

THE ISOLATION AND ATTENUATION OF A VIRUS CAUSING RHINOTRACHEITIS IN TURKEYS IN SOUTH AFRICA

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ABSTRACT

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In March 1978, a number of turkeys with severe respiratory symptoms affecting over 80 % of the flock were investigated for a possible causative agent. With the standard techniques used for the isolation of bacteriae, mycoplasmae and viruses, only *Mycoplasma gallisepticum*, *Mycoplasma meleagridis* and Newcastle disease virus were isolated. Tracheal organ cultures were subsequently prepared from 27-day-old turkey embryos and inoculated with sinus exudate from affected turkeys. After an incubation period of 4 days a virus was isolated with which the typical symptoms, as observed in the field, could be reproduced in susceptible turkeys after 3-5 days. Following primary isolation in tracheal organ cultures, the virus grew readily in embryonated eggs and Vero cells. With the electron microscope, virus-like particles, varying in size from 40 nm-500 nm, were observed, having a pleomorphic shape and studded with fine surface projections. The virus seems to fall into the family Paramyxoviridae. A vaccine produced from attenuated virus in embryonated eggs afforded good protection against mortalities due to airsacculitis that normally follows on to turkey rhinotracheitis infection. The serological and clinical effects of the virus on chickens are also reported on.

INTRODUCTION

In March 1978, turkeys with respiratory symptoms were received from an integrated turkey operation. Clinically, the disease started from 7-14 days of age with sneezing followed by rales and a watery nasal discharge. As the disease progressed the discharge became thicker and eventually blocked the nares, causing the birds to breathe with an open mouth. The sinuses also soon became distended with exudate. On farms most turkeys died from *Escherichia coli* airsacculitis which followed the initial respiratory symptoms. The disease was highly contagious, with morbidity usually over 90 % and total mortalities in many instances going up to 30 %. Treatment with antibiotics for the secondary bacterial infections had limited success.

In 1975, Blalock, Simmons, Muse, Gray & Derieux reported on one of the viruses isolated in 1971-1972 from turkeys with respiratory symptoms. Morbidity in these flocks were as high as 80 % but with only 2 % mortality. With an uncloned adenovirus isolated in embryonated eggs via the allantoic sac route, they were able to reproduce clinical symptoms in 100 % of the infected turkeys with a 50 % mortality in 1973, but in 1975 they could produce only mild symptoms with the original uncloned and cloned virus. Simmons, Miller, Gray, Blalock & Colwell (1976) also reported on a respiratory disease in turkeys with a morbidity rate of 30-50 % and mortalities between 4-6 %. Since no susceptible turkeys were available the pathogenicity of this adenovirus isolate could not be established.

In 1978-79, a respiratory disease occurred on farms in Israel. Characteristic symptoms were mild rales to severe respiratory distress, including nasal discharge, sinusitis, conjunctivitis and pneumonia. Morbidity reached 100 %, with mortalities between 5 % and 90 %. A haemagglutinating yucaipa paramyxovirus was isolated which serologically seemed to be associated with the disease in turkeys (Lipkind, Weisman, Shikmanter, Shoham & Aronovici, 1979). In 1979, Simmons, Gray, Rose, Dillman & Miller reported on the isolation of a bacterium with which

they were able to reproduce rhinotracheitis. The causative agent was originally identified as *Alcaligenes faecalis*, now called *Bordetella avium*.

In June 1985, an acute and rapidly spreading respiratory disease of turkeys, designated turkey rhinotracheitis (TRT), appeared in England and Wales (Anon., 1985). Subsequently, McDougall & Cook (1986), Wilding, Baxter-Jones & Grant (1986) and Wyeth, Gough, Chettle & Eddy (1986) isolated a highly pleomorphic virus which was thought to be an orthomyxovirus or a coronavirus (McDougall & Cook, 1986). Wyeth *et al.*, (1986) suggested it to be a member of the Paramyxoviridae family. Jones, Baxter-Jones, Savage, Kelly & Wilding (1987) were able to produce mild clinical signs in 3-week-old broilers, but could not re-isolate the virus. Serologically infected and contact birds developed antibodies against TRT as determined with the enzyme-linked immunosorbent analysis (ELISA) test. When day-old specific pathogen-free (SPF) chickens were infected, mild respiratory signs developed between 4 and 7 days and the virus could be re-isolated.

In this article, a more detailed account of the initial report on the isolation of a viral agent designated 91/78 causing turkey rhinotracheitis (Buys & Du Preez, 1980) in South Africa is given. Furthermore, in view of the findings with chickens in the United Kingdom, we decided—

- (a) to repeat our work in broilers, SPF chickens and turkeys
- (b) to observe the growth of the virus in Vero cells and confirm its presence by means of electron microscopy
- (c) and with the assistance of Dr P. Wilding to investigate the presence or absence of antibodies against TRT in commercial broilers and broiler breeder flocks.

MATERIALS AND METHODS

Samples of sinus exudate were collected from turkeys with rhinitis and sinusitis symptoms for the following isolation procedures.

Mycoplasma isolation

Sinus exudate was streaked onto C-agar (Chalquest, 1962) and inoculated into Frey's Medium No. 4 (Frey, Hanson & Anderson, 1968). Identification

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of mycoplasmae was done by means of direct immuno-fluorescence with monospecific sera (Buys, 1976).

Bacteriological isolation

Sinus material was streaked onto blood tryptose agar (10 % horse blood) and McConkey's agar.

Virus isolation

Embryonated SPF eggs

Sinus material was collected and mixed with cleaning fluid containing penicillin, streptomycin, neomycin and fungizone. This was inoculated into embryonated specific pathogen-free (SPF) eggs via the following routes:

- | | | |
|-------------------------------|--------------|-------------------|
| (a) Allantoic | — 9-day-old | embryo-nated eggs |
| (b) Yolk sac | — 6-day-old | embryo-nated eggs |
| (c) Chorio allantoic membrane | — 10-day-old | embryo-nated eggs |
| (d) Intravenous route | — 12-day-old | embryo-nated eggs |

Since a lentogenic Newcastle disease strain was consistently isolated from all samples, it was decided to passage sinus material through susceptible turkeys. For this purpose, day-old poults were received from a commercial hatchery and raised in filtered air-positive pressure (FAPP) isolators.

At 2 weeks of age sinus material was inoculated into the infraorbital sinus of 10 poults. These turkeys were then sacrificed as soon as a nasal discharge appeared. Their sinus exudate was then inoculated into the infraorbital sinuses of the next group of turkeys. This procedure was repeated for a third time.

The sinus material from the 3rd passage was harvested and diluted 1:1 with phosphate buffered saline without any antibiotics and stored in small aliquots in liquid nitrogen (N₂). These aliquots were then used as follows:

- The sinus exudate was filtered through a 0,22 µm pore-size filter. This material was then inoculated into the infraorbital sinuses of 3-week-old turkeys, SPF White Leghorns and commercial broilers raised from day-old in FAPP isolators.
- The sinus exudate was mixed with cleaning fluid and inoculated into embryonated SPF eggs via the allantoic sac route, yolk sac route, chorio allantoic membrane route and the intravenous route. Eggs were candled daily and after 7 days the embryos were chilled at 4 °C and allantoic fluid, yolk material, chorio allantoic membranes and embryo livers harvested for further inoculations. This procedure was repeated 3 times, after which the different samples harvested as 3rd generation material were inoculated into the infraorbital sinuses of susceptible 3-week-old turkeys.

Tracheal-ring organ cultures (TROC)

TROC were prepared from 27-day-old turkey embryos according to the method described by Cherry & Taylor-Robinson (1970). Organ cultures were left for 48 h before use. For tracheal organ cultures Eagle's basal medium with 200 units of penicillin, 0,2 mg streptomycin, 0,002 mg fungizone per ml and 0,05 ml N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) buffer was used. The pH of the medium was adjusted to 7,0 with 0,2 M NaOH.

