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Isolation and characterization of rotavirus from feral pigeon in mammalian cell cultures

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SUMMARY

Avian rotaviruses were isolated from feral pigeon faeces treated with trypsin using roller tube cultures of mammalian cells. Two pigeon strains, designated as strains PO-8 and PO-13, produced a marked cytopathic effect (CPE), small intracytoplasmic inclusion bodies and high titres of infectious particles in infected MA-104 and MDBK cell lines without cell adaptation and roller drum apparatus. The pigeon rotaviruses shared a common group specific antigen with the Lincoln strain of bovine rotavirus by indirect immunofluorescence, but differed from both the Lincoln strain and the Wa strain of human rotavirus in neutralization tests.

The RNA segment profile of this virus on polyacrylamide gel electrophoresis differed from that of group A mammalian rotaviruses. The results of a serological survey suggested that antibody to pigeon rotaviruses was widespread in avian species in Japan.

INTRODUCTION

Rotaviruses have been isolated from a wide variety of mammalian and avian species with enteritis and diarrhoea, and also from asymptomatic animals throughout the world (McNulty, 1987; Flewett & Woode, 1978; McNulty *et al.* 1979; Fukusho, Shimizu & Ito, 1981; Sato *et al.* 1981; Hoshino *et al.* 1982; Hoshino *et al.* 1983).

Rotavirus from different hosts have a number of common properties, e.g. similar morphology (Flewett, Bryden & Davies, 1973; McNulty, 1978) and clinical signs (Flewett & Woode, 1978), share a common group specific antigen (Woode *et al.* 1976; Thouless *et al.* 1977), cross protection (Gaul *et al.* 1982) and inter-species transmissions (Tzipore & Makin, 1978; Dagenais *et al.* 1981). However, despite these findings which indicate that not all rotaviruses are species-specific, there is no direct evidence of rotavirus infection between animal species and man as a zoonosis.

Recently, we reported that the prevalence of antibody against human rotavirus detectable by immune adherence haemagglutination (IAHA) in dogs increases in parallel with the frequency of intimate contact with man (Sugiyama *et al.* 1984). This preliminary observation supports the hypothesis that pet and wild animals

which have close contacts with man may possibly play an important role in rotavirus infections of human beings.

Therefore, an attempt was made to isolate rotavirus from feral pigeons, since they have lived in close association with man in various places such as public parks and shrines.

This paper describes the isolation of cytopathic rotaviruses from feral pigeons in a continuous mammalian cell line and reports some biological and serological characteristics of the isolated virus.

MATERIALS AND METHODS

Source of pigeon faecal specimens

The faecal samples tested obtained from pigeons inhabiting public parks and storehouses for animal feed in Aichi and Gifu prefectures in Central Japan. All were apparently normal rectal contents or fresh excreta collected with sterile cotton swabs. For virus isolation, approximately 10% faecal suspensions were made in Hanks solution supplemented with 0·5% lactalbumin hydrolysate, 400 μg/ml of gentamicine, 60 μg/ml of kanamycin and 10 μg/ml of fungizone. The suspensions were clarified by centrifugation at 2500 rev./min. for 10 min and the supernatants were stored at −80 °C until needed.

Cell culture

An established cell line derived from fetal rhesus monkey kidney (MA-104) was used for virus isolation, titration and neutralization tests. Culture cells were grown to confluency in test tubes or in 96-well plates using Eagle's minimum essential medium containing 10% inactivated calf serum, 0·295% tryptose phosphate broth and antibiotics. The cell monolayers were usually inoculated 5–7 days after seeding. A continuous line of bovine kidney (MDBK) cells and primary cultures of chick embryo kidney (CEK) cells were also used for virus propagation or reisolation.

Virus isolation and infectivity assay

Isolation of rotavirus from faecal specimens was attempted by the method of Fukusho, Shimizu & Ito (1981), which included the pretreatment of virus with 10 μg/ml of crystalline trypsin (bovine origin, Type III, Sigma, USA), the incorporation of 0·5 μg/ml of trypsin in the maintenance medium and the use of a roller drum apparatus. Serum-free medium was used for maintenance. The cultures were examined daily for the development of a cytopathic effect (CPE). If CPE was not seen, the monolayers were harvested by freezing and thawing after incubation for 1 week, and the cell lysates were inoculated onto fresh cell cultures by the same procedures. If the monolayers had not shown CPE by the third blind passage, the cultures were checked for virus growth by indirect immunofluorescence (IF) with an immune serum to the Lincoln strain of bovine rotavirus.

