GITC

17.6 ml 0.75 M sodium citrate (pH 7.0)

26.4 ml 10% N-lauryl sarcosinate

293 ml DEPC-treated water

before use, add 360 μl mercaptoethanol / 50 ml of above solution.

9). Hybridising buffer

4ml 20 X SSC

2 ml blocking stock solution

2 μl N-laurylsarcosine

1 μl SDS

10). 20 X SSC buffer (pH 7.0)

175.32 g sodium chloride

88.23 g sodium citrate

1000 ml distilled water

11). Blocking stock solution

10 g blocking reagent powder

100 ml buffer 1

12). Buffer 1 (pH 7.5)

11.61 g maleic acid

8.77 g sodium chloride

1000 ml distilled water

13). Buffer 2

Blocking stock solution diluted 1:10 in buffer 1

14). Buffer 3 (pH 9.5)

12.14 g Tris

5.84 g sodium chloride

10.17 g $MgCl_2$

1000 ml distilled water

REFERENCES

Alexander, D.J. 1989. Newcastle disease. p114-120. In: A Laboratory Manual for the Isolation and Identification of Avian Pathogens (H.G. Purchase, L.H. Arp, C.H. Domermuth, and J.E. Pearson, eds). American Association of Avian Pathologists, Iowa. 227pp.

Alexander, D.J., Borland, E.D., Bracewell, C.D., Chettle, N.J., Gough, R.E., Lister, S.A., and Wyeth, P.J. 1986(a). A preliminary report of investigations into turkey rhinotracheitis in Great Britain. State Veterinary Journal 40: 161-169.

Alexander, D.J., Gough, R.E., Wyeth, P.J., Lister, S.A., and Chettle, N.J. 1986(b). Viruses associated with turkey rhinotracheitis in Great Britain. Veterinary Record 118: 217-218.

Andral, B., Louazis, C., Trap, D., Newman, J.A., Bennejean, G., and Gaumont, R. 1985. Respiratory disease (rhinotracheitis) in turkeys in Brittany, France, 1981-82: field observations and serology. Avian Diseases 29: 26-34.

Andreasen, J.R., Jackwood, M.W., and Hilt, D.A. 1991. Polymerase chain reaction amplification of the genome of infectious bronchitis virus. Avian Diseases 35: 216-220.

Anon. 1985. Turkey rhinotracheitis of unknown aetiology in England and Wales. Veterinary Record 117: 653-654.

Anthony, C.E. 1992. Turkey rhinotracheitis (TRT) and swollen head syndrome (SHS). Proceedings of the 27th Meeting on Poultry Health and Processing, Maryland, USA: 1-3.

Baxter-Jones, C. 1992. TRTV (turkey rhinotracheitis virus): diagnostic aspects. Proceedings of the 27th Meeting on Poultry Health and Processing, Maryland, USA: 4.

Baxter-Jones, C., Cook, J.K.A., Frazier, J.A., Grant, M., Jones, R.C., Mockett, A.P.A., and Wilding, G.P. 1987. Close relationship between TRT isolates. Veterinary Record 121: 562.

Baxter-Jones, C., Grant, M., Jones, R.C., and Wilding, G.P. 1989. A comparison of three methods for detecting antibodies to turkey rhinotracheitis virus. Avian Pathology 18: 91-98.

Baxter-Jones, C., Wilding, G.P., and Grant, M. 1986. Immunofluorescence as a possible diagnostic method for turkey rhinotracheitis. Veterinary Record 119: 600-601.

Beard, C.W. 1989. Influenza. p110-113. In: A Laboratory Manual for the Isolation and Identification of Avian Pathogens (H.G. Purchase, L.H. Arp, C.H. Domermuth, and J.E. Pearson, eds). American Association of Avian Pathologists, Iowa. 227pp.

Beard, C.W., and Hanson, R.P. 1984. Newcastle disease. p452-470. In: Diseases of Poultry (H.J. Barnes, B.W. Calnek, W.M. Reid, and H.W. Yoder, eds). Iowa State University Press, Iowa. 831pp.

Bell, L.G., and Alexander, D.J. 1990. Failure to detect antibody to turkey rhinotracheitis virus in Australian poultry flocks. Australian Veterinary Journal 67: 232-233.

Box, P. 1989. Turkey rhinotracheitis: The end in sight?. Zootecnica International 7: 46-49.

Buys, S.B. 1990. A report on the viruses isolated from TRT and swollen head syndrome cases. Report of the Poultry Session of the South African Veterinary Association Conference. Pretoria. 12pp.

Buys, S.B., and Du Preez, J.H. 1980. A preliminary report on the isolation of a virus causing sinusitis in turkeys in South Africa and attempts to attenuate the virus. Turkeys 28: 36,46.

Buys, S.B., Du Preez, J.H., and Els, H.J. 1989(a). The isolation and attenuation of a virus causing rhinotracheitis in turkeys in South Africa. Onderstepoort Journal of Veterinary Research 56: 87-98.