The TROC cultures were inoculated with sinus exudate diluted 10⁻¹ in cleaning fluid as follows:

The medium was poured off, then 0,1 ml of the sinus exudate mixture was added to each tube and kept in an upright position for 30 min to bring about adsorption. Fresh medium was then added and the organ cultures again placed in a roller drum and incubated at 37 °C. Organ cultures were examined for signs of ciliostasis on a daily basis.

Whenever egg material was inoculated onto organ cultures it was diluted 10⁻¹ in HEPES buffered Eagle's medium. After adsorption the medium was poured off and flushed twice with fresh medium before the final medium was added to the organ cultures.

Organ culture material was serially passaged 26 times in tracheal rings. Titrations of the growth of the virus in TROC were done on generations (#) 2, 10, 19 and 22. The TROC ID₅₀ end-point of the titrations were determined by means of the Reed & Muench method (1938). The 12th organ culture passage was inoculated into embryonated eggs via the chorio allantoic membrane, the allantoic membrane, and yolk sac route. Chorio allantoic membranes, allantoic fluid and yolk sac material were harvested from the different routes. These materials were titrated in tracheal organ cultures and susceptible turkeys were challenged. The chorio allantoic route was then selected to further passage the virus in embryonated eggs.

Tissue cultures

Vero cell monolayers were initially inoculated with chorio allantoic membrane material. A further limited number of serial passages were then done on Vero cells. The same medium as for tracheal organ cultures without the HEPES buffer was used. For the initial growth of the cells to a monolayer 5 % calf foetal serum was added. Thereafter no serum was subsequently added to the medium.

Chorio allantoic membrane material with dilutions 10⁻¹–10⁻³ in Eagle's medium with antibiotics added was used to inoculate Vero cell monolayers. After an adsorption period of 30 min, the supernatant was poured off and the cell cultures flushed twice with medium before the final medium was added to the cells. The tubes were then placed in a roller drum and incubated at 37 °C. The cells were examined daily. Vero cell passages 2, 3 and 5 were inoculated into 4-week-old turkeys.

For electron microscopy Vero cells in 25 ml flasks were each infected with 1 ml of either a 10⁻² or 10⁻³ dilution of the virus.

Electron microscopy

Growth medium from the tracheal rings was centrifuged for 30 min at 50 000 g. The supernatant was discarded and the pellet was resuspended in a small amount of 0,002 M Tris-HCl buffer. Small droplets of 3 % phosphotungstic acid (pH 6) and the virus suspension were mixed on a wax surface and a carbon-coated formvar grid floated onto this mixture for 15 s. The grid was removed and excess fluid drained onto the edge of filter paper. Grids were examined in a Siemens Elmiskop 102 electron microscope. The diameter of particles and surface projections were measured with a Nikon profile projector.

Negative staining was performed on virus-infected Vero cell tissue cultures, showing at least 75 % affected cells. Cells were scraped off and centrifuged

at 1 000 g for 15 min. The supernatant was discarded and the cell pellet gently suspended in distilled water. This suspension was stained as above.

Cell cultures, intended for sectioning, and showing at least 50 % affected cells, were fixed by adding to the medium an equal volume (about 7 ml) of 3 % glutaraldehyde (GA) in Millonig's phosphate buffer, pH 7.2 at room temperature. After 5 min this mixture was poured off and replaced with 5 ml GA, and the cell layer was gently scraped off and centrifuged for 5 min at low speed. The pellets obtained were collected in capillary tubes and centrifuged in a haematocrit centrifuge. They were further processed by standard procedures for embedding in TAAB 812 resin. Thin sections were stained with uranyl acetate and lead citrate.

Haemagglutination test

Chicken, goose, guinea-pig and sheep red blood cells at a concentration of 0.5 % were made in Veronal buffer. Twofold dilutions (50 µl) of the virus was made in microtitre plates and 50 µl of the red blood cell suspension was added. The cells were then kept at 4 °C, 22 °C and 37 °C. Those at 4 °C were kept for 24 h, and the cells at 22 °C and 37 °C for 3 h and 1 h respectively before agglutination, if any, were recorded.

Challenge of susceptible turkeys

Turkeys raised from 1-day-old in FAPP isolators were used from the age of 2 weeks onwards. Poults were challenged with filtered sinus exudate, macerated chorio allantoic membrane, egg yolk material, organ culture supernatant and Vero cell-propagated material. The right sinus of each bird was inoculated with 0.1 ml of the appropriate material. Absence or presence of a nasal discharge was confirmed by pressure on the nostrils. Each group of birds were housed in FAPP cabinets.

Vaccine preparation

Embryonated SPF eggs were inoculated via the chorio allantoic membrane route with the 10th, 17th and 24th egg passage material. Eggs were candled daily and after 7 days' incubation all the remaining embryos were chilled in a refrigerator. The chorio allantoic membranes were collected, mixed with an equal amount of phosphate buffered saline pH 7.2, then homogenized and frozen in liquid N₂ in 10 ml volumes.

Different vaccine trials were then performed. In the first 3 trials the birds were housed in a controlled environmental house with gas brooders, tube feeders and fountain drinkers. The house was subdivided into 24 pens. Each group consisted of 6 pens of which half were stocked with males (180 each) and the other half with females (250 each) which were all randomly distributed throughout the house. The mass of the turkeys were determined at 3, 4, 6 and 8 weeks, mortalities were recorded daily whereas feed consumption was measured weekly.

Vaccine Trial 1

CAM#10 material was used as vaccine. Each group consisted of 1 290 day-old poults.

- Group 1.* Poults were vaccinated subcutaneously at day-old with 0.1 ml of the material.
- Group 2.* Poults were vaccinated at day-old via the cloaca by means of a drop of vaccine of about 0.05 ml.

Group 3 & 4. Unvaccinated control groups.

Vaccine Trial 2

CAM#17 material was used as vaccine. Each group consisted of 1 290 day-old poults.

Groups 1 & 3 were vaccinated at day-old via the cloaca by means of a drop of vaccine of about 0.05 ml.

Groups 2 & 4. Unvaccinated control groups.

At 14 days of age all 4 groups were treated with Linco-Spectin 100¹ (100 g/160 l of drinking water) for 5 days.

Exposure experiment for vaccine Trial 2

At 6 weeks of age 25 females of groups 1-4 and 10-day-old susceptible poults (Group 5) were challenged via the right infraorbital sinus with 0.1 ml of isolate 91/78 (TROC#20). Poults were checked daily for nasal discharge by means of pressure on the nostrils.

Vaccine Trial 3

CAM#24 material was used as vaccine. Each group consisted of 1 290 day-old poults. In each pen 10 % of the poults were inoculated at day-old via the cloaca by means of a drop of vaccine of about 0.05 ml. Groups 1 and 3 were treated with Linco-Spectin 100 (100 g/160 l of drinking water) for 5 days, starting at 15 days of age. Groups 2 and 4 were left unmedicated.

Exposure experiment for vaccine Trial 3

The five groups consisted of:

- (1) Fifty poults from Group 1,
- (2) Fifty poults from Group 2,
- (3) Thirteen susceptible 4-week-old poults,
- (4) Twenty-eight susceptible 4-week-old poults and
- (5) Thirty susceptible day-old poults.

Each group was marked and all the turkeys were kept on shavings in a large pen in a FAPP room. Only Groups 3 and 5 were challenged with 0.1 ml isolate 91/78 (TROC#13) into the right infraorbital sinus. Poults were daily checked for nasal discharge by means of pressure on the nostrils.

Postmortems were also done on all poults that died during the 14-day observation period.

