Isolation and Properties of Viruses from Poultry in Hong Kong which Represent a New (Sixth) Distinct Group of Avian Paramyxoviruses

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SUMMARY

Eight viruses isolated in Hong Kong were shown to be serologically related. One was obtained from the tracheal swab of a chicken and four were from cloacal swabs of ducks sampled at a poultry dressing plant. Three isolations were made from samples taken at a duck farm: two from pond water and one from faeces. Representatives of these isolates were shown to be paramyxoviruses but were serologically distinct from other avian and mammalian paramyxoviruses by haemagglutination inhibition and neuraminidase inhibition tests. Slight variations were seen in the properties of three isolates examined in detail. All three were apathogenic for chickens. The structural polypeptides of one isolate, PMV-6/duck/Hong Kong/199/77, were examined by SDS-polyacrylamide gel electrophoresis. Seven polypeptides were detected, with mol. wt. 180000, 76000, 60000, 55000, 51000, 48000 and 40000. The isolates represent a sixth serologically distinct avian paramyxovirus group.

INTRODUCTION

Paramyxoviruses isolated from domestic poultry in Hong Kong as a result of an influenza surveillance programme (Shortridge et al. 1977) have been shown to be related to Newcastle disease virus (NDV; Shortridge & Alexander, 1978a) or to a serologically distinct group of avian paramyxoviruses, the isolation and properties of which have been described (Shortridge & Alexander, 1978b; Alexander et al. 1979c). Continuation of surveillance has resulted in the isolation of eight serologically related viruses which form a sixth serologically distinct group of avian paramyxoviruses. The isolation of viruses of this group and the properties of three representative viruses are described.

METHODS

Viruses. The reference viruses used and their origins have been described (Alexander & Chettle, 1978; Alexander et al. 1979a, c). The newly isolated viruses described in this report were obtained from two sources: (i) A Hong Kong poultry dressing plant during the course of regular surveillance from November 1975 to June 1979 for influenza viruses in domestic poultry originating from Hong Kong and the south east of the People's Republic of China. As a result of examining 5602 swabs from ducks, 1137 swabs from geese and 1626 swabs from chickens, five isolations of the virus under report were made, one from the tracheal...
swab of a chicken, C54, and four from the cloacal swabs of ducks: D199, D334, D455 and D553. The first isolation was made in July 1977 and the fifth in March 1979. (ii) A domestic duck farm in Hong Kong during monthly sampling of duck faeces and pond water from July 1978 to June 1979. Totals of 33 pond water and 701 faecal samples were examined. Two isolations of the virus were made from pond water samples, D619C and D619D, both in association with influenza A viruses of Hav7 N2 subtype. One isolation, D650, was made from a faeces sample. All three were from samples taken in June 1979.

The protocols for sample collection at the dressing plant and virus isolation in 9- or 10-day-old embryonated fowls' eggs have been described (Shortridge et al. 1977). Faecal samples from the duck farms were obtained by removing a portion of about 2 mm in diam. from the upper side of fresh faeces with a sterile wooden swab stick. These samples were placed in transport medium (Shortridge et al. 1979) and held on ice for about 6 h while they were transported to the laboratory where they were frozen at -70 °C until used. For the last 4 months of sampling, ensamycin (Schering, Burgess Hill, Sussex) was substituted for gentamycin in the transport medium used. Pond water from the duck farm was collected at the pond outflow, held on ice for about 6 h and then overnight at 4 °C. Polyethylene glycol (PEG-6000, Sigma, Poole, Dorset) was used to concentrate 400 ml to 2 ml by the method of Heyward et al. (1977). Transport medium was added to the concentrates which were stored at -70 °C until used, when they were divided into 6 to 12 equal portions and injected into embryonated eggs. All viruses were grown in 9- to 10-day-old embryonated fowls' eggs.

Assays and tests. Sera, serological, haemagglutination and neuraminidase tests were as described (Alexander, 1974; Alexander & Chettle, 1978).

Polyacrylamide gel electrophoresis (PAGE). SDS-PAGE was as described by Alexander et al. (1979a, b).

Haemolytic activity. Viruses were tested for haemolytic activity as described by Kessler et al. (1979) except that 1 ml of virus was added to 2 ml of 1% chicken red blood cells and incubated for 1 h at 37 °C before centrifugation and spectrophotometric estimation of the degree of lysis.

Cell cultures. Experiments using chick kidney (CK) cells were done as described by Alexander et al. (1979c).

Infection of chickens. The pathogenicity of isolates for chickens was tested by infection of 6-week-old white leghorn chickens in groups of 10 by intravenous injection or intranasal instillation of 0·1 ml of diluted allantoic fluid containing approx. 10^7 EID_{50} of virus.