Buys, S.B., Du Preez, J.H., and Els, H.J. 1989(b). Swollen head syndrome in chickens: A preliminary report on the isolation of a possible aetiological agent. Journal of the South African Veterinary Association 60: 221-222.

Cambridge Veterinary Sciences. 1989. Pathasure avian rhinotracheitis. Cambridge Veterinary Sciences ELISA Kit information booklet. 15pp.

Cavanagh, D., and Barrett, T. 1988. Pneumovirus-like characteristics of the mRNA and proteins of turkey rhinotracheitis virus. Virus Research 11: 241-256.

Chanock, R.M. 1979. Parainfluenza viruses. p 611-632. In: Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections (E.H. Lennette, and N.J. Schmidt, eds). American Public Health Association, Washington. 1138pp. .

Chettle, N.J., and Wyeth, P.J. 1988. Turkey rhinotracheitis: Detection of antibodies using an ELISA test. British Veterinary Journal 144: 282-287.

Collins, M.S., and Gough, R.E. 1988. Characterisation of a virus associated with turkey rhinotracheitis. Journal of General Virology 69: 909-916.

Collins, M.S., Gough, R.E., Lister, S.A., Chettle, N., and Eddy, R. 1986. Further characterisation of a virus associated with turkey rhinotracheitis. Veterinary Record 119: 606.

Cook, J.K.A. 1993. Isolation, characterization and serological relatedness of turkey rhinotracheitis viruses from domestic poultry. Proceedings of the Symposium on Newly Emerging and Re-Emerging Avian Diseases: Applied Research and Practical Applications for Diagnosis and Control. American Association of Avian Pathologists, Minneapolis: 56-61.

Cook, J.K.A., and Ellis, M.M. 1990. Attenuation of turkey rhinotracheitis virus by alternate passage in embryonated chicken eggs and tracheal organ cultures. Avian Pathology 19: 181-185.

Cook, J.K.A., Darbyshire, J.H., and Peters, R.W. 1976. The use of chicken tracheal organ cultures for the isolation and assay of avian infectious bronchitis virus. Archives of Virology 50: 109-118.

Cook, J.K.A., Dolby, C.A., Southee, D.J., and Mockett, A.P.A. 1988. Demonstration of antibodies to turkey rhinotracheitis virus in serum from commercially reared flocks of chickens. Avian Pathology 17: 403-410.

Cook, J.K.A., Ellis, M.M., Dolby, C.A., Holmes, H.C., Finney, P.M., and Huggins, M.B. 1989(a). A live attenuated turkey rhinotracheitis virus vaccine: 1. Stability of the attenuated strain. Avian Pathology 18: 511-522.

Cook, J.K.A., Ellis, M.M., and Huggins, M.B. 1991. The pathogenesis of turkey rhinotracheitis virus in turkey poults inoculated with the virus alone or together with two strains of bacteria. Avian Pathology 20: 155-166.

Cook, J.K.A., Holmes, H.C., Finney, P.M., Dolby, C.A., Ellis, M.M., and Huggins, M.B. 1989(b). A live attenuated turkey rhinotracheitis virus vaccine: 2. The use of the attenuated strain as an experimental vaccine. Avian Pathology 18: 523-534.

Cook, J.K.A., Jones, B.V., Ellis, M.M., Jing, L., and Cavanagh, D. 1993a. Antigenic differentiation of strains of turkey rhinotracheitis virus using monoclonal antibodies. Avian Pathology 22: 257-273.

Cook, J.K.A., Kinloch, S., and Ellis, M.M. 1993b. In vitro and in vivo studies in chickens and turkeys on strains of turkey rhinotracheitis virus isolated from the two species. Avian Pathology 22: 157-170.

Dowdle, W.A., Kendal, A.P., and Noble, G.R. 1979. Influenza viruses. p 585-609. In: Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections (E.H. Lennette, and N.J. Schmidt, eds). American Public Health Association, Washington. 1138pp.

Eleazer, T.H. 1991. Swollen heads in broiler breeders. Poultry Digest 50: 71.

FAO. 1992. FAO Quarterly Bulletin of Statistics 5(2): Rome.121pp.

Fenoglio-Preiser, C.M., and Willman, C.L. 1987. Molecular biology and the pathologist. Archives of Pathological Laboratory Medicine 111: 601-618.

Fraser, K.B., and Martin, S.J. 1978. Measles Virus And Its Biology. Academic Press, London. 249pp.

Gaudry, D. 1991. Problems of rhinotracheitis vaccination. Proceedings of the Spring Meeting of the Poultry Veterinary Study Group for the EEC, Portugal: 10-19.

Gaudry, D., and Le Gros, F.X. 1993. Research progress in SHS through so-called "vaccine failures". Proceedings of the Symposium on Newly Emerging and Re-Emerging Avian Diseases: Applied Research and Practical Applications for Diagnosis and Control. American Association of Avian Pathologists, Minneapolis: 67-77.