For virus titration, 0·025 ml of 10-fold dilutions of each isolate were inoculated into four wells of a 96-well microplate containing MA-104 cells, and these were incubated stationary. Based on the appearance of CPE, the mean tissue culture

infective dose ($TCID_{50}$) was calculated by the method of Reed & Muench (1938). Virus dilutions were made in the medium employed for the preparation of the 10% faecal suspension, except that gentamicin and fungizone were omitted.

Viruses

The PO-13 strain, one of the two cytopathic agents, was used mainly for the identification and characterization of virus in this study after 6–8 passages in MA-104 cells. The Wa strain of human rotavirus and the Lincoln strain of bovine rotavirus were also used as reference strains in cross neutralization tests and in co-electrophoresis of viral RNA.

Immunofluorescence (IF) test

The IF test for virus detection on cover-slip cultures was performed as described by Kawamura (1977) with a hyperimmune rabbit serum to the Lincoln strain of bovine rotavirus and FITC-conjugated goat anti-rabbit IgG (Miles Lab., Elkhart, USA).

Physicochemical characterization

Sensitivity to lipid solvents was determined by treating the virus with 20% ether or 10% chloroform at 4 °C for 18 h. Resistance of the isolate to sodium desoxycholate (DOC) and 5-iodo-deoxyuridine (IUdR) was determined by the method of Kurogi *et al.* (1976). The virus particle size was determined by filtration through Millipore filters with a pore size of 450, 100 and 50 nm, and directly by electron microscopic observation of negatively stained virus particles.

Preparation of antiserum

Antisera against the PO-13 strain of isolated virus and the Lincoln strain were prepared in rabbits. The hind footpads were injected with 1·0 ml of purified virus suspension mixed with an equal amount of Freund's complete adjuvant (Difco). The immunized animals were given a similar booster injection 3 weeks later and then bled 1 week after the second injection. Rabbit antiserum against the Wa strain of human rotavirus was kindly supplied by Dr Sakaei of the Aichi prefectural Public Health Laboratory, Nagoya, Japan.

A total of 346 sera collected from feral pigeons living in the warehouses where the PO-13 strain originated, and 489 chick sera, gathered from 20 farms in 7 regions of the Saitama prefecture which is contiguous to Tokyo, were also used in serological survey.

Neutralization test

The neutralization test for virus identification and a serological survey in pigeons was carried out by the method of Sato *et al.* (1981), except that microplates were used instead of tube cultures. The antibody titre was read on a similar principle to virus titrations after incubation at 37 °C for 7 days.

Haemagglutination (HA) and HA inhibition (HI) tests

The ability of the isolated virus to agglutinate erythrocytes from various species of animals was determined by the microtitre method of Inaba *et al.* (1977). The

diluent used was Veronal-buffered saline, containing 0·0125% bovine albumin and 0·001% gelatin. The titres were expressed as the reciprocal of the highest dilution of infected culture fluids that showed complete agglutination. For HI tests, double absorption of serum with 25% kaolin was used to remove non-specific inhibitions from serum (Minamoto *et al.* 1986). Briefly, serum diluted twofold was treated with an equal volume of 25% kaolin for 20 min. After centrifugation to remove kaolin, the supernatant was treated again with a second volume of kaolin and was then absorbed with packed guinea-pig erythrocytes. The pretreated serum was used as a 1 in 8 dilution. Four units of HA antigen in 25 μl were added to 25 μl of serially diluted sera. After incubation at room temperature for 1 h, 0·05 ml of 0·3% guinea-pig erythrocytes was added to the mixtures. The test plates were incubated at room temperature for 2 h. The HI titre was expressed as the reciprocal of the highest serum dilution showing complete HI.

Electron microscopy

The virus suspension in the form of infected culture fluid was initially cleared of cell debris by low-speed centrifugation, and the supernatant was centrifuged at 200000 g for 2 h. The resulting pellets were dissolved in NTE (NaCl-Tris-EDTA) buffer (Sokol *et al.* 1968) to 1/50 the original volume of culture fluid. The concentrated virus suspension was treated with trifluorotrichloroethane (Daiflon-S_3: Daikin Kogyo, Japan). After centrifugation the aqueous phase was harvested, then layered onto saturated caesium chloride of average density of 1·37 g/ml. This mixture was centrifuged at 200000 **g** for 20 h in a Hitachi RPS-40T rotor at 4 °C. Purified virus particles were collected (Fukusho, Shimizu & Ito, 1981) and negatively stained for electron microscopy.

Thin sections of infected MA-104 cells were prepared as described previously (Minamoto *et al.* 1978). The virus and cell preparations were examined in a Hitachi H-800 electron microcope.

Polyacrylamide gel electrophoresis

Double-stranded RNA was extracted from the purified rotavirus by the phenol-chloroform method (Herring *et al.* 1982) and was analysed in a 10% polyacrylamide slab gel by the method of Laemmli (1970).