RESULTS

Preliminary tests

Initial screening tests on the eight isolates showed that they did not react in single radial diffusion tests with antiserum specific for influenza A ribonucleoprotein and that their haemagglutinin activity was not inhibited by chicken antisera specific for NDV or dk/Hong/Kong/D3/75. However, antiserum prepared against isolate D199 in chickens strongly inhibited the haemagglutinin activity of all eight viruses.

Infection of chickens

Infection of 6-week-old chickens with D199, D334 or C54 by intranasal or intravenous routes produced no signs of disease throughout a 21 day observation period. All infected birds showed an immune response to infection. Haemagglutination inhibition (HI) tests on the sera of intravenously infected birds produced titres ranging from 32 to 512 at 3 weeks after infection, with a geometric mean of 104.
Serological relationships

HI and neuraminidase inhibition (NI) tests involving the newly isolated viruses and representatives of avian paramyxovirus serotypes are shown in Table 1. Although the antigenic relatedness of the isolates was demonstrated, no close relationship was seen between these viruses and any of the avian paramyxovirus serotypes tested. Some low level cross reaction was seen between the isolates and PMV-2 serotype representatives. Isolates D199 and D334 were also tested by HI titrations for inhibition by specific antisera to: the avian paramyxovirus PMV-5/budgerigar/Japan/Kunitachi/74, human parainfluenza viruses 1, 2, 3, 4a and 4b, SV5, bovine parainfluenza 3, Sendai virus and mumps virus. In all cases HI titres were less than 10.

Infection of chick kidney cells

Infection of CK cell monolayers with 1 EID₅₀/cell of D199, D334 or C54 produced c.p.e. consisting of small discrete syncytia containing 2 to 6 nuclei which were visible from about 24 h onwards. Estimations of the degree of cell fusion at 72 h p.i. produced figures for the 'fusion events per cell' of 0.87, 1.21 and 0.94 for D199, D334 and C54 respectively.

The growth of D199 in CK cells was not inhibited by 5-iodo 2-deoxyuridine (IdUrd). D199 grew to a titre of 10⁻⁸ EID₅₀/ml by 72 h p.i., both in the absence and presence of 25 μg/ml IdUrd, indicating that the virus possesses an RNA genome. A DNA virus, avian adenovirus CELO-Phelps, grew to a titre, at 72 h p.i., of 10⁻⁶ EID₅₀/ml without IdUrd but only to a titre of 10⁻¹ EID₅₀/ml in the presence of IdUrd.

The three viruses tested, D199, D334 and C54, were able to induce the formation of small syncytia within 3 h of infection with about 1000 EID₅₀ per cell. The 'fusion events per cell' obtained by 'fusion from without' were estimated as: D199, 0.63; D334, 0.56; and C54, 0.47.

Haemolytic activity

D199, D334 and C54 were shown to cause haemolysis of chicken red blood cells and haemolytic activity/haemagglutinin ratios of these and other paramyxoviruses were calculated: D199, 5.5 x 10⁻²; D334, 6.3 x 10⁻³; C54, 4.9 x 10⁻²; NDV-Herts, 1.1 x 10⁻²; Yucaipa, 5.5 x 10⁻³; ty/Wis., 1.1 x 10⁻²; D3/75, 9.6 x 10⁻³.

Properties of neuraminidase activity

The 50 % heat inactivation temperatures of the neuraminidase activity of D199 and D334 after 5 min incubation were estimated as 58 and 55 °C, respectively. The neuraminidase/haemagglutinin ratios were determined for D199, D334 and C54 as 0.076, 0.060 and 0.033, respectively. The pH optima of the neuraminidases were determined as 5.4 for D199 and D334 and 4.9 for C54. All three viruses showed similar pH profiles and the pH values at which 50 % inhibition of activity occurred were: D334, 4.40 and 6.35; D199, 4.40 and 6.20; C54, 4.35 and 5.75.

Heat inactivation of haemagglutinin

Inactivation of haemagglutinin activity at 56 °C produced similar results for D334 and D199 and titres of 2048 for both viruses were reduced to <2 in 30 min. The haemagglutinin of C54 was slightly more heat resistant, a titre of 512 being reduced to 32 by 30 min but was <2 after 45 min incubation.

