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Isolation and Cell Culture Propagation of Rotaviruses From Turkeys and Chickens

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With 3 Figures

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Summary

Rotaviruses were detected by electron microscopy in the faeces of turkey poults and broiler chickens with diarrhoea. Apparently symptomless infection was also observed in broilers. The avian rotaviruses could not be isolated in cell cultures by conventional techniques, but were adapted to serial growth in chick cell cultures following trypsin treatment of the virus and the cells. Immunofluorescence studies showed that the avian and mammalian rotaviruses are antigenically related. Antibodies to rotavirus were widespread in sera collected from turkeys, chickens and ducks.

Introduction

While it is well established that rotaviruses can cause enteritis in a wide variety of mammalian species (6, 15), there are only a few reports of rotavirus infection in avian species (3, 8, 16). The purpose of this paper is to describe the isolation in cell cultures of rotaviruses from chickens and turkeys and to report some serological studies with these viruses.

Materials and Methods

Preparation of Faeces Specimens for Examination by Electron Microscopy

Approximately 15 per cent suspensions of faeces were made in 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.2 (PBS). An equal volume of the fluorocarbon Arcton 113 (Arklone P, ICI Ltd., Runcorn, Cheshire) was added to the suspension and an emulsion formed by thorough mixing. Following centrifugation at $3000 \times g$ for 20 minutes at 4° C, the upper aqueous phase was removed. This was ultracentrifuged for 1 hour at 4° C at 23,000 rpm ($91{,}000 \times g$) using a Beckman SW 41 swing-out rotor. Pellets were resuspended in a few drops of water or 1 per cent ammonium acetate, mounted on 400-mesh carbon-coated grids and stained with methylamine tungstate (EM scope Laboratories, London). Grids were examined in a Philips 301 electron microscope at an accelerating voltage of 80 kV.

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Isolation of Avian Rotaviruses in Cell Cultures

Chick embryo liver (CEL) and chick kidney (CK) cell cultures were prepared and grown as described previously (10). Approximately 15 per cent suspensions of faeces were made in Eagle's (BHK) medium containing 1000 U of penicillin and 1000 μg of streptomycin per ml (ESA). These were extracted with fluorocarbon as described above and the aqueous phase was used to infect cell cultures.

Prior to inoculation, the aqueous phase was incubated for 1 hour at 37° C with trypsin, which was added to give a final concentration of 5 μg/ml. The enzyme was prepared as 1 per cent stock crystalline trypsin in 1 mM HCl (1), and was diluted with ESA immediately before use. The medium was removed from confluent monolayers of CEL or CK cells on 13 mm^2 round coverslips contained in plastic bijou-type bottles (Sterilin Ltd., Teddington, Middlesex) and 0.5 ml of inoculum was added. This was centrifuged on to the monolayer for 1 hour at $2500 \times g$ at room temperature (4). A further 1 ml of ESA containing 5 μg trypsin per ml was then added and the cultures incubated at 37° C. Coverslips were harvested and processed for immunofluorescent staining as described below. If the trypsin removed most of the monolayer, the cells were recovered from the medium by centrifugation, resuspended in a small volume of PBS and smeared on a glass microscope slide. Smears were air-dried at room temperature, then fixed and stained. Culture material for serial passage was held at −70° C until fresh cell cultures were available, then thawed and inoculated as described above.

Cell cultures were prepared for thin-section electron microscopy as described previously (11).

Immunofluorescence

Indirect Staining

Freshly trypsinized PK 15 cells were infected with lamb rotavirus as previously described (13), seeded on to degreased Multispot Teflon-coated microscope slides (C. A. Hendley and Co., Essex) and harvested after 18 hours incubation at 37° C. Alternatively, MDBK cells were infected with isolate 75—447 of bovine rotavirus (14) and harvested 48 hours after infection. Infected cell cultures were fixed in acetone for 10 minutes at room temperature prior to overnight staining at 4° C with turkey and chicken sera. Following washing in PBS, counterstaining was performed with either FITC-conjugated anti-chicken IgG prepared in rabbits or anti-turkey IgG prepared in goats (Nordic Immunological Laboratories, Maidenhead, Berks). After a further wash in PBS, the material was mounted in buffered glycerol and examined using ultra-violet illumination.