RESULTS

Isolation and identification of pigeon rotavirus in cultured cells

Agents which produced CPE in MA-104 cells were isolated from 2 of 55 pigeon faecal samples collected in March 1983, by a method using trypsin treatment and rolling the cultures. In the first case the CPE, consisting of increased cell granularity, cell-rounding and eventual cell detachment from the glass, was observed 2 days after inoculation in the second passage (Fig. 1). In the second case, a similar CPE began to appear 7 days after inoculation in the third passage. The isolates were designated as strain PO-13 and PO-8, respectively. Specific fluorescence varying from small granular to coalesced foci was detected with rabbit immune serum against the Lincoln strain of bovine rotavirus in the cytoplasm of MA-104 cells infected with ether strain (Fig. 2). No such fluorescence

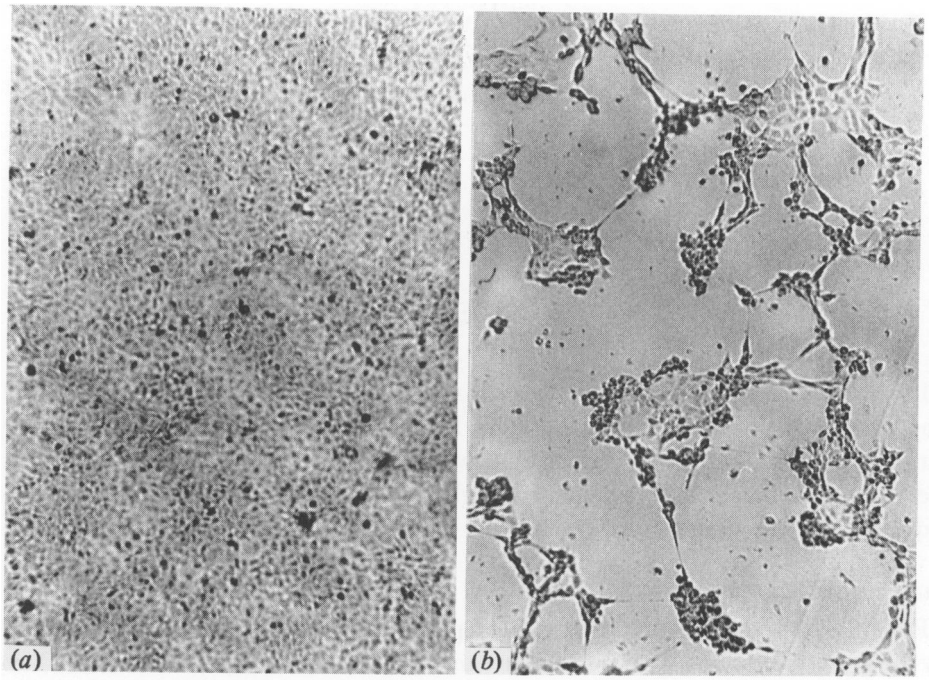

Fig. 1. Cytophatic effect induced by the PO-13 strain of pigeon rotavirus in MA-104 cells, 3 days post-infection. a, Uninfected control cells; b, infected cells.

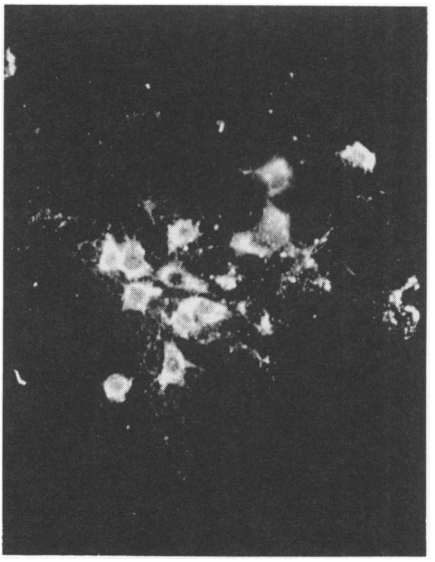

Fig. 2. MA-104 cells infected with pigeon rotavirus (PO-13 strain) showing cytoplasmic fluorescence.

was demonstrated by hyper-immune serum to an avian reovirus. None of the remaining 53 faecal samples produced CPE or rotavirus IF antigen in MA-104 cells during three blind passages. For later experiments to characterize these pigeon rotaviruses, the PO-13 strain was used as prototype.