Morphology

Negative contrast electron microscopy of isolates D199, D334 and C54 showed all three to have typical paramyxovirus morphology. In each case the virus particles were pleomorphic and filamentous particles were present. Large amounts of free nucleocapsid could be
Table 1. *Haemagglutination inhibition and neuraminidase inhibition tests with representatives of the newly isolated viruses and other avian paramyxoviruses*

<table>
<thead>
<tr>
<th>Antisera</th>
<th>PMV</th>
<th>NDV</th>
<th>Yucaipa</th>
<th>PLOC-9</th>
<th>Bangor</th>
<th>Robin</th>
<th>Ty/Wis</th>
<th>D3/75</th>
<th>D199</th>
<th>D334</th>
<th>D455</th>
<th>C54</th>
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<tr>
<td></td>
<td></td>
<td>HI</td>
<td>NI</td>
<td>HI</td>
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</tr>
<tr>
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<td>620</td>
<td>—</td>
<td>—</td>
<td>10</td>
<td>—</td>
<td>80</td>
<td>20</td>
<td>—</td>
<td>—</td>
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</tr>
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<td>Ck/California/Yucaipa/56</td>
<td>2</td>
<td>—</td>
<td>160</td>
<td>240</td>
<td>160</td>
<td>80</td>
<td>40</td>
<td>—</td>
<td>10</td>
<td>20</td>
<td>10</td>
<td>—</td>
</tr>
<tr>
<td>Weaver/Senegal/PLOC-9/76</td>
<td>2</td>
<td>—</td>
<td>80</td>
<td>1280</td>
<td>640</td>
<td>160</td>
<td>—</td>
<td>—</td>
<td>20</td>
<td>—</td>
<td>10</td>
<td>10</td>
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<tr>
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<td>2</td>
<td>—</td>
<td>80</td>
<td>1280</td>
<td>640</td>
<td>&gt; 2000</td>
<td>80</td>
<td>—</td>
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<td>—</td>
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<tr>
<td>Robin/Hiddensee/Ty/Wisconsin/19/75</td>
<td>2</td>
<td>—</td>
<td>80</td>
<td>320</td>
<td>160</td>
<td>640</td>
<td>550</td>
<td>—</td>
<td>—</td>
<td>20</td>
<td>—</td>
<td>10</td>
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<td>Ty/Wisconsin/68</td>
<td>3</td>
<td>20</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>320</td>
<td>200</td>
<td>—</td>
<td>—</td>
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<td>—</td>
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<tr>
<td>Dk/Hong Kong/D3/75</td>
<td>4</td>
<td>10</td>
<td>—</td>
<td>—</td>
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<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>320</td>
<td>160</td>
<td>—</td>
</tr>
<tr>
<td>D199</td>
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<td>10</td>
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<td>10</td>
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<td>1280</td>
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<tr>
<td>D334</td>
<td>—</td>
<td>10</td>
<td>10</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1100</td>
<td>640</td>
<td>640</td>
</tr>
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</table>

* All antisera were prepared in chickens and treated with receptor destroying enzyme before use. Results are the means of four HI and two NI tests.
† Titre less than 10. Blanks represent not done.
A sixth avian paramyxovirus serotype

Fig. 1. Comparison of the structural polypeptides of (a) ck/California/Yucaipa/56, (b) dk/Hong Kong/199/77 and (c) NDV strain Ulster. Purified viruses were disrupted with 2% (w/v) SDS and 2% (w/v) dithiothreitol and the polypeptides separated by electrophoresis on 7.5% (w/v) polyacrylamide gels. The gels were stained with Coomassie brilliant blue. The numbers represent the estimated mol. wt. $\times 10^{-3}$ of the polypeptides.
Table 2. Serological groups of avian paramyxoviruses

<table>
<thead>
<tr>
<th>Group</th>
<th>Isolate</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMV-1</td>
<td>Newcastle disease virus</td>
<td>Hanson (1978)</td>
</tr>
<tr>
<td>PMV-2</td>
<td>ck/California/Yucaipa/56</td>
<td>Bankowsk et al. (1962)</td>
</tr>
<tr>
<td></td>
<td>ck/Tula-USSR/6889/68</td>
<td>Isachenko et al. (1975)</td>
</tr>
<tr>
<td></td>
<td>finch/N. Ireland/Bangor/73</td>
<td>McFerran et al. (1974)</td>
</tr>
<tr>
<td></td>
<td>parrot/England/0121/74</td>
<td>Collins et al. (1975)</td>
</tr>
<tr>
<td></td>
<td>robin/Hiddensee/19/75</td>
<td>Starke et al. (1977)</td>
</tr>
<tr>
<td></td>
<td>weaver/Senegal/PLOC-9/76</td>
<td>Fleury (1978)</td>
</tr>
<tr>
<td></td>
<td>wren/Czechoslovakia/Olomouc-1/77</td>
<td>Fleury &amp; Alexander (1979)</td>
</tr>
<tr>
<td>PMV-3</td>
<td>ty/Ontario/6661/67</td>
<td>G. Lang (personal communication); Tumova et al. (1979a)</td>
</tr>
<tr>
<td></td>
<td>ty/Wisconsin/68</td>
<td>Tumova et al. (1979a)</td>
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<td></td>
<td>parakeet/Netherlands/449/75</td>
<td>Smit &amp; Rondhuis (1976)</td>
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<td>PMV-4</td>
<td>dk/Hong Kong/D3/75</td>
<td>Shortridge &amp; Alexander (1978b); Alexander et al. (1979c)</td>
</tr>
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<td></td>
<td>dk/Mississippi/406/75</td>
<td>Webster et al. (1976)</td>
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<td></td>
<td>budgerigar/Japan/Kunitachi/1/74</td>
<td>Alexander et al. (1979b)</td>
</tr>
<tr>
<td>PMV-5</td>
<td>budgerigar/Japan/II/75</td>
<td>Nerome et al. (1978)</td>
</tr>
<tr>
<td>PMV-6</td>
<td>dk/Hong Kong/199/77</td>
<td>Yoshida et al. (1977)</td>
</tr>
</tbody>
</table>