Control experiments showed that antibody to rotavirus was present in both FITC conjugates to a titre of approximately 1/30. However, this was no longer detectable when they were tested at their working dilutions of 1/80 to 1/100. Subsequent work has shown that in some batches of conjugate the titre of the antibody to rotavirus was higher than that of the anti-species antibody.

Specimens of sera were collected from 15 turkeys which had experienced rotavirus-associated diarrhoea 2—3 weeks previously. Equal volumes of these sera were pooled to provide a pooled convalescent turkey serum.

Many of the chicken and turkey field sera had to be adsorbed with bovine liver powder to eliminate non-specific staining. Liver powder was added to the sera at approximately 0.05 g per ml and the two were mixed overnight at 4° C. Following centrifugation at $3000 \times g$ for 30 minutes the supernatant was removed. This process was repeated before the sera were examined for antibody to rotavirus.

Direct Staining

Hyperimmune sera to calf, lamb and pig rotaviruses were prepared in rabbits, conjugated with FITC and used as described previously (13).

Serum Neutralisation Test for Antibody to Calf Rotavirus

Doubling dilutions of turkey and chicken sera were made in Eagle's (BHK) medium, buffered with 33 mM bicarbonate and containing 100 U of penicillin and

100 μg of streptomycin per ml (EBA). Serum dilutions (0.5 ml) were added to equal volumes of EBA containing 100 $TCID_{50}$ of isolate 75—447 of calf rotavirus (14). The serum-virus mixtures were incubated overnight at 4° C and then inoculated into roller tube cultures of MDBK cells. Two tubes were used for each serum dilution. The tubes were rolled at 37° C for 1 hour to allow the virus to adsorb to the cells before the cultures were refed with EBA. The cultures were incubated at 37° C in a roller apparatus and examined daily for 7 days for evidence of a cytopathic effect.

Results

Detection of Rotaviruses in Avian Faeces by Electron Microscopy

We have previously reported the detection by electron microscopy of rotavirus particles in the faeces of 5 week old turkey poults with diarrhoea (16). More recently, we have observed large numbers of rotavirus particles in the caecal contents of 3 to 7 day old poults affected with a syndrome characterised clinically by diarrhoea, inflammation of the vent and vent pecking.

In a search for rotaviruses in chickens, specimens of pooled caecal and large intestinal contents from 54 broiler chickens aged between 19 and 36 days were examined by electron microscopy. These broilers were from flocks free from obvious disease and the intestinal contents were normal in volume, consistency and colour. Rotaviruses were observed in very small numbers in 6 of these specimens. Rotavirus particles have been observed in greater numbers in the faeces of 42 day old birds from a broiler flock with a diarrhoea problem.

The avian rotaviruses were morphologically indistinguishable from those which infect mammalian species. Intact double-shelled particles averaged 66 nm in diameter. Single-shelled particles were about 10 nm smaller (Fig. 1).

Isolation of Turkey and Chicken Rotaviruses in Cell Cultures

Faecal material from 6 day old turkeys with diarrhoea was treated with trypsin and inoculated into cultures of CK cells which were maintained on trypsin-containing medium. Electron microscopic examination of the faecal material showed it contained numerous rotavirus particles. Viral replication was followed in early passages by indirect immunofluorescent staining using the pooled convalescent turkey serum. Later passages were stained with an FITC-conjugated antiserum to calf rotavirus.