The physicochemical properties of the isolated virus were investigated as

Table 1. *Cross-neutralization among pigeon, human and bovine rotaviruses*

Rotavirus (strain)	Neutralizing titre of hyperimmune antiserum* to indicated rotavirus (strain)		
	Pigeon (PO-13)	Human (Wa)	Bovine (Lincoln)
PO-13	1024	11·3	< 8
Wa	11·3	3526	287
Lincoln	< 8	18·8	9736

* Rabbit serum.

follows. The infectivity of PO-13 strain was almost completely resistant to 20% ethyl ether, 10% chloroform and 0·1% DOC. The nucleic acid of the isolate was found to be RNA since its replication was not inhibited by 50 μg/ml IUdR. In addition, the virus passed readily through membrane filters with pore sizes of 450 and 100 nm, but was almost completely excluded by a 50 nm membrane filter.

Haemagglutination by isolated virus

Pigeon rotavirus agglutinated guinea-pig, human O, human A, rabbit and goat erythrocytes but not bovine, horse and goose erythrocytes, at 37 °C, room temperature and 4 °C. Of those tested, guinea-pig erythrocytes gave the highest HA titre and the clearest HA pattern at room temperature.

Cross-neutralization test

To examine the immunological relationship between the pigeon rotaviruses and other rotaviruses, cross-neutralization tests were done using the inhibition of CPE as the indicator. The results indicated that pigeon rotavirus was clearly distinguishable from the Wa strain of human rotavirus and the Lincoln strain of bovine rotavirus, although there was a low level of heterologous reaction between the PO-13 and the Wa strains (Table 1).

Electron microscopy

Isolated virus at the eighth passage level in tissue culture was concentrated by high-speed centrifugation and fractions with densities between 1·36 and 1·37 g/ml obtained by CsCl equilibrium density gradient centrifugation were negatively stained to examine the shape and size of virus particles.

The preparations revealed numerous virus particles with typical rotavirus morphology (Fig. 3). Single and double-shelled particles had an average diameter of about 50 nm and 65 nm, respectively.

Thin sections of infected MA-104 cells revealed virus particles within distended cisternae of rough endoplasmic reticulum (Fig. 4).

Growth curve of isolated virus in cell cultures

The growth characteristics of pigeon rotavirus were examined in stationary cultures of MA-104, MDBK and primary chick embryo kidney cells. Typical virus growth curves in both mammalian cell lines infected with the PO-13 strain at a multiplicity of infection of 5·0 are shown in Fig. 5.

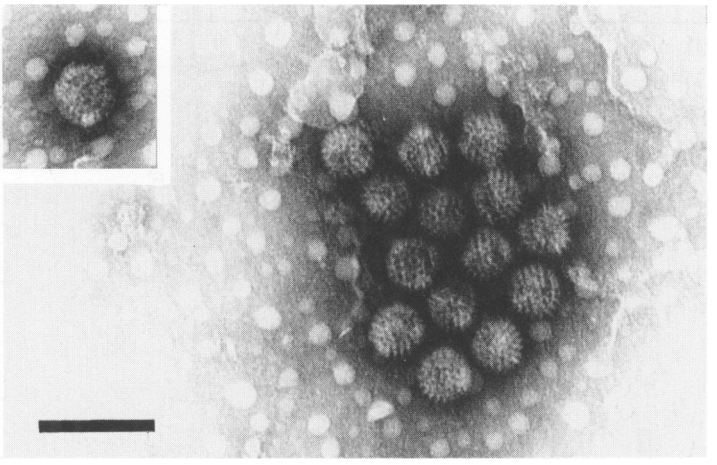

Fig. 3. Electron micrograph of viral particles obtained from the fourth passage of the PO-13 strain in MA-104 cells. Single-shelled particles are seen. Inset: a double shelled particle. Phosphotungstic acid-negative strain. Bar, 100 nm.

