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Definition of Two New Groups of Atypical Rotaviruses

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SUMMARY

Comparative antigenic and nucleic acid analyses were carried out on two new atypical rotavirus isolates coming respectively from chickens (D/132) and pigs (E/DC-9). Indirect immunofluorescence showed that each virus carried different group antigens which were also distinct from those of previously described rotavirus groups. By genome profile analysis each virus had a pattern of genomic RNAs clearly distinct from those of the other rotavirus groups. Comparative terminal fingerprinting of corresponding genome segments from the two viruses showed large differences between them, indicating that all of their genomic RNAs had significant differences in sequence both from each other and from the three previously defined rotavirus groups. On the basis of these results, extension of the number of rotavirus groups from three to five is proposed, with isolates D/132 and E/DC-9 being the type members of groups D and E respectively.

INTRODUCTION

In recent years rotaviruses have become recognized as the major aetiological agents of acute viral gastroenteritis in humans and all the major species of domestic livestock (Flewett & Woode, 1978; Du-Pont, 1984). Their medical importance is illustrated by a survey in India which identified rotaviruses as the agents most frequently isolated from hospitalized cases of acute gastroenteritis (Mata, 1983). Furthermore, evidence now suggests that rotaviruses may also be involved in causing severe gastroenteritis in adults, particularly the very old (Echeverria *et al.*, 1983; Cubitt & Holzel, 1980). These observations have stimulated work on vaccines against rotavirus infection (Snodgrass & Wells, 1976; Sheridan *et al.*, 1983; Vesikari *et al.*, 1984). However, effective vaccines depend on a detailed understanding of the epidemiology of the disease and extent of viral antigenic diversity.

Serological analysis, using a wide range of techniques, has shown that the majority of rotavirus isolates possess a common group antigen on the inner of the two capsid shells irrespective of their species of origin (Woode *et al.*, 1976; Flewett & Woode, 1978). However, recent reports have appeared describing viral agents associated with gastroenteritis in several species which morphologically resemble rotaviruses but do not possess the previously defined group antigen (Bridger, 1980; Saif *et al.*, 1980; McNulty *et al.*, 1981; Rodger *et al.*, 1982). These viruses have been variously termed pararotaviruses, non-group A rotaviruses or simply atypical rotaviruses. Whilst in comparison to 'typical' rotaviruses their isolation is still rare, they are beginning to appear with increasing frequency (Dimitrov *et al.*, 1983; Hung *et al.*, 1983; Espejo *et al.*, 1984; McNulty *et al.*, 1984; Snodgrass *et al.*, 1984). Furthermore, the description of a large outbreak of rotavirus diarrhoea predominantly amongst adults in China (Hung *et al.*, 1983, 1984), associated with a virus that did not carry the typical group antigen has served to emphasize the potential medical and epidemiological importance of these non-group A rotaviruses and the need for their fuller characterization.

In a previous study we compared two atypical rotaviruses with a 'typical' rotavirus using both nucleic acid and serological analyses. This led to the definition of three distinct rotavirus groups (Pedley *et al.*, 1983). Those isolates carrying the previously characterized group antigen were termed group A rotaviruses. The other two groups, B and C, were defined using porcine virus isolates obtained in the United Kingdom (Bridger, 1980) and United States (Saif *et al.*, 1980) respectively. In this report we describe results in which a combined serological and nucleic acid approach has been used to define two additional rotavirus groups, D and E.

METHODS

Viruses. The tissue culture-adapted OSU strain of porcine rotavirus (A/OSU) obtained originally from Dr E. H. Bohl was used as a representative group A rotavirus. This virus was propagated in MA 104 cells as previously described for bovine rotavirus (McCrae & Faulkner-Valle, 1981). Representative viruses for groups B and C were B/NIRD-1 (Bridger *et al.*, 1982) and C/Cowden (Saif *et al.*, 1980) respectively. The atypical strain of chicken rotavirus (D/132) was kindly supplied by Dr M. S. McNulty. The E/DC-9 strain was identified during routine genome profile analysis of field isolates of porcine rotavirus from the United Kingdom (Chasey *et al.*, 1985).

Antisera. Convalescent antisera to the porcine viruses were taken 3 weeks after oral inoculation of gnotobiotic piglets (Bridger & Brown, 1984). They were raised against two group A viruses, A/SW1-2 and A/SW20-21 (Woode *et al.*, 1976), the group B virus B/NIRD-1 (Bridger *et al.*, 1982), two group C viruses, C/Cowden (Bohl *et al.*, 1982) and C/37030, and E/DC-9. In addition, convalescent antiserum to the bovine virus A/UK was obtained by infection of a gnotobiotic calf (Bridger & Brown, 1984). Hyperimmune antiserum to the avian virus D/132 (McNulty *et al.*, 1981) was kindly supplied by Dr M. S. McNulty.