Giraud, P., Bennejean, G., Guittet, M., and Toquin, D. 1986. Turkey rhinotracheitis in France: Preliminary investigations on a ciliostatic virus. Veterinary Record 119: 606-607.

Giraud, R., Le Gros, F.X., Guittet, M., Bouquet, J.F., Toquin, D., and Bennejean, G. 1987. Turkey rhinotracheitis: isolation of a viral agent and first trials with experimental inactivated or attenuated vaccines. Proceedings of the 30th Western Poultry Disease Conference. Davis, California: 94-95.

Goren, E. 1985. Een 'nieuwe ziekte' bij de kip; diagnostische bevindingen. Tijdschrift Voor Diergeneeskunde 110: 1076-1077.

Gough, R.E., and Collins, M.S. 1989. Antigenic relationships of three turkey rhinotracheitis viruses. Avian Pathology 18: 227-238.

Gough, R.E., Collins, M.S., Cox, W.J., and Chettle, N.J. 1988(a). Experimental infection of turkeys, chickens, ducks, geese, guinea fowl, pheasants and pigeons with turkey rhinotracheitis virus. Veterinary Record 123: 58-59.

Gough, R.E., Collins, M.S., and Hancock, R.D. 1988(b). Isolation of turkey rhinotracheitis virus from five-day-old turkeys. Veterinary Record 122: 370-371.

Grant, M., Baxter-Jones, C., and Wilding, G.P. 1987. An enzyme-linked immunosorbent assay for the serodiagnosis of turkey rhinotracheitis infection. Veterinary Record 120: 279-280.

Grow, A. 1992. A serologic occurrence of viral turkey rhinotracheitis. Foreign Animal Disease Report 20: 1,4.

Hawkes, R.A. 1979. General principles underlying laboratory diagnosis of viral infections. p 3-48. In: Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections (E.H. Lennette, and N.J. Schmidt, eds). American Public Health Association, Washington. 1138pp.

Hill, S.A. 1984. Methods in Plant Virology. Blackwell Scientific, Oxford. 167pp.

Horner, R.F., Bishop, G.C., and Haw, C. 1992. An upper respiratory disease of commercial chickens resembling infectious coryza, but caused by a V factor-independent bacterium. Avian

Pathology 21: 421-426.

Jackwood, M.W., Kwon, H.M., and Hilt, D.A. 1992. Infectious bronchitis virus detection in allantoic fluid using the polymerase chain reaction and a DNA probe. Avian Diseases 36: 403-409.

Jing, L., Cook, J.K.A., Brown, T.D.K., Shaw, K., and Cavanagh, D. 1993. Detection of turkey rhinotracheitis virus in turkeys using the polymerase chain reaction. Avian Pathology 22: 771-783.

Jones, R.C. 1993. Viral rhinotracheitis: Pathogenesis and immunity in laboratory challenge studies. Proceedings of the Symposium on Newly Emerging and Re-Emerging Avian Diseases: Applied Research and Practical Applications for Diagnosis and Control. American Association of Avian Pathologists, Minneapolis: 62-66.

Jones, R.C., Baxter-Jones, C., Wilding, G.P., and Kelly, D.F. 1986. Demonstration of a candidate virus for turkey rhinotracheitis in experimentally inoculated turkeys. Veterinary Record 119: 599-600.

Jones, R.C., Baxter-Jones, C., Savage, C.E., Kelly, D.F., and Wilding, G.P. 1987. Experimental infection of chickens with a ciliostatic agent isolated from turkeys with rhinotracheitis. Veterinary Record 120: 301-302.

Jones, R.C., Naylor, C.J., Bradbury, J.M., Savage, C.E., Worthington, K., and Williams, R.A. 1991. Isolation of a turkey rhinotracheitis-like virus from broiler breeder chickens in England. Veterinary Record 129: 509-510.

Jones, R.C., Williams, R.A., Baxter-Jones, C., Savage, C.E., and Wilding, G.P. 1988. Experimental infection of laying turkeys with rhinotracheitis virus: Distribution of virus in the tissues and serological response. Avian Pathology 17: 841-850.

Jordan, F.T.W. 1990. Poultry Diseases (3rd ed.). Balliere Tindall. 497pp.

Kwok, S., and Higuchi, R. 1989. Avoiding false positives with PCR. Nature 339: 237-238.

Kwon, H.M., Jackwood, M.W., Brown, T.P., and Hilt, D.A. 1993. Polymerase chain reaction and a biotin-labeled DNA probe for detection of infectious bronchitis virus in chickens. Avian Diseases 37: 149-156.

Lensing, H.H. 1973. Some remarks on Newcastle disease live virus vaccine testing. p189-194. In: Developments in Biological Standardization (C.H. Merieux, E.C. Hulse, D. Gaudry, W.H. Allan, and R.H. Regamey, eds). S Karger, Basel, Switzerland. 445pp.