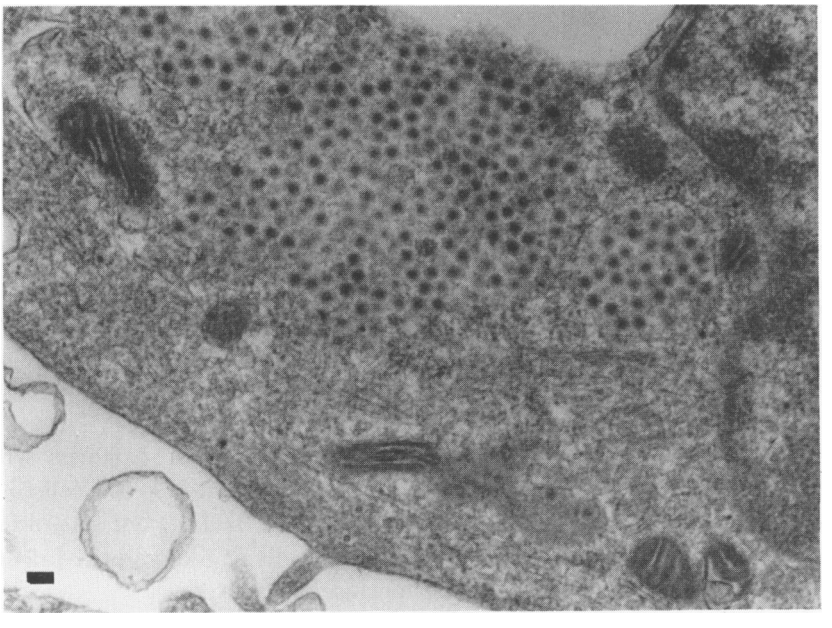

Fig. 4. Thin section electron micrograph showing a number of virus particles within distended rough endoplasmic reticulum. Bar, 100 nm. ×36000.

In infected MA-104 cells, the virus yield increased logarithmically, reaching almost maximum levels by 16 h post-infection, and remained at the same level up to 96 h. The maximal titre of virus produced was 9·2 $\log_{10}$ $TCID_{50}$/ml. HA activity reacting with guinea-pig erythrocytes in culture fluid was first detected 12 h post-infection, and gradually increased in parallel with infectivity. The maximum HA titre was 512.

The CPE, characterized by cellular granulation and rounding-up, began to

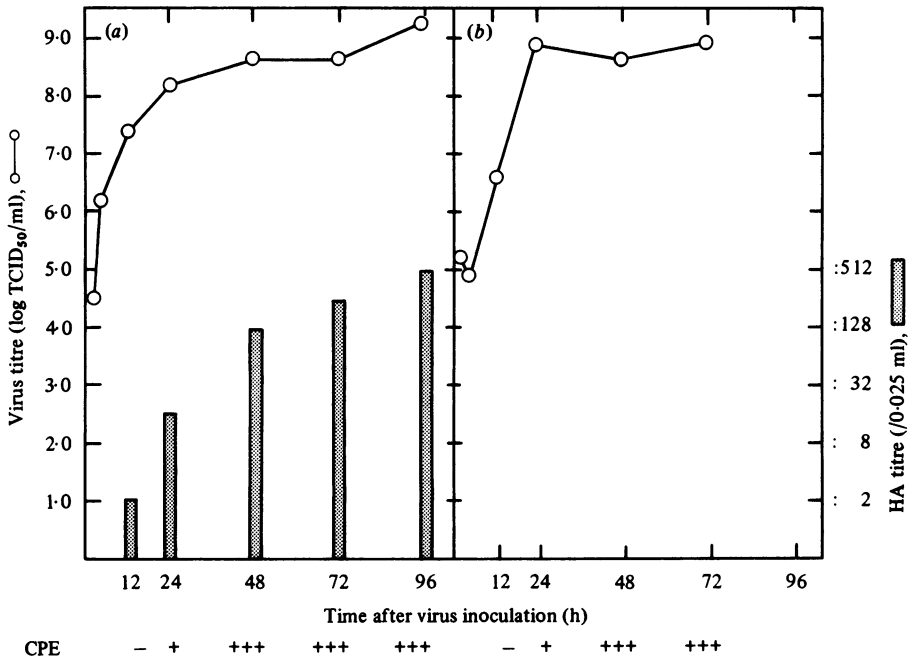

Fig. 5. Development of infectivity, HA and CPE in mammalian cells infected with pigeon rotavirus, strain PO-13 (multiplicity of infection = 5·0). a; MA-104 cells; b, MDBK cells.

appear 16 h post-infection when the production of infectious virus was almost maximum; the number of degenerated cells in the monolayers then rapidly increased to 48 h post-infection when nearly all the cells had detached from the glass. When cell monolayers showing CPE were fixed in Bouin's solution and stained with haematoxylin and eosin, eosinophilic inclusion bodies of various sizes surrounded by a halo were observed in the cytoplasm of many cells (not shown).

In MDBK cells, the isolate showed similar replication patterns with high susceptibility and induction of CPE to those observed in MA-104 cells.

In contrast, although a maximum titre of 7·1 $\log_{10}$ $TCID_{50}$/ml was obtained in culture fluid from infected chick embryo kidney cells at 5 days post-infection, no CPE was seen throughout a cultivation period of 10 days.

The above experiments on the isolation and replication of pigeon rotavirus in cell cultures were all carried out in the presence of trypsin. However, pigeon rotavirus was also found to replicate in MA-104 cell cultures without trypsin in the maintenance medium. In the absence of trypsin, almost the same yield of infectious virus as that in the presence of trypsin was eventually achieved.

Polyacrylamide gel electrophoresis of viral RNA

The isolated virus was shown to have an RNA genome consisting of 11 segments. As shown in Fig. 6, the electrophoretic migration profile was markedly different from that of mammalian rotaviruses.