Indirect immunofluorescence. The antigens used were MA 104 cells infected with A/OSU, sections of frozen intestinal tissue taken from gnotobiotic piglets infected with B/NIRD-1, C/Cowden or E/DC-9 viruses, and chick embryo liver cells infected with D/132 virus kindly supplied by Dr M. S. McNulty. Antigens were fixed in acetone, stained with dilutions of test antisera for 1 h at room temperature followed by the appropriate fluorescein-conjugated anti-species immunoglobulin (Nordic Immunological Laboratories, Maidenhead, U.K.). A positive serum and a conjugate control were routinely included.

Preparation, fractionation and one-dimensional terminal fingerprint analysis of end-labelled dsRNA. The starting material for these analyses was infected MA 104 cells for A/OSU and D/132 (McCrae & Faulkner-Valle, 1981), faeces from gnotobiotic piglets infected with B/NIRD-1 or C/Cowden (Pedley *et al.*, 1983), and for the porcine virus E/DC-9, the original faecal material submitted to the Central Veterinary Laboratory, Weybridge.

Extraction of viral genomic dsRNA and labelling of it at the 3' termini with cytidine 3',5'-[5'-^{32}P]bisphosphate using T4 RNA ligase were all carried out as described by Clarke & McCrae (1981).

Analytical and preparative fractionation of the 3' end-labelled RNA and subsequent isolation of individual RNA segments by electro-elution from the polyacrylamide gels were all carried out as previously described (Pedley *et al.*, 1983). Similarly, the conditions used for production of the terminal fingerprints were as previously described (Pedley *et al.*, 1983).

RESULTS

Antigenic studies

The antigenic relationships of the two new atypical viruses both to each other and to the previously defined rotavirus groups was investigated using indirect immunofluorescence of either infected gut sections or infected tissue culture slides as appropriate. Using antisera to A/SW20-21, B/NIRD-1, C/Cowden, D/132 and E/DC-9, this analysis showed that each of the two new viruses carried its own group antigen that gave no serological cross-reaction with each other or with the group antigens of the type members of the three rotavirus groups defined to date (Table 1). These results were confirmed with antisera to further isolates, A/SW1-2, A/UK and C/37030.

Genome profile analysis

The genome profiles of the isolates D/132 and E/DC-9 were compared with those of the group A, B and C viruses on 7·5% polyacrylamide gels (Fig. 1). In common with the group B and C viruses both new viral isolates lacked the tight segment 7–8–9 triplet characteristic of group A rotaviruses, but both were distinct in having a more even distribution of the medium and lower molecular weight segments throughout the gel. A feature of the D/132 isolate was the presence of five genome segments in the higher-molecular weight region of the gel compared to the more usual four (Fig. 1).
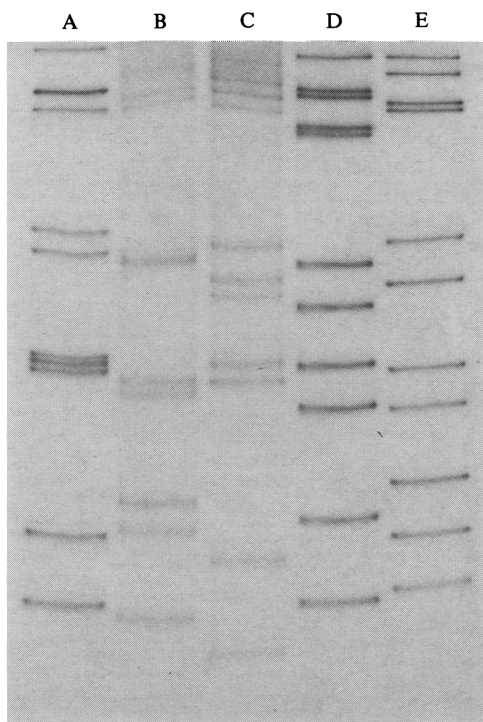
Fig. 1. Comparative genome profile analysis of D/132 (group D) and E/DC-9 (group E) with other rotavirus groups. Genomic dsRNA was extracted either from purified virions (groups A and D) or from the faeces of infected animals as described in Methods. Following 3′ end-labelling with [^{32}P]pCp it was analysed as previously described (Pedley *et al.*, 1983). The isolates used were: group A, OSU strain of porcine rotavirus; group B, B/NIRD-1; group C, C/Cowden; group D, D/132; group E, E/DC-9.