Ling, R., and Pringle, C.R. 1988. Turkey rhinotracheitis virus: In vivo and in vitro polypeptide synthesis. Journal of General

Virology 69: 917-923.

Lister, S.A., and Alexander, D.J. 1986. Turkey rhinotracheitis: a review. Veterinary Bulletin 56: 637-663.

Lu, Y.S., Shien, Y.S., Tsai, H.J., Tseng, C.S., Lee, S.H., and Lin, D.F. 1994. Swollen head syndrome in Taiwan - isolation of an avian pneumovirus and serological survey. Avian Pathology 23: 169-174.

Lukert, P.D. 1989. Virus identification and classification. p182-185. In: A Laboratory Manual for the Isolation and Identification of Avian Pathogens (H.G. Purchase, L.H. Arp, C.H. Domermuth, and J.E. Pearson, eds). American Association of Avian Pathologists, Iowa. 227pp.

McDougall, J.S., and Cook, J.K.A. 1986. Turkey rhinotracheitis: preliminary investigations. Veterinary Record 118: 206-207.

Metselaar, D., and Simpson, D.I.H. 1982. Practical Virology. Oxford University Press, Oxford. 496pp.

Morley, A.J., and Thomson, D.K. 1984. Swollen-head syndrome in broiler chickens. Avian Diseases 28: 238-243.

Murtaugh, M.P. 1993. Applications in avian medicine and diagnostics: polymerase chain reaction. Vineland Update 44: 4pp.

Naylor, C.J., Al-Ankari, A.R., Al-Afaleq, A.I., Bradbury, J.M., and Jones, R.C. 1992. Exacerbation of Mycoplasma gallisepticum infection in turkeys by rhinotracheitis virus. Avian Pathology 21: 295-305.

Naylor, C.J., and Jones, R.C. 1993. Turkey rhinotracheitis: a review. Veterinary Bulletin 63: 439-449.

O'Brien, J.D.P. 1985. Swollen-head syndrome in broiler breeders. Veterinary Record 117: 619-620.

O'Loan, C., and Allan, G.M. 1990. The detection of turkey rhinotracheitis virus antigen in formalin fixed, paraffin embedded tissue using a streptavidin-biotin-immunoperoxidase method. Avian Pathology 19: 401-407.

O'Loan, C.J., Allan, G., Baxter-Jones, C., and McNulty, M.S. 1989. An improved ELISA and serum neutralisation test for the detection of turkey rhinotracheitis virus antibodies. Journal of Virological Methods 25: 271-282.

O'Loan, C.J., Allan, G.M., McNair, J., Mackie, D.P., and McNulty, M.S. 1990. TRT virus serology: Discreptancy between ELISA and indirect immunofluorescence. Avian Pathology 19: 173-180.

O'Loan, C.J., Curran, W.L., and McNulty, M.S. 1992. Immuno-gold labelling of turkey rhinotracheitis virus. Journal of Veterinary Medicine 39: 466-469.

Pages, A., Ramis, A., and Majo, N. 1992. T.R.T.-like virus particles in purified viral preparation from hen swollen head syndrome, using electron microscopy. Proceedings of the 19th World Poultry Congress (vol. 1): 418-419.

Parrott, R.H., Kim, H.W., Brandt, C.D., Beem, M.O., Richardson, L., Gerin, J.L., and Chanock, R.L. 1979. Respiratory syncytial virus. p 695-708. In: Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections (E.H. Lennette, and N.J. Schmidt, eds). American Public Health Association, Washington. 1138pp.

Pattison, M., Chettle, N.J., Randall, C.J., and Wyeth, P.J. 1989. Observations of swollen head syndrome in broiler and broiler breeder chickens. Veterinary Record 125: 229-231.

Peake, I. 1989. The polymerase chain reaction. Journal of Clinical Pathology 42: 673-676.

Pearson, J.E., Senne, D.A., Panigrahy, B., and Mixson, M.A. 1993. Viral turkey rhinotracheitis and swollen head syndrome of chickens: The current status of these diseases in the United States. Proceedings of the Symposium on Newly Emerging and Re-Emerging Avian Diseases: Applied Research and Practical Applications for Diagnosis and Control. American Association of Avian Pathologists, Minneapolis: 78-83.

Peleteiro, M. 1991. An overview of the swollen head syndrome in

poultry. Proceedings of the Spring Meeting of the Poultry Veterinary Study Group, Portugal: 1-6.

Perelman, B., Meroz, M., and Samberg, Y. 1988. "Swollen head syndrome" in broiler breeders in Israel. Veterinary Record 123: 444.

Picault, J.P., Giraud, P.D., Guittet, M., Bennejean, G., Lamande, J., Toquin, D., and Gueguen, C. 1987. Isolation of a TRTV-like virus from chickens with swollen-head syndrome. Veterinary Record 121: 135.

