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**Characterization of a Virus Associated with Turkey Rhinotracheitis**

**By M. S. Collins* and R. E. Gough**

Poultry Department, Central Veterinary Laboratory, New Haw, Weybridge, Surrey KT15 3NB, U.K.

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**SUMMARY**

A virus associated with turkey rhinotracheitis was purified and its morphology and structural polypeptides were compared with those of the bovine, human and murine members of the genus *Pneumovirus*. The isolate possessed surface projections 13 to 14 nm in length and a helical nucleocapsid 14 nm in diameter with a pitch of 7 nm. Approximately seven presumed viral polypeptides were observed. Their apparent molecular weights were 200 x 10^3 (200K), 84K, 54K, 42K, 37K, 31K and 14K; two of these, the 84K and 54K polypeptides, were glycosylated. The virus was shown to possess many features that were similar to established pneumoviruses and can therefore be regarded as a possible member of this genus.

An acute upper respiratory tract disease of turkeys termed turkey rhinotracheitis (TRT) was first reported in Great Britain during 1985 (Anon., 1985; Alexander et al., 1986). A similar disease has been reported in many other countries during the past 20 years and several aetiological agents have been implicated (Lister & Alexander, 1986). Investigations in this country have resulted in the isolation of a virus with the morphological and biological properties of a pneumovirus (Wyeth et al., 1986). Similar myxovirus-like agents have been isolated from TRT-infected birds in other laboratories using avian tracheal organ cultures, in Britain (McDougall & Cook, 1986; Jones et al., 1986), France (Giraud et al., 1986) and South Africa (Buys & du Preez, 1980).

The generic name *Pneumovirus* has been adopted to include the respiratory syncytial virus (RSV) of humans and bovids and the pneumonia virus (PVM) of mice (Andrewes et al., 1978). These viruses have nucleocapsid dimensions of about 14 nm diameter with a helical pitch of 6-5 nm which distinguishes them from other paramyxoviruses (Joncas et al., 1969; Bäch & Howe, 1973; Compans et al., 1967).

Polyacrylamide gel electrophoresis, in the presence of SDS, of radiolabelled RSV has demonstrated between seven and ten presumed viral polypeptides (Bernstein & Hruska, 1981; Dubovi, 1982). At least six virus-specific proteins ranging from 25K to 48K have been described for the human and bovine viruses which had very similar polypeptide profiles (Wunner & Pringle, 1976; Cash et al., 1977). Three polypeptides of PVM (43K, 40K and 28K) had electrophoretic mobilities which corresponded to the viral polypeptides of the human RSV, but the apparent absence of glycopolypeptides equivalent to VP48 and VP32 suggested that PVM was quite distinct from RSV (Cash et al., 1977). Two envelope glycopolypeptides (90K and 70K) of RSV have been identified (Peeples & Levine, 1979). The 70K polypeptide was composed of two polypeptides, 50K and 20K, linked by disulphide bonds (Fernie & Gerin, 1982) and was considered to be the protein involved in cell fusion since one monoclonal antibody binding to it inhibited syncytial formation (Walsh & Hruska, 1983). On the basis of its location and Mr, it has been suggested that the 90K glycopolypeptide is analogous to the haemagglutinin of other paramyxoviruses (Fernie & Gerin, 1982).

Using SDS-PAGE, one of the TRT isolates (CVL 14/1) was characterized by comparing its polypeptide profile with those of other viruses of known taxonomy. These included avian...
Table 1. Dimensions and buoyant densities of pneumoviruses and the virus associated with turkey rhinotracheitis

<table>
<thead>
<tr>
<th>RNP</th>
<th>Diameter (nm)</th>
<th>Pitch (nm)</th>
<th>Pleomorphic*</th>
<th>Length of projections (nm)</th>
<th>Particle size (spherical) (nm)</th>
<th>Buoyant density (g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRT agent</td>
<td>14</td>
<td>7-0</td>
<td>+</td>
<td>13-14</td>
<td>(80-200)</td>
<td>1-21</td>
</tr>
<tr>
<td>Human RSV</td>
<td>14 (13-5)‡</td>
<td>7-0 (6-5)‡</td>
<td>+</td>
<td>15 (15)‡</td>
<td>(100-350)‡</td>
<td>(1-16-1-26)§</td>
</tr>
<tr>
<td>Bovine RSV</td>
<td>14 (14)‖</td>
<td>6-5 (6-5)‖</td>
<td>+</td>
<td>14-15 (12)‖</td>
<td>(80-400)‖</td>
<td>(1-16-1-26)§</td>
</tr>
<tr>
<td>PVM</td>
<td>13-14</td>
<td>-</td>
<td>+</td>
<td>12 (8-9)¶</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDV Ulster</td>
<td>18</td>
<td>5-5</td>
<td>+</td>
<td>12</td>
<td>100-400</td>
<td>1-20</td>
</tr>
</tbody>
</table>

* +, Very pleomorphic.
† Wyeth et al. (1986).
‡ Joncas et al. (1969).
§ Stott & Taylor (1985).
‖ Andrewes et al. (1978).
¶ Compans et al. (1967).