Table 1. *Comparison of D/132 and E/DC-9 with rotavirus groups A, B and C by indirect immunofluorescence*

	Virus				
Antiserum	A/OSU	B/NIRD-1	C/Cowden	D/132	E/DC-9
A/SW20-21	2560*	<20	<40	<40	<20
B/NIRD-1	<10	2000	<20	<20	<20
C/Cowden	<10	<10	2000	<20	<20
D/132	NT†	<40	<40	>1000	<20
E/DC-9	<20	<20	<20	<20	320

* Highest dilution at which fluorescence was clearly visible.
† NT, Not tested.

Terminal fingerprint analysis

Previous studies have shown that the migration properties of individual RNA segments on polyacrylamide gels give no indication of sequence relationship (Clarke & McCrae, 1982). Therefore, terminal fingerprinting was used for detailed comparative analysis of individual RNA segments of the two isolates. In these experiments, due to the limited availability of dsRNA from the group C and particularly the group B viruses, the D/132 and E/DC-9 dsRNA segments were only compared directly with those of a group A virus. The results obtained on comparing the segment 10 RNAs is shown in Fig. 2(*a*). For the purposes of interpretation the

(a)   Segment 10   (b)   Segment 4   Segment 5

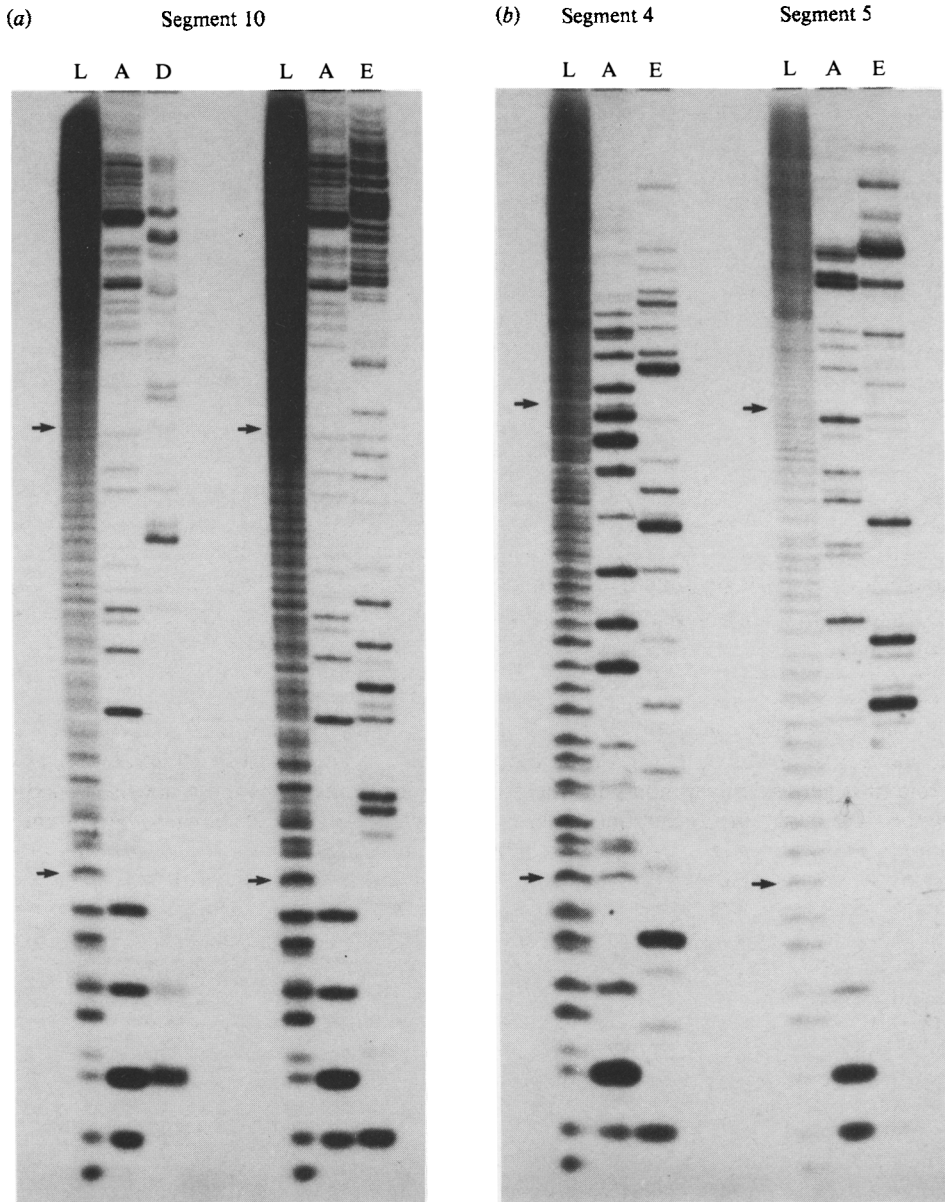L   A   D   L   A   E   L   A   E   L   A   E