Field Trials

CAM#17 was used to vaccinate 5 flocks of turkeys each consisting of 10 400 day-old poults. Again only 10 % of the flock were vaccinated at day-old via the cloaca by means of a drop of vaccine of about 0.05 ml. Each flock was treated at about 15 days of age with Linco-Spectin 100 (100 g/160 l drinking water) for 5 days. The mortalities in these 5 flocks were compared with 6 flocks (46 500 poults in total) that did not become infected with TRT and 6 flocks (67 620 poults in total) that naturally became infected with TRT. From 1 of the vaccinated flocks a number of poults were taken at 4 weeks of age to be challenged by contact exposure.

Exposure experiment for field trial

The 6 groups consisted of:

- (1) Nineteen susceptible 4-week-old unvaccinated turkeys,

¹ Upjohn (Pty) Ltd., P.O. Box 246, Isando 1600

- (2) Nine susceptible 4-week-old unvaccinated turkeys,
- (3) Ten susceptible day-old poults,
- (4) Twenty-five 8-week-old turkeys from a flock that recovered from a natural outbreak of TRT at 2 weeks of age,
- (5) Eight 3-week-old poults that had recovered from a challenge with 91/78 (TROC#20) at day old and
- (6) One hundred 4-week-old turkeys vaccinated at day-old with 91/78 CAM#17.

Each group was marked and all the turkeys were kept on shaving in a large pen in a FAPP room. Groups 2 and 3 were challenged with 0,1 ml of isolate 91/78 (TROC#20) into the right infraorbital sinus.

Convalescent serum

Turkeys, SPF and broiler chickens were raised in isolation from day-old up to 4 weeks of age. All the birds were then bled and infected with the isolate designated 91/78 that was in its 6th egg passage via the chorio allantoic membrane route. Symptoms were recorded and after 21 days all the turkeys and chickens were bled. After separation of the sera from the clot the sera were centrifuged and filtered through a 0,45 µm pore size filter into sterile vials, and frozen.

These sera were sent to Dr P. Wilding, British United Turkeys (B.U.T.), United Kingdom for the determination of antibodies against TRT by means of the ELISA test. End of cycle bleeds from broiler breeders (1979 onwards) and broilers (1981 onwards) were also analysed by B.U.T. by means of the ELISA test for antibodies against TRT.

RESULTS

Field cases

Fig. 1 a & b shows typical field cases of TRT with swollen infraorbital sinuses and blocked outer nares, causing the birds to breathe through their mouths.

Mycoplasma isolation

M. gallisepticum and *M. meleagridis* were isolated from the original sinus exudate material. Isolations done on sinus material after 3 passages through poults were negative for mycoplasmae.

Bacteriological isolation

Apart from *E. coli* no other specific pathogenic bacteria could be isolated.

Virus isolations

The sinus material collected from field outbreaks of TRT were consistently found to be contaminated with lentogenic Newcastle disease vaccine virus. After this material was passaged 3 times through susceptible turkeys it was found to be free from Newcastle disease virus. After 3 passages through SPF eggs via the different routes, no specific changes were observed in the embryonated eggs. The egg material harvested from the 3rd passages of the different routes was inoculated into susceptible turkeys without any symptoms developing.

Tracheal organ cultures

Tracheal organ cultures were observed daily for cilia movement. After 4 days very slight ciliostasis were found in small localized areas. This was followed by focal areas of complete stasis and after 6

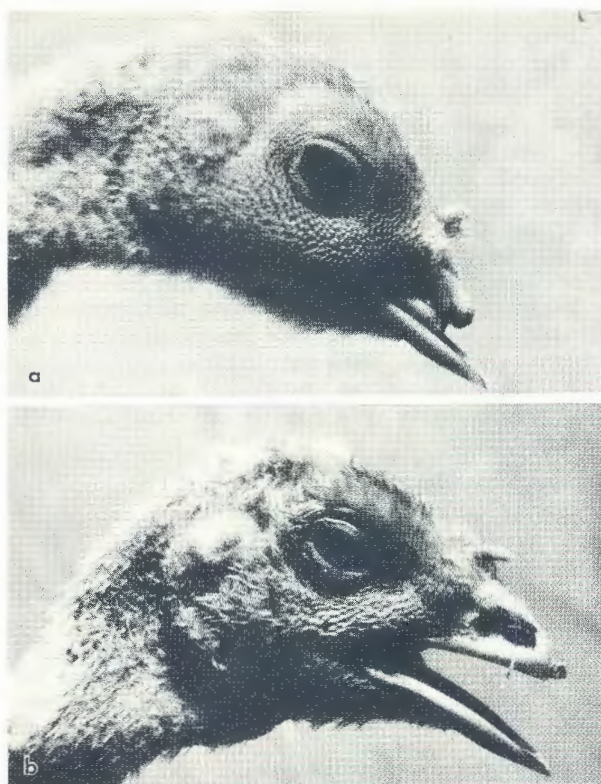


FIG. 1a A turkey poult with typical TRT symptoms. Notice the swelling of the infraorbital sinus below the eye

FIG. 1b Notice the nasal discharge blocking the nostrils

days, more than 75 % of the circumference of the tracheal ring showed ciliostasis.

This material was serially passaged in organ cultures up to the 26th passage. The results of the titrations of the virus in TROC are given in Table 1.

TABLE 1 The results of titrations in tracheal ring organ cultures of the virus causing TRT

Generation	TROC ID ₅₀ /ml
2	10 ^{5.0}
10	10 ^{6.1}
19	10 ^{6.4}
22	10 ^{6.3}

Material from the first up to the 26th passage was able to produce clinical symptoms in turkeys within 4-5 days.

Embryonated egg passages

Titrations in tracheal organ cultures of material harvested from the different passage routes in embryonated eggs yielded the following titres. (expressed as TROC ID₅₀/ml):

- (a) Chorio allantoic membrane 10^{3.5}
- (b) Yolk material 10^{3.0}
- (c) Allantoic fluid 10^{2.5}

All 3 samples also induced clinical symptoms in susceptible turkeys. Up to the 25th blind passage via the chorio allantoic route no changes could be observed in the embryonated eggs.

Growth on Vero cells

A 9th embryo passage virus isolate was inoculated onto Vero cells at dilutions ranging from 10⁰-10⁻⁶. On the 5th day disseminated focal areas of cells