Segment 5 of pigeon rotavirus was widely separated from segment 6 and

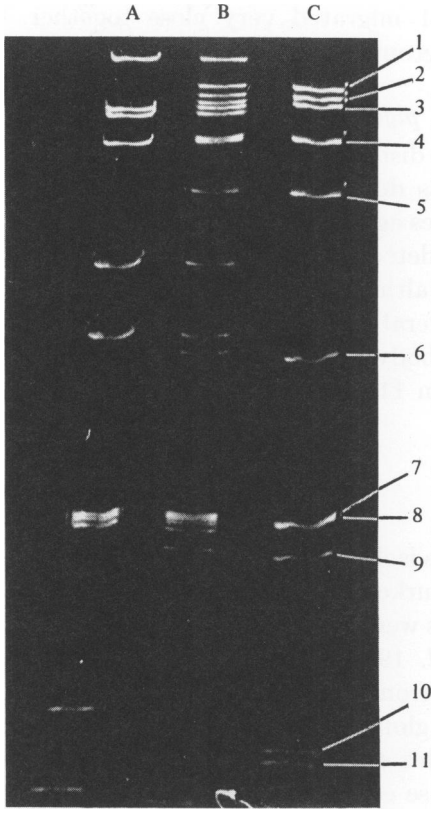

Fig. 6. RNA electrophoretic pattern of pigeon rotavirus in a 10% polyacrylamide gel. The numbers at right designate the segments in decreasing order of molecular weight. A, Lincoln strain of bovine rotavirus; B, Co-electrophoresis of PO-13 and Lincoln strains; C, PO-13 strain of pigeon rotavirus.

Table 2. *Prevalence of HI antibody titres to the PO-13 isolate in pigeons and chickens*

Serum	No. of tested sera	HI antibody titre					No. of positive sera (%)*
		< 8	8	16	32	64	
Pigeon	346	234	97	13	2	0	112 (32·4)
Chicken	489	210	188	87	4	0	279 (57·1)

* Positive: ⩾ 8.

Table 3. *Distribution of serum neutralizing antibody titre to the PO-13 isolate among pigeons*

No. of pigeons	Antibody titre							% of ⩾ 4
	< 4	4	4·7	5·7	6·8	8	22·6	
53	42	5	2	1	1	1	1	20·8

segments 10 and 11 migrated very close together. This RNA profile closely resembled those of group A chicken rotaviruses.

Serological survey in pigeons and chickens

To investigate the distribution of pigeon rotavirus in Japan, a serological survey of avian species was done by HI and neutralization tests. Table 2 shows the frequency of HI titres against the PO-13 strain in pigeon and chick sera. HI titres of 8 or higher were detected in 112 of 346 (32·4%) pigeon sera and in 279 of 489 (57·1%) chick sera, although the titres in both species lay between 8 and 32. Of sera obtained from feral pigeons, 53 random samples were tested for the presence of neutralizing antibody against the isolate. As shown in Table 3, titres of 4 or higher were found in 11 sera (20·8%). The coincidence of sera positive by both tests was 92%.

DISCUSSION

The first rotavirus isolations from avian species were made from faecal samples from chicken and turkeys in Northern Ireland by McNulty *et al.* (1979). Very recently, rotaviruses were also isolated from pheasants (Yason & Schat, 1985) and ducks (Takase *et al.* 1986). It is important to compare the relations between rotaviruses isolated from avian species and man bearing in mind the contribution of avian sources to global epidemics of influenza in man and other mammalian species. To clarify this situation, an attempt was made to isolate rotavirus from pigeons living in close contact with man.

Two avian rotaviruses were isolated from the faeces of clinically asymptomatic feral pigeons. Both induced a marked CPE in roller cultures of MA-104 cells, when the faecal inoculum was treated with trypsin and the enzyme was included in the maintenance medium. Until recently, all avian rotaviruses have been isolated either in a primary chick kidney or embryo liver cells. However, most of these viruses did not induce a marked CPE in infected chick cells. Moreover, the use of primary cell cultures has some drawbacks. Cultures must be prepared each time from chick tissues and examined for miscellaneous adventitious viruses. To counter these disadvantages, we used MA-104 cells which have been used for the isolation and growth of many mammalian rotaviruses (Sato *et al.* 1981; Fukusho, Shimizu & Ito, 1981; Hoshino *et al.* 1982, 1983). More recently, the MA-104 cell line was successfully used for the direct isolation of avian rotavirus by two groups (Theil, Reynolds & Saif, 1986; Kang, Nagaraja & Newman, 1986), despite the fact that most avian viruses do not grow in mammalian cell cultures.