Roberts, R.G., Montandon, A.J., Green, P.M., and Bentley, D.R. 1991. Analysis of genomic sequence variation using amplification and mismatch detection (AMD) and direct sequencing. p 51-66. In: PCR A Practical Approach (M.J. McPherson, P. Quirke, and G.R. Taylor, eds). Oxford University Press, Oxford. 253pp.

Saiki, R.K. 1989. The design and optimization of the PCR. p 7-16. In: PCR Technology: Principles and Applications for DNA Amplification (H.A. Erlich, ed). Stockton Press, New York. 246pp.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Ehrlich, H.A. 1988. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239: 487-491.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular

Cloning: A Laboratory Manual vol 3 (2nd ed). Cold Spring Harbor Laboratory Press, New York.

Shane, S.M. 1992. Laboratorio Avimex Conference: new viral diseases for the poultry industry in the 90's - swollen head syndrome in broilers. Zootecnica International 3: 74-77.

Shane, S.M. 1993. Advances in the control of poultry diseases. Zootecnica International 3: 18-27.

Sigma Chemical Company. 1988. SDS Molecular Weight Markers. Technical Bulletin MWS-877. 8pp.

Spurr, A.R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. Journal of Ultrastructure Research 26: 31-43.

Stuart, J.C. 1986. Field experiences in the UK with turkey rhinotracheitis. Technical Turkeys 34: 24-25.

Taylor, G.R. 1991. Polymerase chain reaction: basic principles and automation. p 1-14. In: PCR A Practical Approach (M.J. McPherson, P. Quirke, and G.R. Taylor, eds). Oxford University Press, Oxford. 253pp.

Vanmarcke, J. 1989. Swollen head syndrome. Rhone Merieux information booklet. 18pp.

Versteeg, J. 1985. A Colour Atlas of Virology. Wolfe Medical Publications Ltd, The Netherlands. 240pp.

Villegas, P. 1987. Laboratory Manual of Avian Virus Diseases. University of Georgia Press, Georgia. 65pp.

Villegas, P., and Purchase, H.G. 1989. Titration of biological suspensions. p186-191. In: A Laboratory Manual for the Isolation and Identification of Avian Pathogens (H.G. Purchase, L.H. Arp, C.H. Domermuth, and J.E. Pearson, eds). The American Association of Avian Pathologists, Iowa. 227pp.

Weisman, Y., Strengel, C., Blumenkranz, R., and Segal, Y. 1988. Turkey rhinotracheitis (TRT) in turkey flocks in Israel: virus isolation and serological response. Processing: Western Poultry Disease Conference: 67-68.

Wilding, P., Baxter-Jones, C., and Grant, M. 1986. Ciliostatic agent found in rhinotracheitis. Veterinary Record 118: 735.

Williams, R.A., Savage, C.E., and Jones, R.C. 1991(a). Development of a live attenuated vaccine against turkey rhinotracheitis. Avian Pathology 20: 45-55.

Williams, R.A., Savage, C.E., Worthington, K.J., and Jones, R.C. 1991(b). Further studies on the development of a live attenuated vaccine against turkey rhinotracheitis. Avian Pathology 20: 585-596.

Wyeth,P.J. 1990. Turkey rhinotracheitis, swollen head syndrome cause heavy loss. Poultry Digest 49: 16-21.

Wyeth, P.J., and Alexander, D.J. 1989. Pneumovirus infections. p121-123. In: A Laboratory Manual for the Isolation and Identification of Avian Pathogens (H.G. Purchase, L.H. Arp, C.H. Domermuth, and J.E. Pearson, eds). The American Association of Avian Pathologists, Iowa. 227pp.

Wyeth, P.J., Chettle, N.J., Gough, R.E., and Collins, M.S. 1987. Antibodies to TRT in chickens with swollen head syndrome. Veterinary Record 120: 286-287.

Wyeth, P.J., Gough, R.E., Chettle, N.J., and Eddy, R. 1986. Preliminary observations on a virus associated with turkey rhinotracheitis. Veterinary Record 119: 139.

Yu, Q., Davis, P.J., Barrett, T., Binns, M.M., Boursnell, M.E.G., and Cavanagh, D. 1991. Deduced amino acid sequence of the fusion glycoprotein of turkey rhinotracheitis virus has greater identity with that of human respiratory syncytial virus, a pneumovirus, than that of paramyxoviruses and morbilliviruses. Journal of General Virology 72: 75-81.

Yu, Q., Davis, P.J., Brown, T.D.K., and Cavanagh, D. 1992. Sequence and in vitro expression of the M2 gene of turkey rhinotracheitis pneumovirus. Journal of General Virology 73: 1355-1363.

Yu, Q., Davis, P.J., Li, J., and Cavanagh, D. 1992. Cloning and sequencing of the matrix protein (M) gene of turkey rhinotracheitis virus reveal a gene order different from that of respiratory syncytial virus. Virology 186: 426-434.