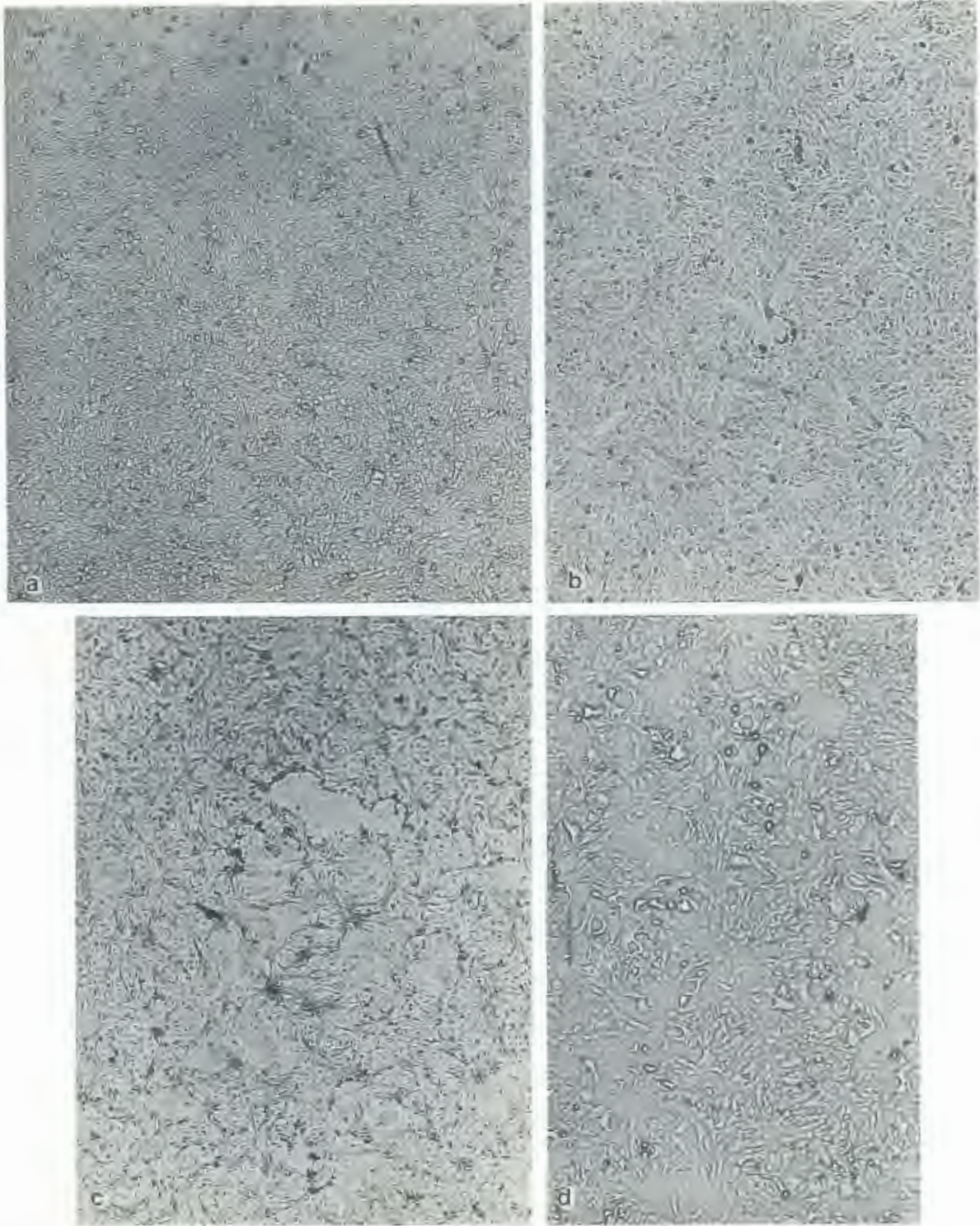


FIG. 2a Normal uninfected Vero cells

FIG. 2b Vero cells infected with isolate 91/78 showing areas of cell necrosis and syncytia formation

FIG. 2c Infected Vero cells showing large syncytia and areas with cell necrosis

FIG. 2d More advanced stage of cell necrosis with several small syncytia noticeable

rounding off were seen. These were followed by small syncytia forming on the 6th day, and on the 7th day large areas were transformed into syncytia. With further passages syncytia could already be found on the 3rd day (see Fig. 2 a, b, c & d).

Haemagglutination

The virus was not able to haemagglutinate chicken, goose, guinea-pig or sheep red blood cells in a buffer at pH 7.5.

Electron microscopy

(a) Negative staining

Material from the 3rd organ culture passage as well as viral suspensions of Vero cell cultures was used. Various virus-like particles were observed, having a pleomorphic shape and studded with regularly spaced fine surface projections, measuring about 14 nm in height. No clear symmetry could be observed. Although negatively stained particles

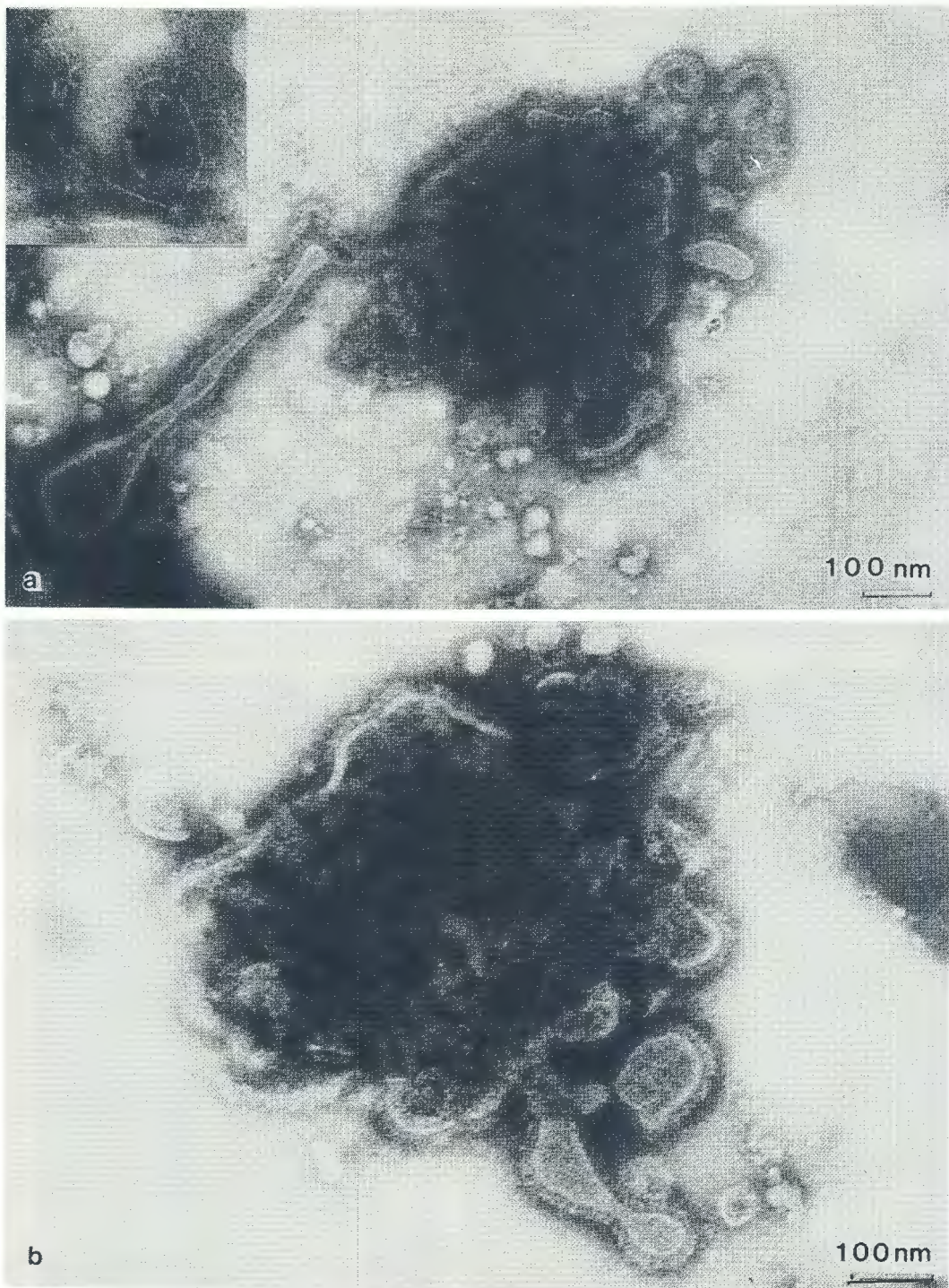


FIG. 3a & b Virus particles present in tracheal organ culture supernatant of cultures infected with the agent causing TRT. The particles are extremely pleomorphic forming bizarre shapes and filamentous forms. They clearly show the distinct fringe or surface projections. The inset shows a thin ribbon-like structure which does not resemble the typical nucleocapsid helix structures

seem to rupture easily, no internal nucleoprotein helixes (herring-bone structures), similar to that of Newcastle disease virus, were released from the particles or could be observed within mature virus particles. The virus-like particles varied considerable in size from 40 nm to 500 nm (Fig. 3a & b). In negatively stained preparations of Vero cell tissue cultures virus particles were observed having more quasi-spherical shapes (Fig. 4a) as well as forming complexes up to several micrometres in size. Although better definition of the virus particles with their fine

surface projections were obtained (compare Fig. 3 & 4a) they were still difficult to discern with negative staining.