Following an initial passage of 24 hours duration, less than 1 per cent of CK cells contained cytoplasmic antigen which reacted with the pooled convalescent serum. However, the number of infected cells increased with successive 24 hour passages (Fig. 2). After 10 serial passages in CK cells, the turkey virus was passaged twice in CEL cells. A stock pool of the virus was then prepared in CEL cells. A working pool was grown up from the stock pool in CEL cells maintained in 25 sq cm plastic bottles. All passages of the virus in CEL cells were of approximately 48 hours duration. When the working pool was used to infect CEL cells, about 70 per cent of the cells consistently contained viral antigen when harvested 48 hours after infection (Fig. 2d).

Faecal material containing rotavirus particles from broiler chickens with diarrhoea was similarly inoculated into CEL cultures and passaged at 48 hourly inter-

vals. Synthesis of viral antigen was again followed by immunofluorescence using the antisera to the turkey and calf rotaviruses. As with the turkey virus, the number of infected cells increased with each successive passage. At the 4th passage about 50 per cent of the cells in the monolayer contained rotavirus antigen (Fig. 2c).

Attempts to isolate the turkey and chicken rotaviruses from the faecal preparations used above were unsuccessful when trypsin treatment was not employed. Synthesis of viral antigen was demonstrated initially, but the number of fluorescing cells markedly decreased with each passage and fluorescence was not observed after 4 serial passages.

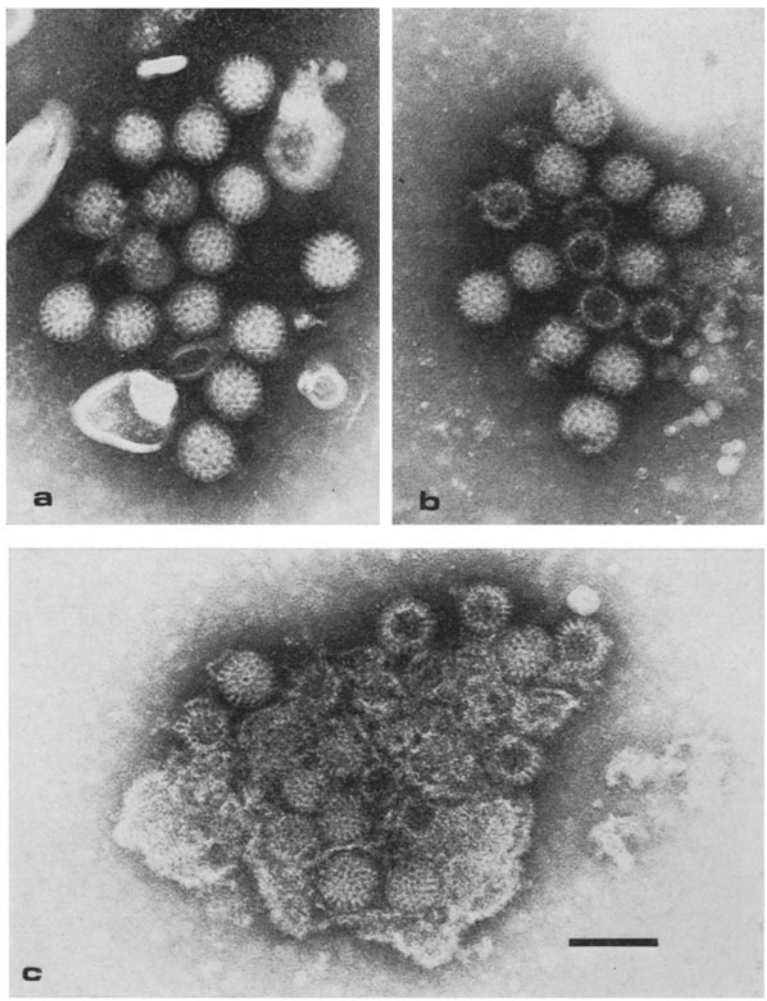

Fig. 1. Negative contrast electron microscopy of avian rotaviruses. Bar represents 100 nm. *a* Double-shelled rotavirus particles from turkey faeces. *b* Single-shelled particles from turkey faeces. *c* Single- and double-shelled particles from CEL cells infected with chicken rotavirus

After 10 passages in CK cells and then 5 passages in CEL cells, the turkey virus produced what appeared to be a cytopathic effect in CEL cells. Cells in infected cultures became granular and detached from the glass. This change was not observed in uninfected trypsin-treated cultures.