seen in each preparation. Intact particles of D199 (excluding filamentous forms) had a mean diameter of about 160 nm and a range of 100 to 300 nm, the nucleocapsid was about 18 nm in diam.

**Structural polypeptides**

The structural polypeptides of D199 were examined by SDS-PAGE under reducing conditions. A typical gel stained with Coomassie brilliant blue is shown in Fig. 1 with gels of NDV strain Ulster and Yucaipa virus which were run in parallel. All three viruses had similar but distinct polypeptide profiles. Seven polypeptides with mol. wt. 180,000, 76,000, 60,000, 55,000, 51,000, 48,000 and 40,000 were detected in gels of D199.

**DISCUSSION**

The results obtained in the present study confirm that the isolates are paramyxoviruses serologically distinct from representatives of the other known serotypes of avian paramyxoviruses. Surveillance of domestic poultry in Hong Kong has proved an extremely fruitful source of influenza A viruses (Shortridge et al. 1977, 1979; Shortridge, 1980), NDV (Shortridge & Alexander, 1978a), paramyxoviruses related to dk/Hong Kong/D3/75 (Shortridge & Alexander, 1978b) and now a further serological group of avian paramyxoviruses. The number of isolations of influenza viruses: NDV: D3/75-like viruses: D199-like viruses are 353: 144: 34: 4 from ducks (5602 swabs), 7: 49: 14: 1 from chickens (1626 swabs) and 12: 9: 6: 0 from geese (1137 swabs). These considerable differences in isolation rates over 41 months of continuous surveillance probably represent the extent of the presence of the different viruses in the domestic poultry of the region although the isolation techniques employed may possibly favour one virus more than another.

Hinshaw et al. (1979) reported that influenza viruses could be isolated from the water and shores of Canadian lakes where wild ducks had congregated prior to winter migration. While it was predictable that avian paramyxoviruses, which may also replicate in the intestine of waterfowl and are shed in high titres in the faeces, would also be isolated in lake or pond water used by infected birds the present study is the first report of such isolations.
A sixth avian paramyxovirus serotype

Comparison of the properties of three of the isolates, D199, D334, and C54, indicated a very close relationship between D199 and D334 but some differences were seen between these and C54. The heat resistance of C54 haemagglutinin was greater than that of the other isolates, the pH optimum of the neuraminidase was lower, 4.9 for C54 compared to 5.4 for D199 and D334, and C54 showed much greater haemolytic activity than the other two isolates.

D199 and the related isolates represent a sixth serologically distinct group of avian paramyxoviruses. In recent years, mainly as a result of influenza surveillance studies in birds, the number of isolations of avian paramyxoviruses and the number of distinct serotypes have shown a marked increase. In the absence of any internationally accepted criteria the nomenclature of these viruses has caused considerable problems. There has been a tendency to adopt the method of nomenclature recommended for influenza viruses (WHO report, 1971) so that a virus is named by: species or type of bird, country or geographical location of origin, reference number or casual name and year of isolation. Tumova et al. (1979b) have further suggested that the serotype of the virus should be designated by the prefix PMV-1, PMV-2 etc., and have proposed that the prototype species of the first four groups should be NDV, Yucaipa, ty/Wis and D3/75. Using this method, Kunitachi-like viruses (Nerome et al. 1978) would represent group PMV-5 and D199-associated viruses PMV-6. The serotype groups and known isolates from each group are listed in Table 2. In an earlier paper, Alexander et al. (1979a) grouped serologically related paramyxoviruses but these groups are not directly comparable to those proposed here and by Tumova et al. (1979b) as, in their paper, group 2 consisted of ty/Wis and related viruses and group 3 of Yucaipa-like viruses.

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