(b) Sections of Vero cell tissue cultures

Various developing forms of the virus were observed in thin sections as accumulation of virus nucleoprotein (electron dense material) on the plasma membrane changing it to a more electron dense complex. It then matures and buds through the plasma membrane (Fig. 4).

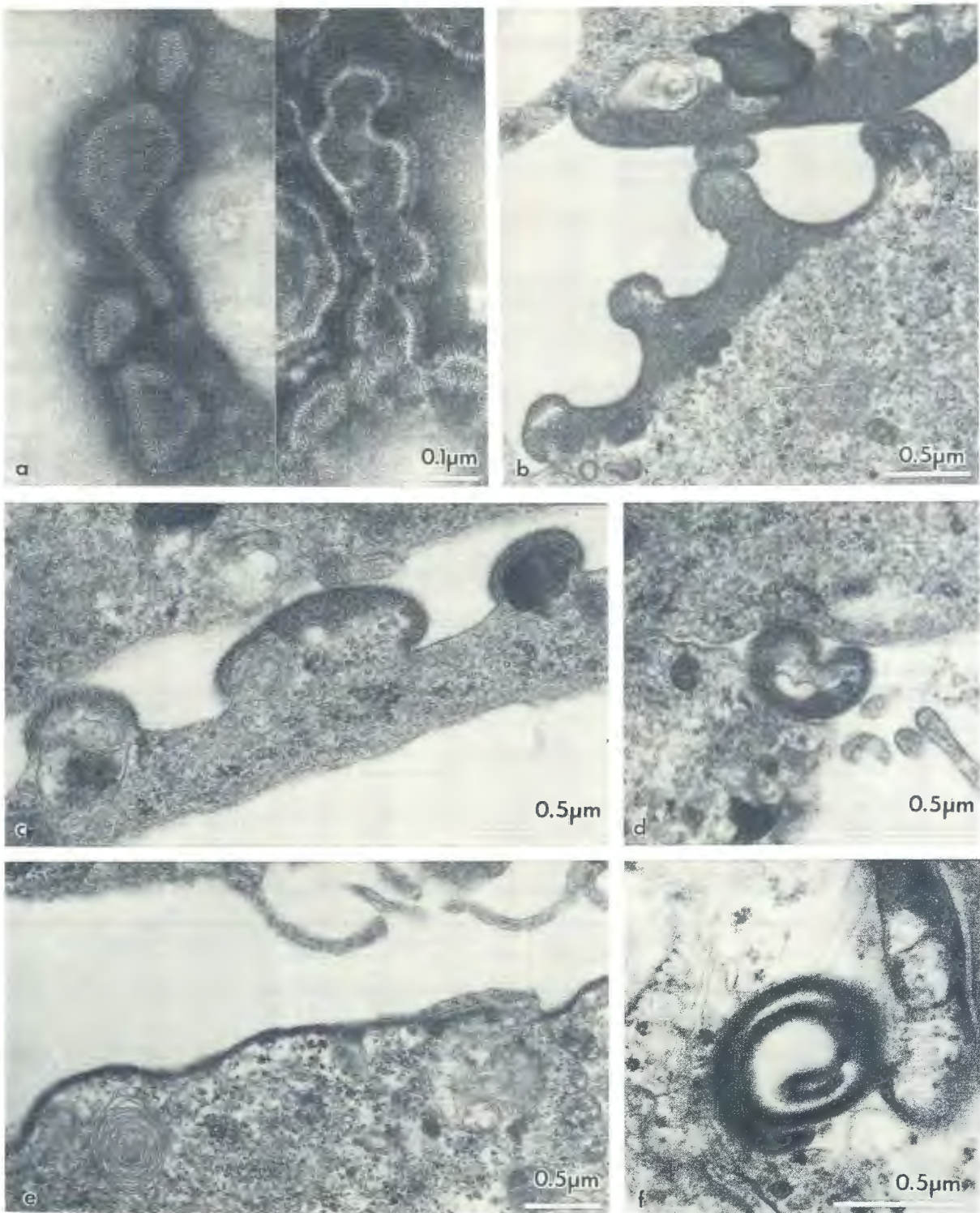


FIG. 4 Composite micrograph showing various forms of TRTV in Vero cell tissue cultures

(a) Negative staining

The distinct fringe is clearly visible on the filamentous and semi-spherical forms. Note $100 \text{ nm} = 0.1 \mu\text{m}$.

(b) Viral nucleoprotein accumulated on the cell membrane as well as 4 virions or a single pleomorphic virion in the process of budding from the plasma membrane

(c), (d), (e) & (f) Various stages of the budding process as well as the formation of filamentous forms along and outside a region of a cell surface. Note fine fringe on budding virions.

Results of chicks and poults challenged with sinus exudate and isolate 91/78

After 3 passages in 14-day-old poults the sinus material was diluted 1:1 with phosphate-buffered saline and filtered through a $0.22 \mu\text{m}$ pore size filter.

After being frozen in liquid N_2 , this material was

inoculated into 14-day-old susceptible poults, 14-day-old SPF White Leghorn chickens and 14-day-old broilers. After 4 days the poults started to show signs identical with those of field cases, as can be seen in Fig. 1. After 6 days 100% were effected. Up to 10 days post-inoculation no symptoms developed in the SPF or broiler chicks (Table 2).

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TABLE 2 The clinical results of 14-day-old poults, SPF and broiler chickens challenged with sinus exudate and tracheal organ culture material 91/78 (TROC#3)

Type of bird	No.	Inoculum	Number of birds with sinusitis post-challenge									
			Days									
			1	2	3	4	5	6	7	8	9	10
Turkeys	10	Sinus exudate	0	0	0	1	5	10	9	9	8	8
SPF Leghorn	10	Sinus exudate	0	0	0	0	0	0	0	0	0	0
Broilers	10	Sinus exudate	0	0	0	0	0	0	0	0	0	0
Turkeys	10	(TROC)	0	0	4	10	10	10	10	10	9	9
SPF Leghorn	10	(TROC)	0	0	0	0	0	0	0	0	0	0
Broilers	10	(TROC)	0	0	0	0	0	0	0	0	0	0

TABLE 3 The number of poults, SPF and broiler chickens challenged with isolate 91/78 propagated in embryonated eggs and on Vero cells, and displaying a nasal discharge. All birds were 4 weeks of age at challenge

Type of bird	No.	Inoculum	Number of birds with sinusitis post-challenge									
			Days									
			1	2	3	4	5	6	7	8	9	10
Turkeys	10*	CAM#6†	0	0	0	0	8	10	10	10	9	9
Turkeys	10	CAM#10	0	0	0	0	0	2	5	5	5	5
Turkeys	10	Yolksac#9	0	0	0	0	0	1	4	4	4	4
Turkeys	10	Vero#2	0	0	0	0	0	0	2	4	4	4
Turkeys	10	Vero#3	0	0	0	0	0	0	0	0	0	0
Turkeys	10	Vero#5	0	0	0	0	0	0	0	0	0	0
SPF chickens	10*	CAM#6†	0	0	0	0	0	0	0	0	0	0
Broilers	10	CAM#6	0	0	0	0	0	0	0	0	0	0

* The birds in these groups had a pre-bleed and a post-bleed 21 days post challenge

† These specific ampoules had been kept in liquid N₂ for 6 years before being used as inoculum

TABLE 4 Mortality, mass gain and feed intake at 4 weeks of age of poults vaccinated with 91/78 (CAM#10)

Group No.	Treatment	4 weeks		For the period 2-4 weeks	
		No./group	Mortality %	Mass gain (g)	Feed intake (g)
1	0,1 ml S/C	1 290	19,6	592,4 (ab)	538 (a)
2	0,05 ml cloaca	1 290	20,0	605,3 (a)	551 (a)
3	Contact control	1 290	34,6	573,8 (bc)	506 (b)
4	Contact control	1 290	25,5	565,5 (c)	513 (b)