Electron microscopic examination of CEL cells infected with the working pool of turkey rotavirus revealed the presence of numerous rotavirus particles

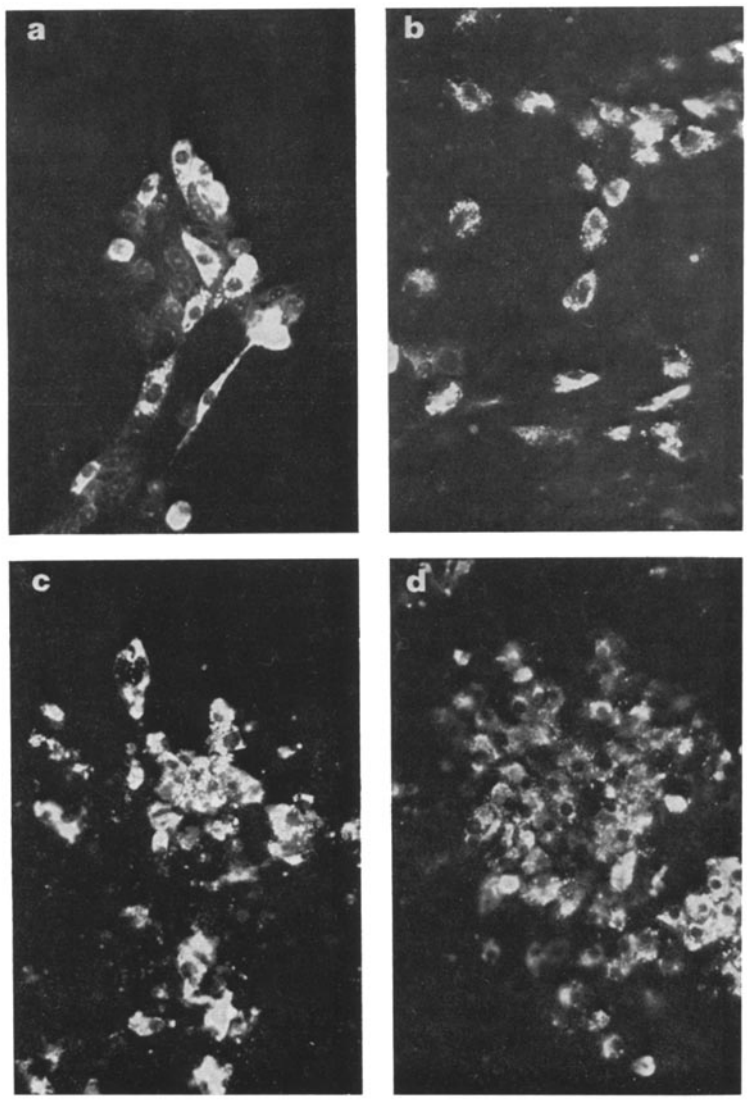

Fig. 2. Immunofluorescence. *a* PK 15 cells infected with lamb rotavirus, stained with pooled convalescent turkey serum. *b* CK cells infected with 9th passage turkey rotavirus, stained with pooled convalescent turkey serum. *c* CEL cells infected with 4th passage chicken rotavirus, stained with antiserum to calf rotavirus. *d* CEL cells infected with working pool of turkey rotavirus, stained with antiserum to lamb rotavirus. (All × 200)

within dilated cisternae of the rough endoplasmic reticulum (Fig. 3). Rotavirus particles were also detected by negative contrast electron microscopy in lysates of CEL cultures infected with either the turkey or chicken rotavirus isolate (Fig. 1c).