Paramyxoviruses types 1 and 3, two avian coronavirus, an avian orthomyxovirus and bovine RSV, a member of the genus *Pneumovirus*. The results of that study were not conclusive but indicated similarities between the TRT virus and bovine RSV rather than the other viruses (Collins et al., 1986).

In this report we present the results of characterization studies using electron microscopy and SDS-PAGE to compare the morphology and polypeptides of a TRT virus and representatives of the *Pneumovirus* genus, including PVM, a bovine and a human RSV.

TRT isolate CVL 14/1 (Wyeth et al., 1986) was passaged three times at limiting dilution in chicken embryo fibroblast cultures. Cell cultures were prepared by conventional methods using embryos from a specific pathogen-free flock. When the cells in roller bottles were confluent, they were infected with virus at an approximate m.o.i. of 0-01 to 0-1 TCID$_{50}$/cell. The infected cultures were maintained at 37 °C until extensive c.p.e. was visible (approximately 80 h) at which time they were subjected to one freezing and thawing cycle.

Mouse pneumovirus strain PVM/6a was obtained from Dr A. J. Easton (Department of Biological Sciences, University of Warwick, Coventry, U.K.). Confluent monolayers of BHK-21 cells were infected at high m.o.i. in roller bottles containing the Glasgow modification of Eagle's medium supplemented with 5% foetal calf serum. The cultures were frozen and thawed 6 to 7 days after infection when maximum c.p.e. was observed.

Isolates of bovine RSV (strain 127) and human RSV (strain A2) were obtained from Dr E. J. Stott (AFRC Institute for Research on Animal Diseases, Compton, U.K.) and cultivated in primary calf kidney cells by conventional methods. When widespread c.p.e. was apparent, cultures were again subjected to one freeze–thaw cycle before concentration and purification of the virus.

The Ulster 2C strain of Newcastle disease virus (NDV) held at the Central Veterinary Laboratory was inoculated into 9-day-old embryonated chicken eggs. About 10$^5$ 50% egg infectious doses in 0-1 ml was inoculated into the allantoic cavity. After 5 to 6 days the virus was harvested and purified. Viral purification was performed by centrifugation at 3000 g for 10 min to remove cell debris. Each supernatant was then centrifuged at 30000 g for 60 min and the resulting pellet suspended in a small volume of deionized water. The viral concentrate was placed on a 30 ml step gradient comprising 30% (w/w) sucrose (25 ml) and 55% (w/w) sucrose (5 ml). After centrifugation at 53000 g for 1 h the band at the interface was diluted, pelleted and suspended in 0-01 M-Tris–HCl buffer pH 7-0 for SDS–PAGE and electron microscopy.

Isopycnic densities were calculated by further centrifugation of concentrated virus on 30 ml continuous 30 to 55% (w/w) sucrose gradients for 18 h at 83000 g. Fractions were collected for refractive index measurements and estimations of viral densities. The peak buoyant density of 1-21 g/ml compares favourably with the buoyant density measurements of pneumoviruses in sucrose gradients (Table 1).
Viral preparations were applied to a carbon-coated Formvar grid, stained using 3% sodium silicontungstate pH 9.0 and examined using a Philips EM 300 microscope. Examination of the TRT agent, which had been purified by a single centrifugation in a sucrose gradient, revealed a preparation apparently free of cellular debris containing many intact and extremely pleomorphic particles with a large number of projections. Spherical virions were generally 80 to 200 nm in diameter but occasionally very large (Fig. 1a). Filamentous forms were also seen, frequently 1 to 2 μm or more in length (Fig. 1c). The TRT virions possessed closely spaced projections 13 to 14 nm in length; the helical nucleocapsids were 14 nm in diameter with an estimated pitch of 7 nm (Fig. 1d). These measurements are in close agreement with those obtained for pneumoviruses (Andrewes et al., 1978; Compans et al., 1967; Joncas et al., 1969).

Paramyxoviruses in the genera Paramyxovirus and Morbillivirus, however, have helical nucleocapsids of larger diameter usually about 18 nm and with a smaller pitch (Table 1).

Variations in the morphology of the TRT virus and established pneumoviruses were also observed. The generally more rigid nucleocapsid structure of the bovine and human RSVs (Fig. 1e) was not apparent with the TRT agent whose helical component appeared less distinct and somewhat stretched and extended (Fig. 1b, d), resembling more that found in PVM (Fig. 1f) (Compans et al., 1967). Estimations of the pitch were therefore made over only five turns of the helix, since longer sections of non-deformed nucleocapsid were not readily observed, and can only be regarded as approximate. In contrast the well defined surface projections of the TRT agent resembled those found on the human and bovine RSVs (Fig. 1g) rather than the shorter and less distinct fringe of PVM (Fig. 1h).