TABLE 5 The number of poults with sinusitis post-challenge with 91/78 (TROC#20). Poults vaccinated 6 weeks prior with 91/78 (CAM#17)

Group	Treatment	No./group	Cumulative number with sinusitis post-challenge (days)													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	0,05 ml cloaca	25	0	0	0	5	6	8	10	10	10	10	10	10	10	10
2	Contact vaccinated	25	0	0	0	0	2	4	5	6	6	6	6	6	6	6
3	0,05 ml cloaca	25	0	0	0	0	3	4	5	5	5	5	5	5	5	5
4	Contact vaccinated	25	0	0	0	0	4	5	7	7	7	7	7	7	7	7
5	Unvaccinated	10	0	0	3	9	10	10	10	10	10	10	10	10	10	10
1-4		100	0	0	0	5	12	10	10	13	3	1	1	0	0	0
5		10	0	0	3	9	10	10	10	10	10	9	3	1	0	0

Total mortalities for each group after 6 weeks were:

Group 1 7,2 % Group 3 6,1 %
 Group 2 6,2 % Group 4 7,5 %

The 3rd organ culture passage was also inoculated in 14-day-old poults, SPF chickens and broilers. Again, typical TRT symptoms developed in the turkeys within 3 days, but no symptoms were observed in chickens (Table 2). The 5th, 10th, 15th, 20th and 26th organ culture passages induced typical TRT symptoms in 14-day-old turkey poults with no indication of a loss in pathogenicity.

Isolate 91/78 CAM#6 induced rhinitis and sinusitis in 100 % of the birds while CAM route #10 and yolk sac route #9 induced symptoms in only 50 % and 40 % respectively of the poults challenged.

Only Vero#2 could induce rhinitis in 40 % of the poults. At no stage could any symptoms be induced in SPF White Leghorns or broilers (Tables 2 & 3).

Results of vaccine trials*(a) Vaccine Trial 1*

The weekly mortalities, the mass gains and feed intake are tabulated in Table 4.

Respiratory symptoms were found 14 days post inoculation in Groups 1 and 2. Within a further 24 h symptoms could be found in the contact controls.

TABLE 6 The number of poult with sinusitis and septicaemia post-challenge with 91/78 (TROC#13). Poults vaccinated 4 weeks prior with 91/78 (CAM#24)

Group	Treatment	No./group	Cumulative number with sinusitis post-challenge (days)													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	0,05 ml cloaca (medicated)	50	0	0	0	0	0	0	20	44	46	48	49	49	49	49
2	0,05 ml cloaca (unmedicated)	50	0	0	0	0	0	6	33	47	50	50	50	50	50	50
3	Unvaccinated ¹	13	0	0	0	1	3	8	10	10	10	12	12	12	12	12
4	Unvaccinated ²	28	0	0	0	0	0	0	12	24	27	28	28	28	28	28
5	Unvaccinated ³	30	0	0	2	14	24	24	28	29	29	29	29	29	29	29
			Number with sinusitis post-challenge (days)													
1	0,05 ml cloaca (medicated)	50	0	0	0	0	0	0	20	43	43	43	42	33	22	4
2	0,05 ml cloaca (unmedicated)	50	0	0	0	0	0	6	33	47	50	44	43	33	13	1
3	Unvaccinated ¹	13	0	0	0	1	3	8	10	10	9	10	9	2	0	0
4	Unvaccinated ²	28	0	0	0	0	0	0	12	24	27	26	24	23	22	9
5	Unvaccinated ³	30	0	0	2	2	14	24	24	28	29	29	28	26	11	6
			Cumulative mortality due to septicaemia (days)													
1	0,05 ml cloaca (medicated)	50	0	0	0	0	0	0	0	0	2	2	2	2	2	2
2	0,05 ml cloaca (medicated)	10	0	0	0	0	0	0	1	1	1	2	2	3	4	4
3	Unvaccinated ¹	13	0	0	0	0	0	0	0	0	0	0	1	1	1	1
4	Unvaccinated ²	28	0	0	0	0	0	0	0	0	1	3	5	6	7	7
5	Unvaccinated ³	30	0	0	0	0	0	0	0	0	0	1	2	8	11	13

¹ Unvaccinated 4-week-old challenged I/S 91/78 (TROC#13)² Unvaccinated 4-week-old³ Unvaccinated day-old challenged I/S 91/78 (TROC#13)

The respiratory symptoms (sinusitis, sneezing and wet rales) were markedly more pronounced in the contact controls, and lasted longer. At 28 days post-inoculation very few birds with symptoms could be found. Most of the poults that died showed marked coli-septicaemia. The mortality of Groups 1 and 2 were better than those of Groups 3 and 4. The mass gain and feed intake of Group 2 differed highly significantly from groups 3 and 4 (Table 4).

*(b) Vaccine Trial 2**Clinical symptoms after vaccination with CAM#17*

Symptoms after 14 days appeared in Group 1 and were followed the following day by symptoms in Groups 2, 3 and 4. Medication of all groups with Linco-Spectin started on Day 14. Respiratory symptoms reached a peak at Days 16 and 17. From Day 22 onwards very few poults with respiratory symptoms could be found.

The number of birds showing clinical signs after challenge with a virulent isolate of 91/78 is given in Table 5.

Protection at 6 weeks of age as measured by means of absence of clinical signs after challenge (Table 5)

- | | |
|-------------------------------------|------|
| 1. Vaccinated via cloaca | 70 % |
| 2. Vaccinated via horizontal spread | 74 % |
| 3. Unvaccinated controls | 0 % |

On the 8th day post-challenge only 13 % of the vaccinated poults exhibited clinical signs compared with the 100 % of the unvaccinated poults.

*(c) Vaccine Trial 3**Clinical observations after vaccination*

Clinical signs of sneezing and rales started on Day 13. Approximately 5 % of the birds developed sinus exudate compared with approximately 80 % with CAM#17. After the 22nd day no clinical signs could be seen or heard. The number of birds showing clinical signs after challenge with a virulent isolate of 91/78 is shown in Table 6.

Clinical signs after challenge

It is evident from Table 6 that only 2 % of the vaccinated and none of the unvaccinated poults were protected against sinusitis at 4-weeks of age. Of the vaccinated poults, 88 % were protected against mortality due to septicaemia compared with 74 % in unvaccinated poults. Nasal discharge, sinusitis, sneezing and rales were marginally less in vaccinated poults than in unvaccinated. Recovery also was marginally quicker in the vaccinated poults. As far as feed intake, mass gain and mortalities are concerned, no significant difference could be found between medicated and non-medicated poults after vaccination with 91/78 (CAM#24), an indication that the vaccine did not have marked side-effects on the poults (see Table 7).

TABLE 7 Feed intake, mass gain and mortality at 4 weeks of age of the group treated with antibiotics compared with those of the untreated group. Both groups were vaccinated at day-old with 91/78 (CAM#24)

Group No.	Treatment	Feed intake (g)	Mass gain (g)	Mortality %
1	Linco-Spectin	864	671	4.7
2	Unmedicated	856	655	4.8

*Field trial**Clinical symptoms after vaccination of the 5 flocks with 91/78 (CAM#17)*

In all the flocks a water medication with Linco-Spectin in the drinking water was given for 5 days from Day 13 post vaccination (PV). Symptoms of sneezing, rales, lacrimation and nasal discharge started at the 11th day. The males consistently developed symptoms 2 days before the females. Within 3 days after the first symptoms (Day 14 PV) approximately 80 % or more of the poults would have developed the typical TRT symptoms. On Day 16 PV poults started to recover and on Day 20 PV very few affected poults could be found. Five flocks with a

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TABLE 8 The number of poult with sinusitis and septicaemia post-challenge with 91/78 (TROC#20). Some of the poult were vaccinated 4 weeks prior with 91/78 (CAM#17) as part of a field trial