Antigenic Relationship Between Avian and Mammalian Rotaviruses

The antigenic relationship between avian and mammalian rotaviruses was investigated by immunofluorescence. The pooled turkey convalescent serum reacted with turkey, chicken, calf and lamb rotavirus antigens in infected cell cultures. Similarly, turkey and chicken rotavirus antigens were stained by FITC-conjugated hyperimmune sera to calf, lamb and pig rotaviruses. Some of these reactions are illustrated in Figure 2. Fluorescence was not detected when uninfected cell cultures were used.

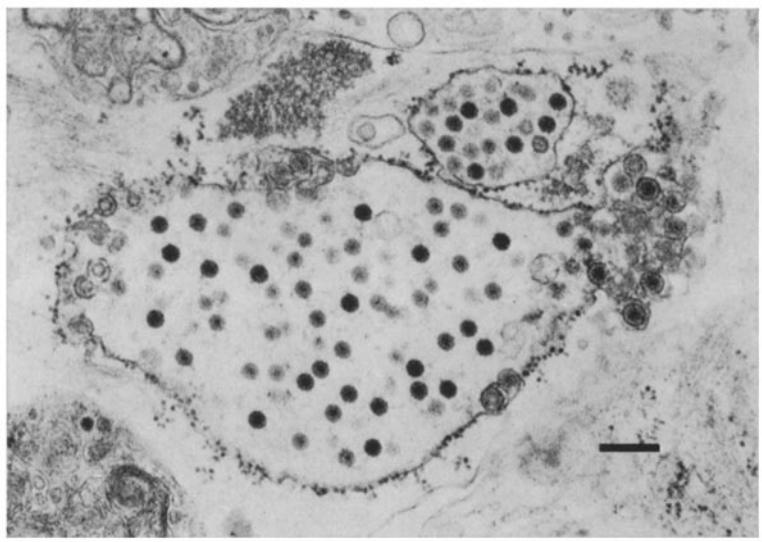

Fig. 3. Thin section of part of cytoplasm of CEL cell infected with working pool of turkey rotavirus. Note virus particles within dilated cisternae of rough endoplasmic reticulum. Bar represents 200 nm

Survey of Avian Sera for Antibody to Rotavirus

The antigenic relationship between mammalian and avian rotaviruses enabled us to carry out a survey of avian sera for antibody to rotavirus, using indirect immunofluorescent staining of PK15 cells infected with lamb rotavirus. Sera were collected from parent turkey and chicken flocks when the birds were approximately 20 weeks old. In the initial screening only 1 serum per farm was examined, and sera were tested at a dilution of 1/20. Antibody to rotavirus was detected in 9 of 26 (35 per cent) turkey sera and in 14 of 35 chicken sera (40 per cent) examined. Of 59 turkey sera collected from 5 farms identified as antibody-

positive by the initial screening procedure, 53 (90 per cent) possessed antibody. Similarly, antibody was detected in 25 of 42 (59 per cent) chicken sera from 5 positive farms. No antibody was detected in sera collected from the laboratory's specific pathogen free chicken flock. This flock is maintained in positive pressure filtered air isolation houses and is regularly monitored for infection with a number of avian pathogens. This has included direct electron microscopic examination of faeces and rotaviruses have not been observed.

Indirect immunofluorescence antibody titres were relatively high in both turkey and chicken sera. Of the 23 turkey and chicken sera identified as antibody-positive when screened at a dilution of 1/20, 22 were still positive when tested at a dilution of 1/100. The indirect immunofluorescence titre of the pooled convalescent turkey serum was 640. The geometric mean titre of 10 randomly selected positive chicken sera was 394.

We have also examined sera from adult ducks by indirect immunofluorescence, 10 of 21 sera screened at a dilution of 1/20 had antibody to rotavirus.

Chicken and turkey sera with varying indirect immunofluorescence antibody titres were tested for neutralising antibody to isolate 75—447 of bovine rotavirus. Significant levels of neutralising activity were found in many sera with indirect immunofluorescence titres in excess of 40. Some of the results are shown in Table 1. Generally speaking, neutralisation titres were lower than indirect immunofluorescence titres, but some sera, e.g. turkey serum 8, were exceptions to this rule.