The results presented here also clearly demonstrated that this continuous cell line can be a very useful tool for isolating and propagating pigeon rotaviruses. The highest virus titre reached by the PO-13 strain in stationary MA-104 cell cultures was approximately 9·0 $\log_{10}$ $TCID_{50}$/ml. As far as we know this is the highest amount of infectious virus yielded in any combination of rotaviruses and cells *in vitro*. Therefore, this isolate will greatly help fundamental work on rotaviruses.

A further interesting finding was that both MA-104 and MDBK cells showed a high susceptibility to the PO-13 strain together with an obvious CPE, whereas replication of the isolate in chick embryo kidney cells was at a lower level than in

the two continuous cell lines and was not accompanied by CPE. Although further work is needed, finding that pigeon rotavirus has a higher affinity for cells from mammalian than avian origin, makes us suspect that this isolate may have evolved from a mammalian rotavirus. As well as the PO-13 strain having a common antigen and similar physicochemical and morphological properties to some mammalian rotaviruses, this hypothesis is supported by a serological survey in man and animals using the haemagglutination inhibition (HI) test which detects type-specific rather than group-specific antibody (Minamoto *et al.* 1986), even if rotaviruses from different animal species may belong to the same serotype (Hoshino *et al.* 1984). The results of the survey indicated that antibody reacting with the pigeon rotavirus was widespread in human and animal populations in Japan because HI antibody titres of 8 or higher were detected in 57·1% of chick sera, 32·4% of pigeon sera, and 35·6% of human sera.

On the other hand, some results incompatible with this hypothesis were also obtained in this study. One was the obvious genomic difference between the pigeon and conventional group A mammalian rotaviruses. The electrophoretic migration profile of genomic RNA from our isolate most probably corresponds to the electrophero-group one described by Todd & McNulty (1986), although comparative co-electrophoresis experiments were not done. Another was the antigenic differences revealed by cross-neutralization tests between the pigeon virus and mammalian rotavirus. These discrepancies could not be explained by this study. One possible explanation includes antigenic or genomic drift *in vivo* or diversity of serotypes (McNulty *et al.* 1980). In any event, much more work is required to obtain direct evidence for a zoonotic role for rotavirus.

Finally, the present study has shown that avian rotaviruses, closely resembling those isolated in Northern Ireland and the United States, were also widespread in avian species in Japan.

Further investigations on the antigenic analysis of the PO-13 strain using monoclonal antibodies are now in progress.

REFERENCES

Dagenais, L., Schwers, A., Pastoret, P. P. & Chappuis, G. (1981). Propagation of bovine rotavirus by dogs. *Veterinary Record* **109**, 187.

Flewett, T. H., Bryden, A. S. & Davies, H. A. (1973). Virus particles in gastroenteritis. *Lancet* ii, 1497.

Flewett, T. H. & Woode, G. N. (1978). The rotaviruses. *Archives of Virology* **57**, 1–23.

Fukusho, A., Shimizu, Y. & Ito, Y. (1981). Isolation of cytopathic porcine rotavirus in cell roller culture in the presence of trypsin. *Archives of Virology* **69**, 49–60.

Gaul, S. K., Simpson, T. F., Woode, G. N. & Fulton, R. W. (1982). Antigenic relationships among some animal rotaviruses: virus neutralization in vitro and cross-protection in piglets. *Journal of Clinical Microbiology* **16**, 495–503.

Herring, A. J., Inglis, N. F., Ojen, C. K., Snodgrass, D. R. & Menzies, J. D. (1982). Rapid diagnosis of rotavirus infection by direct detection of viral nuclei acid in silver-stained polyacrylamide gels. *Journal of Clinical Microbiology* **16**, 473–477.

Hoshino, Y., Wyatt, R. G., Scott, F. W. & Appel, M. J. (1982). Isolation and characterization of a canine rotavirus. *Archives of Virology* **72**, 113–125.

Hoshino, Y., Wyatt, R. G., Greenberg, H. B., Kalica, A. R., Flores, J. & Kapikian, A. Z. (1983). Isolation and characterization of an equine rotavirus. *Journal of Clinical Microbiology* **18**, 585–591.

HOSHINO, Y., WYATT, R. G., GREENBERG, H. B., FLORES, J. & KAPIKIAN, A. Z. (1984). Serotypic similarity and diversity of rotaviruses of mammalian and avian origin as studied by plaque-reduction neutralization. *Journal of Infectious Diseases* **149**, 694–702.

INABA, Y., SATO, K., TAKAHASHI, E., KUROGI, H., SATODA, K., OMORI, T. & MATUMOTO, M. (1977). Hemagglutination with Nebraska calf diarrhea virus. *Microbiology and Immunology* **21**, 531–534.