Zander, D.V. 1984. Principles of disease prevention: diagnosis and control. p1-37. In: Diseases of Poultry (M.S. Hofstad, J.H. Barnes, B.W. Calnek, W.M. Reid, and H.W. Yoder, eds). Iowa State University Press, Iowa. 831pp.

Zanella, A., and Marchi, R. 1982. Inactivated vaccines in oil emulsion in the control of the infectious diseases of poultry. p19-32. In: Developments in Biological Standardization (Y. Moreau, and W. Hennessen, eds). S Karger, Basel, Switzerland. 333pp.

Zellen, G. 1988. Swollen-head syndrome in broiler chickens. Canadian Veterinary Journal 29: 298.

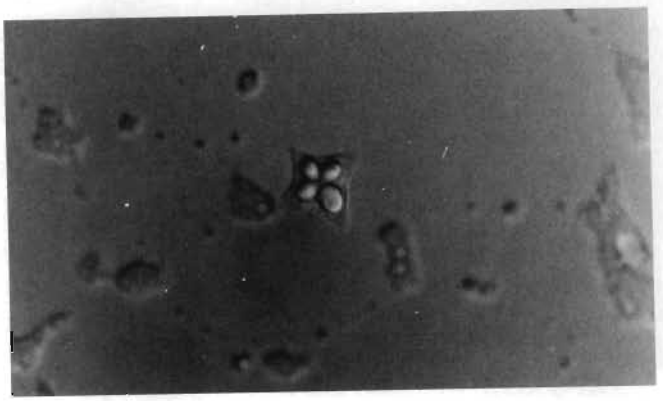


FIG 1: Typical *Kudoa thyrsites* spore

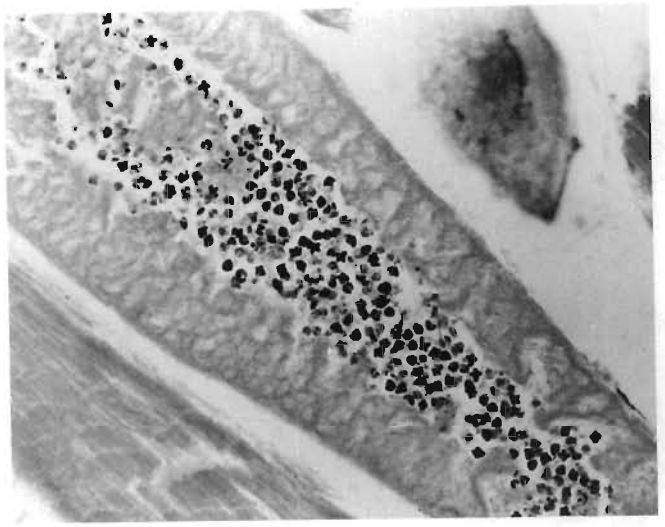


FIG 2: Accumulation of *Kudoa thyrsites* spores within muscle bundle

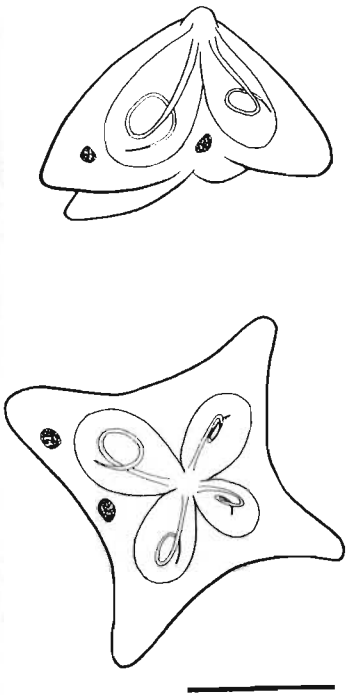


FIG 3: Diagrams of antero-posterior and lateral views of *Kudoa thyrsites* spores (bar = 5 μm). (Reprinted from Langdon [1991] Myoliquefaction post mortem ('milky flesh') due to *Kudoa thyrsites* (Gilchrist) (Myxosporea: Multivalvulida) in mahi mahi, *Coryphaena hippurus* L)

home and laid on a cold stone floor before preparation for cooking. On returning to the fish 20 hours later, extensive muscle degeneration was apparent, the fish resembling a 'bag of bones'. The fish was submitted to the MAFF Veterinary Investigation Centre at Penrith for further examination.

Muscle smears were made on to glass slides and agar-coated slides, while samples of muscle were fixed in 10 per cent buffered formalin and histological sections cut and stained with haematoxylin and eosin and Giemsa. Numerous myxosporean spores were seen on direct smear. These conformed with published descriptions of *K thyrsites,* showing four pyriform polar capsules arranged tetrahedrally. One polar capsule was larger than the remaining three, their apices met centrally and the overall spore shape was stellate (Figs 1 and 2).