Table 1. *Comparison of rotavirus antibody titres of avian sera measured by indirect immunofluorescence and neutralisation tests*

	Indirect FA titre	Neutralisation titre
Pooled turkey convalescent serum	640	60
Turkey serum 4	<20	<20
Turkey serum 8	160	>640
Turkey serum 11	160	80
Chicken serum 2	320	20
Chicken serum 4	>640	>640
Chicken serum 6	160	30
Chicken serum 11	<20	30

Discussion

Rotaviruses have been detected in avian species only comparatively recently (3, 8, 16). As far as we are aware, this is the first account of the isolation of these viruses from turkeys and chickens. Like their mammalian counterparts, avian rotaviruses are difficult to isolate and grow in cell cultures using conventional techniques. However, mammalian rotaviruses can be serially propagated in cell cultures if the virus inoculum is treated with proteolytic enzymes (2, 18), or if trypsin is incorporated into the medium after infection (1). However, it is not

clear from these studies whether the effect of the enzyme is on the virus or on cellular receptors for the virus. Consequently we used both types of treatment in our isolation attempts. Rotaviruses were isolated from both chicken and turkey faeces using this approach, whereas no isolates were made without trypsin treatment. The isolation of these viruses in cell cultures should greatly facilitate investigation of their biological and biochemical characteristics.

It is tempting to suggest that avian and mammalian rotaviruses have a similar level of pathogenicity. Indeed, previous reports of rotavirus infection in turkeys and hens have associated infection with outbreaks of diarrhoea (3, 8, 16). However, infection with rotavirus does not invariably result in illness in mammalian species (5, 12, 17). This would also appear to be the case with avian species, as shown by the symptomless infection of broiler chickens described in this paper. The excretion of very small numbers of virus particles in the faeces is apparently a common feature of asymptomatic rotavirus infection in chickens and human neonates (5).

Mammalian rotaviruses possess a group antigen demonstrable by immunofluorescence or complement fixation tests (9, 19, 20). Our immunofluorescence studies indicate that this antigen is also present in the turkey and chicken rotaviruses. This was unexpected as most avian viruses do not cross-react by immunofluorescence with their mammalian counterparts. There was therefore a possibility that the viruses we isolated from turkeys and chickens represented a chance infection of these species with mammalian rotaviruses. However, the finding that antibody reacting with the mammalian rotavirus group antigen was widespread in turkey, chicken and duck sera argues against this. Furthermore, preliminary analysis of the RNA segments of these avian viruses by polyacrylamide gel electrophoresis has revealed a feature not previously described for any of the mammalian rotaviruses (TODD and McNULTY, unpublished observations).

The serological suvey also showed that the percentage of birds with antibody to rotavirus on infected turkey farms was higher than that on chicken farms. This may be related to the system of poultry husbandry in Northern Ireland. Parent chicken flocks are derived from birds reared on a number of different farms and transferred to breeding farms at about 17 weeks of age. On the other hand, turkeys are more often reared and bred on the same farm. Assuming that rotavirus infections occur predominantly in young birds, one would expect infected parent turkey flocks to be almost 100 per cent positive for antibody, whereas the percentage of birds with antibody in the parent chicken flocks would depend on the proportions of birds supplied from sero-positive and sero-negative rearing farms. Given this situation, our figure of 40 per cent for parent chicken flocks with antibody is probably an underestimate as only one serum per farm was examined.

Significant levels of neutralising activity against isolate 75—447 of bovine rotavirus were detected in some of the turkey and chicken sera (Table 1). This is probably due to the low level of cross-reaction which occurs between rotaviruses from different animal species (19). However, the considerable variation in the ratios of indirect immunofluorescence and neutralisation titres raises the possibility that turkeys and chickens can be infected with more than one serotype of rotavirus. This would not be surprising, as several serotypes of rotavirus capable of infecting humans have been shown to exist (7).

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