Group	Treatment	No./group	Cumulative number with sinusitis post-challenge (days)													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	Unvaccinated	19	0	0	0	0	0	1	9	15	19	19	19	19	19	
2	Unvaccinated	9	0	0	0	1	6	6	7	9	9	9	9	9		
3	Unvaccinated	10	0	0	0	9	10	10	10	10	10	10	10	10		
4	Natural outbreak	25	0	0	0	0	0	0	0	0	0	0	0	0		
5	TROC#20	8	0	0	0	0	0	0	1	2	2	2	2	2		
6	Vaccinated	100	0	0	0	0	1	5	6	8	17	24	33	35		
			Number with sinusitis post-challenge (days)													
1	Unvaccinated	19	0	0	0	0	0	1	8	14	18	17	15	14	10	4
2	Unvaccinated	9	0	0	0	1	6	6	7	9	5	4	4	1	0	
3	Unvaccinated	10	0	0	0	9	10	10	10	10	7	6	4	1	1	
4	Natural outbreak	25	0	0	0	0	0	0	0	0	0	0	0	0	0	
5	TROC#20	8	0	0	0	0	0	0	1	2	2	2	2	2	2	
6	Vaccinated	100	0	0	0	0	1	4	4	6	15	21	19	21	15	9
			Cumulative mortality due to septicaemia (days)													
1	Unvaccinated	19	0	0	0	0	0	0	1	1	1	2	4	5	7	8
2	Unvaccinated	9	0	0	0	0	0	0	0	0	3	3	4	4	4	5
3	Unvaccinated	10	0	0	0	0	0	0	0	0	2	2	2	2	2	3
4	Natural outbreak	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	TROC#20	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	Vaccinated	100	0	0	0	0	0	0	0	0	0	0	1	1	2	2

1. Susceptible unvaccinated
2. Susceptible unvaccinated, challenged I/S 91/78 (TROC#20)
3. Susceptible unvaccinated day-olds, challenged I/S 91/78 (TROC#20)
4. Poults 8-weeks-old. Recovered after a natural outbreak at 2 weeks of age
5. Poults 3-weeks-old, that have recovered after being challenged with 91/78 (TROC#20) at day old

total of 52 000 poult were vaccinated by administering 91/78 (CAM#17) to 10 % of the poult via the vent route. From one of the vaccinated flocks 100 poult were taken at 4 weeks of age and challenged together with controls (see Table 8).

Poult that recovered from a natural outbreak were 100 % protected against the development of sinusitis and mortality after challenge. Of the contact-vaccinated poult 65 % were protected against the development of sinusitis and 98 % against mortality due to septicaemia after challenge. Unvaccinated poult were 100 % susceptible to the development of sinusitis and 42 % died from septicaemia.

TABLE 9 Mortality in commercial poult up to 8 weeks of age

Group No.	Number	History	Mortality %
1	46 500	Non-infected poult	6,4
2	67 620	Naturally infected poult	29,7
3	52 000	Contact vaccinated with 91/78 (CAM#17)	10,0

TABLE 10 The distribution of antibodies against TRT in commercial broiler breeder and broiler flocks

Origin	Average titre	Range
1		
Breeder flock 1979	8	4-10
Breeder flock 1981	10	9-11
Breeder flock 1984	5	1-8
Breeder flock 1985	6	0-10
Breeder flock 1986	4	1-5
Breeder flock 1987	8	0-12
2		
Broiler flock 1981	2,6	0-9
Broiler flock 1982	6,8	0-9
Broiler flock 1983	1,9	0-9
Broiler flock 1987	1,6	0-8

1 + 2. Birds bled at end of production cycles

Of the birds that had recovered from a challenge with 91/78 (TROC#20), 25 % showed mild clinical signs, but none died from septicaemia. Mortalities in the vaccinated flocks were 3,6 % higher than in uninfected flocks but were 19,7 % lower than in naturally infected susceptible flocks (see Table 9).

Serological results

Dr P. Wilding tested sera from commercial broiler breeders, broilers and laboratory infected poult and chickens by means of the ELISA test for the presence of antibodies against TRT (Baxter-Jones, Cook, Frazier, Grant, Jones, Mockett & Wilding, 1987) (see Tables 10 & 11).

TABLE 11 Serological results of experimentally infected poult and SPF chickens tested by means of the ELISA test for the presence of antibodies against TRT

Species	No.	Average titre log base 2		
		Pre-bleed	Post-bleed	Range
Turkey	7 ¹	0	8	4-11
Chicken	3 ¹	0	12	11-13

¹ Clinical symptoms of these groups recorded in Table 3

DISCUSSION

Setting up tracheal organ cultures proved to be the best procedure for the isolation of the virus causing TRT. These cultures not only supported multiplication of the virus but also exhibited ciliostasis, which is a sensitive indicator of viral growth. In these cultures there was only a 1 log increase in ID₅₀ titre from generation 2-10 and the titre subsequently stabilised on about 10^{6.4} TROC ID₅₀/m^l. Titres for embryonated egg virus were significantly lower at 10^{3.5} ID₅₀/m^l of which the CAM route gave the highest titre. Virus, propagated via the CAM route and kept

in liquid N₂ since 1981, was still able 6 years later to evoke TRT symptoms in 10 out of 10 turkeys when challenged with this material.

In our first report on this virus (Buys & Du Preez, 1980) it was thought to belong to the families Orthomyxo or Paramyxoviridae. McDougall & Cook (1986) suggested an orthomyxovirus and even considered a coronavirus for this agent. The electron microscopic description by Wyeth *et al.* (1986) of their strain CVL14/1 closely resembles the description of our strain 91/78. They suggested that their strain might be a pneumovirus which belongs to the family Paramyxoviridae. Likewise, Collins & Gough (1988) came to the same conclusion that the agent associated with TRT is a member of the *Pneumovirus* genus.

The *Pneumovirus* genus of the Paramyxoviridae family includes the important member respiratory syncytial virus (RSV), the type species. The virus causing turkey rhinotracheitis shares an important property with RSV in that it characteristically produces large syncytial masses in tissue culture. Like RSV, the turkey virus has a number of properties that separate it from Paramyxovirus and Morbillivirus, the other 2 genera of the Paramyxoviridae family. It apparently possesses longer surface projections of about 14 nm in length, which are regularly spaced and club-shaped in appearance but lack haemagglutinin, neuraminidase or hemolytic activity (McDougall & Cook, 1986; Wyeth *et al.*, 1986).

In *Pneumovirus*, the nucleocapsid is apparently narrower and more delicate, measuring 12–15 nm in diameter. Collins & Gough (1988) demonstrated a nucleocapsid strand with negative staining which appears less distinct and of a slightly different structure from that of a *Paramyxovirus*. Electron microscopy of the virus grown in TROC and Vero cells in our laboratory has not revealed any discernible internal component with the herring bone pattern which is characteristic of this family. Only a ribbon-like structure could be observed, similar to the structures described by Collins, Gough, Lister, Chettle & Eddy (1986) (see insert, Fig. 3).

In negative stained preparations from primary tracheal organ cultures, highly pleomorphic virions with bizarre shapes, including spherical and filamentous forms (Fig. 3) similar to RSV were seen. The elongated filamentous forms observed can have a total length of several microns and are in agreement with Wyeth *et al.* (1986) and Collins *et al.* (1986). These filamentous forms of the turkey virus observed may be thought of as an artefact due to negative staining or may be derived from host material (Collins *et al.*, 1986), but our sections of Vero cells infected with 91/78 clearly demonstrate that assembly and budding of filamentous forms of these virions are indeed part of the morphogenesis cycle of these virions (Fig. 4). As is shown in Fig. 4, it appears that the cellular membrane is incorporated into the virion from which budding occurs, and appears as a dense outer layer. These limited observations on the structure and morphogenesis of the virus causing TRT are in agreement with the observations of Compans, Harter & Choppin (1967) on pneumonia virus of mice. The appearance, size and shape of the virus-like particles found with negative contrast preparations and thin sections as seen in the electron microscope, together with the fact that the virus do not haemagglutinate red blood cells, would indicate a virus in the Paramyxoviridae family of viruses, possibly the genus *Pneumovirus*.