Electrophoretic analysis of viral polypeptides was performed using the procedure of Laemmli (1970). Slab gels composed of 13% acrylamide and 0.35% N,N'-methylenebisacrylamide with a short stacking gel of 3% acrylamide and 0.08% N,N'-methylenebisacrylamide were prepared. Viral preparations were treated with 2% SDS, 2% dithiothreitol and 5% sucrose (final concentrations) before heating for 2 min in a boiling water-bath. Bromophenol blue was then added (0.001%) and 25 μl of each preparation containing approximately 100 μg of protein applied to the gel. Electrophoresis was performed using a constant current of 40 mA. Standards of known molecular weights were also subjected to electrophoresis; these included the NDV strain Ulster (haemagglutinin 76K, ribonucleoprotein (RNP) 55K and matrix protein 40K), phosphorylase a (rabbit muscle, 94K), bovine serum albumin (68K) and IgG (heavy chain 55K; light chain 25K). The band corresponding to the light chain of IgG was just visible in the stained gel but easily detected by scanning using a 626 nm filter. Polypeptides were detected by staining gels with 0.2% Coomassie Brilliant Blue R (CBB) and glycopolypeptides using Schiff's reagent as described by Alexander & Collins (1977).

Seven polypeptides of apparent Mr 54K, 47K, 42K, 37K, 36K, 31K and 14K were clearly discernible when the TRT agent was analysed by SDS-PAGE and stained with CBB (Fig. 2). These accounted for > 70% of the total protein present determined by scanning and integration of the polypeptide profile using a Joyce Loeb Chromoscan 3. The two polypeptides of 42K and 31K were by far the most prominent, representing respectively 26.6% and 18.08% of the total protein. Five faint bands were also observed representing one polypeptide of Mr 200K and four of 10K or less. The low Mr polypeptides may be host cell histones.

The 42K, 37K, 31K and 14K polypeptides may correspond to the structural polypeptides designated 38K, 35K, 30K and 15K in the accompanying communication by Ling & Pringle (1988), who were able to confirm the virus specificity of these polypeptides by immunoprecipitation using specific antisera and by demonstrating their synthesis in vivo and in vitro. Two polypeptides (Mr 47K and 36K) migrated at the same rate as proteins present in the NDV preparation and are probably non-viral, the former apparently corresponding to the polypeptide designated actin in NDV (Wang et al., 1976).

Fig. 2 shows the polypeptides of TRT virus, three pneumoviruses and an avian paramyxovirus. The TRT virus polypeptides of 42K and 31K are comparable in Mr, and may be analogous to the two major non-glycosylated polypeptides VP40-44 and VP27-28 of established pneumoviruses (Stott & Taylor, 1985; Pringle, 1985; Peeples & Levine, 1979). These are the nucleoprotein and matrix polypeptide respectively. The corresponding polypeptides of viruses
Fig. 1. Electron microscopy of the TRT agent and other pneumoviruses. (a) Giant spherical TRT virion with filaments; arrows point to cross-sections of the helical nucleocapsid which appear as rings 14 nm in diameter. (b) Nucleoprotein extruding from a TRT virion. (c) Filamentous forms of the TRT virus. (d) Nucleoprotein of the TRT virion showing the stretching and loops (arrowed) that occur in its
helical component. Areas measured to estimate the helical pitch are also indicated (arrowheads). (e) Bovine RS pneumovirus nucleoprotein. (f) PVM nucleoprotein. (g) Bovine RS pneumovirus. (h) PVM pneumovirus. Bar marker represents 100 nm on micrographs (b), (d), (e), (f), (g) and (h), 200 nm on (a) and 500 nm on (c).
classified in the genera *Paramyxovirus* and *Morbilliviruses* are larger; NDV for example, a member of the first genus, has a nucleoprotein of $M_r$ 53K to 57K and a matrix protein 40K to 44K (Alexander & Collins, 1981).

SDS–PAGE and staining with Schiff's reagent of the virus associated with TRT identified at least two glycopolypeptides of $M_r$ 84K and 54K (Fig. 3). The larger glycoprotein was the most...
abundant although scarcely visible when stained with CBB. The glycopolypeptide of Mr 54K probably corresponded to the broad fairly diffuse band representing the same Mr, visualized when the gel was stained with CBB. Two other areas stained by Schiff's reagent were very heterogeneous and did not appear to correspond to any of the other polypeptides stained with CBB. Ling & Pringle (1988) have radiolabelled the TRT virus with $^3$Hglucosamine and observed an 83K Mr glycoprotein which they considered may correspond to the 84K glycopolypeptide in this report, though this was not conclusive. One of two other glycoproteins they described of Mr 57K and 45K was regarded as possibly analogous to the 54K glycopolypeptide in this study.

The glycoproteins of the other pneumoviruses were not distinguishable by Schiff's staining apart from that at the 50K Mr position in the bovine and human RSV profiles.

In conclusion we believe that the data presented suggest that the agent associated with turkey rhinotracheitis is a member of the *Pneumovirus* genus.

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**REFERENCES**


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