KANG, S. Y., NAGARAJA, K. V. & NEWMAN, J. A. (1986). Primary isolation and identification of avian rotaviruses from turkeys exhibiting signs of clinical enteritis in a continuous MA-104 cell line. *Avian Diseases* **30**, 494–499.

KAWAMURA, A. JR. (1977). *Fluorescent Antibody Techniques and Applications*, 2nd ed., pp. 1–94. Tokyo: University of Tokyo Press.

KUROGI, H., INABA, Y., TANAKA, Y., ITO, Y., SATO, K. & OMORI, T. (1976). Isolation and properties of reovirus from cattle in an outbreak of acute respiratory disease. *National Institute of Animal Health Quarterly* **16**, 39–48.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

MCNULTY, M. S. (1978). Rotaviruses. *Journal of General Virology* **40**, 1–18.

MCNULTY, M. S., ALLAN, G. M., TODD, D. & MCFERRAN, J. B. (1979). Isolation and cell culture propagation of rotaviruses from turkeys and chickens. *Archives of Virology* **61**, 13–21.

MCNULTY, M. S., ALLAN, G. M., TODD, D., MCFERRAN, J. B., MCKILLOP, E. R., COLLINS, D. S. & MCCRACKEN, R. M. (1980). Isolation of rotaviruses from turkeys and chickens: demonstration of distinct serotypes and RNA electropherotypes. *Avian Pathology* **9**, 363–375.

MINAMOTO, N., TAMURA, Y., HIRYAMA, N., SUZUKI, H., KURATA, K., SAZAWA, H. & YAMAMOTO, I. (1978). An electron microscopic study of the surface structures and hemadsorption on chick embryo cells infected with rabies virus. *Archives of Virology* **58**, 95–109.

MINAMOTO, N., HIRAO, M., KINJO, T. & HIRAI, K. (1986). Hemagglutination inhibition test of pigeon rotavirus and its application to serological survey in humans and animals. *Research Bulletin of the Faculty of Agriculture, Gifu University* **51**, 235–242.

LEED, L. J. & MUENCH, H. (1938). A simple method of estimating fifty percent end points. *American Journal of Hygiene* **27**, 493–497.

SATO, K., INABA, Y., SHINOZAKI, T., FUJII, R. & MATUMOTO, M. (1981). Isolation of human rotavirus in MA-104 cells. *Microbiology and Immunology* **25**, 1025–1035.

SATO, K., INABA, Y., SHINOZAKI, T. & MATUMOTO, M. (1981). Neutralizing antibody to bovine rotavirus in various animal species. *Veterinary Microbiology* **6**, 259–261.

SOKOL, F., KUWART, E., WIKTOR, T. J., HUMMLER, K. & KOPROSWKI, H. (1968). Purification of rabies virus growth in tissue culture. *Journal of Virology* **2**, 836–849.

SUGIYAMA, M., MINAMOTO, N., KINJO, T. & HASHIMOTO, A. (1984). A serological survey on rotavirus infection in dogs by immune adherence hemagglutination test. *Japanese Journal of Veterinary Science* **46**, 767–771.

TAKASE, K., NONAKA, F., SAKAGUCHI, M. & YAMADA, S. (1986). Cytopathic avian rotavirus isolated from duck faeces in chicken kidney cell cultures. *Avian Pathology* **15**, 719–730.

THEIL, K. W., REYNOLDS, D. L. & SAIF, Y. M. (1986). Isolation and serial propagation of turkey rotaviruses in a fetal rhesus monkey kidney (MA-104) cell line. *Avian Diseases* **30**, 93–104.

THOULESS, M. E., BRYDEN, A. S., FLEWETT, T. H., WOODE, G. N., BRIDGER, J. C., SNODGRASS, D. R. & HERRING, J. A. (1977). Serological relationships between rotaviruses from different species as studied by complement fixation and neutralization. *Archives of Virology* **53**, 287–294.

TODD, D. & MCNULTY, M. S. (1986). Electrophoretic variation of avian rotavirus RNA in polyacrylamide gels. *Avian Pathology* **15**, 149–159.

TZIPORI, S. & MAKIN, T. (1978). Propagation of human rotavirus in young dogs. *Veterinary Microbiology* **3**, 55–63.

WOODE, G. N., BRIDGER, J. C., JONES, J. M., FLEWETT, T. H., BRYDEN, A. S., DAVIES, H. A. & WHITE, G. B. B. (1976). Morphological and antigenic relationship between viruses (rotaviruses) from acute gastroenteritis of children, calves, piglets, mice and foals. *Infection and Immunity* **14**, 804–810.

YASON, C. V. & SCHAT, K. A. (1985). Isolating and characterization of avian rotaviruses. *Avian Diseases* **29**, 499–508.