Although no indications were found of attenuation in the serial passages in TROC, attenuation could be found in the serial passages in embryonated eggs. It was therefore decided to undertake semi-large scale trials in an isolated test house where feeding trials are normally undertaken. In the experiment where 91/78 CAM#10 was used the severity of symptoms in the 2 contact groups was very similar to that found with the field strain. The severity of symptoms in the infected groups was marginally less, the mortalities of the infected groups were at least 5 % less than those of the contact groups and there were significant differences in the mass gains and feed intake of the infected groups (see Table 4). The fact that symptoms appeared in the contact groups only 24 h after the onset of symptoms in infected groups would indicate a very fast multiplication of the virus in the host with consequent rapid horizontal spread to adjoining pens. CAM#17 gave significantly milder symptoms than CAM#10 and by Day 22 fewer birds with symptoms could be found than on Day 28 with CAM#10. Mortalities at 6 weeks of age varied between 6.1 % and 7.5 % for all groups. The protection against a challenge with virulent isolate 91/78 was on an average 70 % compared to none in susceptible turkeys (see Table 5). Although very mild symptoms were evoked by CAM#24, it did not afford any significant protection against the development of rhinitis and sinusitis when turkeys were challenged. Since there was such a rapid horizontal spread of the virus, it was decided to do a field vaccination trial with CAM#17, infecting only 10 % of the turkeys. It was estimated that together with a Linco-Spectin treatment, administered as soon as the clinical symptoms started, an average of about 3 % mortality could be attributed to a vaccine reaction with CAM#17. Although only 65 % of the birds were protected against the development of clinical symptoms, nearly 100 % were protected against losses caused by septicaemia (Table 8). Turkeys that recovered from a natural outbreak were refractile to re-infection 6 weeks later. Vaccinations with CAM#17 gave acceptable results in the field if the 10 % mortalities are compared with the nearly 30 % in natural cases (Table 9).

When our strain 91/78 was passaged in Vero cells, we found that it lost its pathogenicity between the 2nd and 3rd passages. McDougall (personal communication, 1986) found the same result, and in addition that poults inoculated with the 3rd Vero Cell passage resisted subsequent challenge with the virulent virus causing TRT, and suggested this as a possible venue to pursue in the preparation of a vaccine. Since the only major turkey producer ceased production of turkeys in 1983, no further refinement was done on our chicken embryo origin vaccine.

Hatching eggs and day-old turkeys were received from a commercial hatchery on a weekly basis for 24 months. The turkeys were raised in a FAPP room to serve as susceptible poults for various experiments. At no time did any of these poults, originating from naturally infected recovered parents, ever show any clinical signs to indicate egg transmission. Neither could any poults from day-old up to 14-day-old be found that were refractory to challenge. This could mean that either no maternal antibodies are transmitted from recovered parents or that maternal antibodies are not able to prevent the development of clinical symptoms. Judging from the successful isolation of the virus by means of TROC maternal

antibodies do not prevent the growth of the virus in tracheal explants.

One of the reasons for our renewed interest in the virus was the possibility that the virus causing TRT might play a role in the respiratory disease complex of broilers. In the course of our work, SPF chickens and broilers were challenged on 4 separate occasions with infective sinus exudate, TROC material and embryonated egg origin material without any clinical symptoms ever developing. Serologically, it could be shown, however, that SPF chickens did respond to a challenge with 91/78 (Table 11). Sera collected from broiler breeders in 1979 and broilers in 1981 already had antibodies against TRT (see Table 10). Unfortunately, we did not have any sera dating back any further. These results still do not exclude the possibility that this virus in combination with other respiratory viruses could play a role in the broiler respiratory disease complex, especially in view of the findings by Jones *et al.* (1987), who were able to produce mild clinical signs with their strain in SPF chickens.

Further work on the classification of our strain 91/78 is now in progress in our laboratory.

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REFERENCES

- ANON., 1985. Turkeyrhinotracheitis of unknown aetiology in England and Wales. *Veterinary Record*, 117, 653-654.
- BAXTER-JONES, C., COOK, J. K. A., FRAZIER, J. A., GRANT, M., JONES, R. C., MOCKETT, A. P. A. & WILDING, G. P., 1987. Close relationship between TRT virus isolates. *Veterinary Record*, 120, p. 562.
- BLALOCK, H. G., SIMMONS, D. G., MUSE, K. E., GRAY, G. G. & DERIEUX, W. T., 1975. Adenovirus respiratory infection in turkey poults. *Avian Diseases*, 19, 707-716.
- BUYS, S. B., 1976. The isolation of *Mycoplasma synoviae* from chickens with infectious synovitis and air-sacculitis in the Republic of South Africa. *Onderstepoort Journal of Veterinary Research*, 43, 39-42.
- BUYS, S. B. & 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*, June, pp. 36 & 46.
- CHALQUEST, R. R., 1962. Cultivation of the infectious synovitis type pleuropneumonia-like organisms. *Avian Diseases*, 6, 36-43.
- CHERRY, J. D. & TAYLOR-ROBINSON, D., 1970. Large quantity production of chicken embryo tracheal organ cultures and use in virus and mycoplasma studies. *Applied Microbiology*, 1970, 658-662.
- COLLINS, M. S. & 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. R. & EDDY, R., 1986. Further characterisation of a virus associated with turkey rhinotracheitis. *Veterinary Record*, 119, p. 606.
- COMPANS, R. W., HARTER, D. H. & CHOPPIN, P. W., 1967. Studies of pneumonia virus of mice in cell culture. II. Structure and morphogenesis of the virus particle. *Journal of Experimental Medicine*, 126, 267-285.
- FREY, M. L., HANSON, R. P. & ANDERSON, D. P., 1968. A medium for the isolation of avian mycoplasmas. *American Journal of Veterinary Research*, 29, 2163-2171.
- JONES, R. C., BAXTER-JONES, C., SAVAGE, C. E., KELLY, D. F. & WILDING, G. P., 1987. Experimental infection of chickens with a ciliostatic agent isolated from turkeys with rhinotracheitis. *Veterinary Record*, 120, 30-302.
- LIPKIND, M. A., WEISMAN, Y., SHIKMAN, E., SHOHAM, D. & ARONOVICI, A., 1979. The isolation of a yucaipa-like paramyxovirus from epizootics of a respiratory disease in turkey poultry farms in Israel. *Veterinary Record*, 105, 577-578.
- MCDUGALL, J. S. & COOK, J. K. A., 1986. Turkey rhinotracheitis: preliminary investigations. *Veterinary Record*, 118, 206-207.
- REED, L. J. & MUENCH, H., 1938. A simple method for estimating fifty percent endpoints. *American Journal of Hygiene*, 27, 493-497.
- SIMMONS, D. G., MILLER, S. E., GRAY, J. G., BLALOCK, H. G. & COLWELL, W. M., 1976. Isolation and identification of a turkey respiratory adenovirus. *Avian Diseases*, 20, 65-74.
- SIMMONS, D. G., GRAY, J. G., ROSE, L. P., DILLMAN, R. C. & MILLER, S. E., 1979. Isolation of an etiologic agent of acute respiratory disease (rhinotracheitis) of turkey poults. *Avian Diseases*, 23, 194-203.
- WILDING, G. P., BAXTER-JONES, C. & GRANT, M., 1986. Ciliostatic agent found in rhinotracheitis. *Veterinary Record*, 118, p. 735.
- WYETH, P. J., GOUGH, R. E., CHETTLE, N. & EDDY, R., 1986. Preliminary observations on a virus associated with turkey rhinotracheitis. *Veterinary Record*, 119, p. 139.