Myxosporean spores were also readily detected in histological sections both within and between muscle fibres. There was little or no associated inflammatory reaction and no evidence of pseudocyst formation, encapsulation or pigment reaction (Fig 3). This is in marked contrast to fish such as Pacific hake, *Merluccius productus,* which develops a focal, chronic inflammatory response that ultimately destroys the parasite (Morado and Sparks 1986).

The lack of inflammatory reaction noted in salmonids is thought to allow the parasite to continue multiplying within the flesh of the fish. *K thyrsites* is presumed to excrete histiolytic enzymes which diffuse into the surrounding muscle. Whether these enzymes are released in response to the death of the host in order to facilitate spore release from infection sites, or whether they are excreted continuously but removed by vascular perfusion in the live fish is not clear.

The method of transmission of *Kudoa* species between fish hosts is unknown. It appears to be a strictly fish parasite, carrying no known human health risk. No drugs are commercially available to treat *Kudoa* infections.

It is likely that salmonid species represent aberrant hosts. However, its potential significance for salmon farming in both Ireland and Scotland due to the unappealing appearance and poor quality flesh of the resultant product must not be underestimated.

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References

BARJA, J. L. & TORANZO, A. E. (1993) *Bulletin of the European Association of Fish Pathologists* **13**, 86
KABATA, Z. & WHITAKER, D. J. (1989) *Canadian Journal of Zoology* **67**, 341
LANGDON, J. S. (1991) *Journal of Fish Diseases* **14**, 45
MORADO, J. F. & SPARKS, A. (1986) *Journal of Fish Diseases* **9**, 445
WHITAKER, D. J. & KENT, M. L. (1991) *Journal of Aquatic Animal Health* **3**, 291

Isolation of an avian pneumovirus-like agent from broiler breeder chickens in South Africa

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Veterinary Record (1994) **134**, 525-526

TURKEY rhinotracheitis (TRT) is an acute upper respiratory tract infection of poultry (Collins and others 1986). Concurrent with the appearance of TRT in turkeys, a disease condition known as swollen head syndrome, was observed in chickens worldwide (Buys and others 1989). Several researchers isolated a non-haemagglutinating myxo-like virus from turkeys with TRT which was named TRT virus (TRTV) (Naylor and Jones 1993). A TRTV-like virus was subsequently isolated from chickens with swollen head syndrome (Picault and others 1987, Buys and others 1989).

The aetiological agent of TRT is believed to be a pneumovirus

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TABLE 1: ELISA results of the two affected flocks, and SPF birds inoculated with yolk sac material, before and after challenge

Source of serum	ELISA index value	
	Pre-infection	Post infection
Flock 1	0·22	0·45 (14)*
Flock 2	0·25	0·84 (21)*
SPF birds†	0·18	0·91 (10)*

* Days after infection
† SPF birds inoculated with third yolk sac material

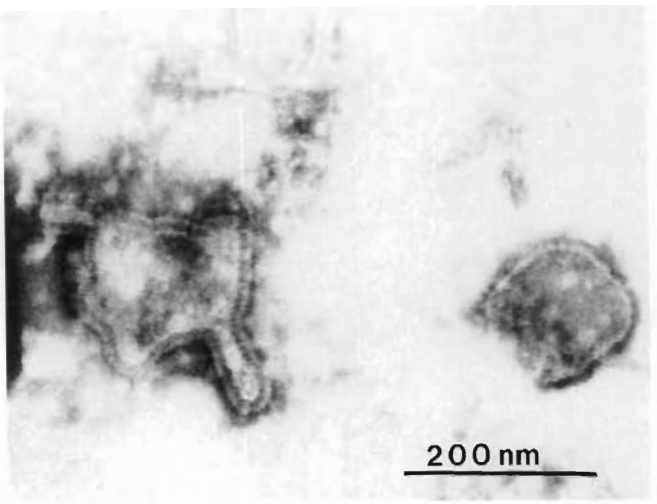

FIG 1: Pleomorphic virus-like particle with a fringe of spikes

of the Paramyxoviridae family (Collins and Gough 1988). This is the first report on the isolation of a TRTV-like agent from broiler breeders with sinusitis and swollen head syndrome in South Africa.

During August 1991, two adult broiler breeder flocks, housed in close proximity to one another in the Natal midlands, exhibited acute sinusitis and periorbital swelling. Approximately 10 per cent of the flock exhibited signs, while egg production declined between 5 and 10 per cent over a two-week period. During the early stages of the disease, virus isolation was attempted by killing five affected birds and aseptically removing the infraorbital sinus fluid with a syringe. The sinuses were then washed with cold tryptose phosphate broth containing antibiotics.

The antibody status of the two affected flocks, flocks 1 and 2, against TRTV just before the appearance of clinical signs, as determined by ELISA (Pathasure, Cambridge), was negative, while sera drawn 14 and 21 days after infection revealed a significant rise in antibodies against TRTV (Table 1).

Sinus fluids were pooled into a single vial and clarified by centrifugation. The material was then inoculated into the allantoic sac of nine-day-old embryonated specific pathogen free (SPF) eggs. The allantoic fluid was harvested seven days after inoculation and then inoculated via the yolk sac of six-day-old embryonated SPF eggs, and also on to Vero cell monolayers. No evidence of virus activity was detected in the first allantoic sac and first yolk sac passages. However, embryo mortality occurred nine days after inoculation in the third yolk sac passage. Affected embryos were congested, stunted and oedematous. After two Vero cell passages, a few loose cells were observed floating in the media. Loose floating cells were also observed when chicken embryo kidney cells were inoculated with third passage Vero cell material. The third Vero cell passage material was also inoculated into the yolk sac of SPF eggs, causing congestion and stunting of the embryos.

The third yolk sac passage material was inoculated into the sinus of three four-week-old SPF birds housed in an isolator. The birds were bled before inoculation and 10 days after inoculation. None of the inoculated birds displayed any clinical signs of infection. The birds seroconverted from a negative ELISA value pre-inoculation, to a highly positive value 10 days after inoculation (Table 1).

Electron microscopy of sixth yolk sac passage allantoic fluid, revealed the presence of a few highly pleomorphic virus-like particles (Fig 1) 112 to 297 nm in diameter with a fringe of spikes approximately 12 nm in length. The isolate did not agglutinate chicken red blood cells and was completely inactivated at 56°C for one hour and by chloroform. The isolate differed from that of Buys and others (1989), in that it failed to produce clinical signs of infection in SPF birds. This could be due to it being a non-pathogenic strain or to the lack of secondary pathogenic organisms.

The characteristics of this virus suggests that it could be related to TRTV which has already been classified as a pneumovirus.

References

BUYS, S. B., DU PREEZ, J. H. & ELS, H. J. (1989) *Journal of the South African Veterinary Association* **60**, 221
COLLINS, M. S. & GOUGH, R. E. (1988) *Journal of General Virology* **66**, 909
COLLINS, M. S., GOUGH, R. E., LISTER, S. A., CHETTLE, N. & EDDY, R. (1986) *Veterinary Record* **119**, 606
NAYLOR, C. J. & JONES, R. C. (1993) *Veterinary Bulletin* **63**, 439
PICAULT, J. P., GIRAUD, P. D., GUITTET, M., BENNEJEAN, G., LAMANDE, J., TOQUIN, D. & GUEGUEN, C. (1987) *Veterinary Record* **121**, 135

Abstracts

Expiration of hydrogen by cats given xylose orally

THE mean rate of expiration of hydrogen by 11 healthy cats after the oral administration of 0·75 g/kg xylose was 0·18 ml/hour and was little different from the rate before the administration of the xylose. In contrast, the rate of expiration of hydrogen by five cats with chronic diarrhoea and/or vomiting was much higher before the administration of the same dose of xylose, and in four of them increased considerably after its administration, indicating some degree of failure of absorption of carbohydrate. One of the sick cats had a hiatus hernia and three had inflammatory bowel disease; large numbers of *Clostridium* species were isolated from samples of small intestinal fluid taken from all the sick cats, but from only one of the eight healthy cats sampled. However, it was not possible to decide whether a specific bacterial pathogen was responsible for the increased expiration of hydrogen by the sick cats.

MUIR, P., GRUFFYDD-JONES, T. J., CRIPPS, P. J., PAPASOULIOTIS, K. & BROWN, P. J. (1994) *Journal of Small Animal Practice* **35**, 86

Treatment of endotoxin-induced mastitis with isotonic or hypertonic sodium chloride

EIGHT lactating Holstein cows received 1 mg endotoxin by intramammary infusion into the left fore quarter. Four hours later each cow received either isotonic (IS) 0·9 per cent sodium chloride at 5 ml/kg bodyweight (n=4) or hypertonic (HS) 7·0 per cent sodium chloride, 5 ml/kg (n=4). This model closely mimicked naturally acquired Gram-negative bacterial mastitis, causing a consistent inflammatory reaction in the udder and a severe, transient shock syndrome which resolved in two days. Plasma volume decreased at three hours after inoculation, and expanded consistently both in HS and IS treated cows at six (peak values), 12, 24 and 48 hours after endotoxin infusion. Serum protein and haematocrit values decreased. Neither haemoglobinuria nor haematuria was observed. In contrast to other studies using an intravenous endotoxin induction model, serum activity of hepatic and muscle-specific enzymes was unchanged or decreased in this model. Azotaemia, increased serum urea nitrogen or creatinine concentration was lacking.

TYLER, J. W., WELLES, E. G., ERSKINE, R. J., LIN, H.-C., WILLIAMS, M. A., SPANO, J. S., GASLIN, T. & McCLURE, K. A. (1994) *American Journal of Veterinary Research* **55**, 278