Fig. 2. (a) Terminal fingerprint analysis for genome segment 10 of a group A rotavirus and the two atypical viruses D/132 and E/DC-9. The individual genomic RNAs were isolated, subjected to partial T1 RNase digestion and the digestion products fractionated as described in Methods. Lane designations are: L, a partial alkaline hydrolysis reference ladder of end-labelled RNA to allow nucleotide position relative to the termini to be measured; A, the fingerprint given by the group A rotavirus, in this case the OSU strain of porcine rotavirus; D, the fingerprint from isolate D/132; E, the fingerprint given by E/DC-9. Arrows next to the reference ladders are shown at 10 and 40 nucleotides in from the termini. (b) Comparative terminal fingerprint analysis of the genomic segments 4 and 5 of isolate E/DC-9 and the group A virus A/OSU. Lane designations and nucleotide position arrows are described in (a).

terminal fingerprints can be divided into two regions: a short terminal region which is absolutely conserved across all 11 RNA segments for groups A and C (Clarke & McCrae, 1982; McCrae & McCorquodale, 1983; Pedley *et al.*, 1983), internal to which is a region extending up to approximately 40 nucleotides in from the termini whose pattern is diagnostic of a particular RNA segment (Clarke & McCrae, 1983). The fingerprints of D/132 and E/DC-9 are distinct from each other over both of these regions and also dissimilar to the fingerprints from the corresponding segment of the group A virus. By reference to Pedley *et al.* (1983), comparison of these fingerprints with those of group B and C viruses also failed to reveal any homology. This type of analysis was carried out on the other RNA segments of these viruses (results not shown) and these confirmed the conclusions reached from Fig. 2(*a*). Comparison of the RNA fingerprints obtained for all 11 segments of D/132 confirmed that, in common with the group A and C viruses, there is a region of apparent absolute sequence conservation within the terminal 10 nucleotides (results not shown). Similar analysis of the results from E/DC-9 failed to reveal any conservation at the termini (Fig. 2*b*). Thus, only a single band at nucleotide position 3 (this band is very weak on the segment 5 fingerprint in Fig. 2*b*) was found to be common to all of the E/DC-9 segments.

DISCUSSION

Using established serological techniques for detecting the group antigen of rotaviruses, we have previously been able to define three distinct antigenic groups: the group A, B and C rotaviruses (Pedley *et al.*, 1983). In this paper we have characterized two additional viruses which are morphologically identical to rotaviruses but lack the group A common antigen present in the majority of rotavirus isolates. The serological analysis using indirect immunofluorescence showed that both of these virus isolates possessed a group antigen unrelated to each other or to any of the three previously described groups. These results therefore provide serological criteria for the definition of two new rotavirus groups, D (D/132) and E (E/DC-9).

The genome profile analysis of D/132 and E/DC-9 resolved 11 segments of dsRNA with a molecular range similar to that of the other three rotavirus groups, supporting their inclusion in the rotavirus genus. In common with the other atypical rotaviruses these new isolates did not have the tight triplet of medium molecular weight segments (7, 8, 9) shown to be present in all group A rotaviruses analysed to date. It will be of interest to see whether genome profile analysis of additional atypical isolates as they are found will continue to allow this tight triplet of RNA segments to be used as a diagnostic feature of group A rotaviruses. The results of our genome profile analysis agree well with those of McNulty *et al.* (1981) except that we achieved a greater separation of RNA segments 8 and 9, but this is probably a reflection simply of differences in the electrophoresis systems used. The RNA segment 5 of the D/132 virus migrated much closer to segment 4 than in any of the representative viruses for the other rotavirus groups, but this does not appear to be a diagnostic feature of group D viruses, as some avian group A rotaviruses show a similar genome profile (Todd *et al.*, 1980).

Terminal RNA fingerprinting of the RNA segments of D/132 and E/DC-9, performed to investigate the extent of divergence between these isolates, showed no homology between the fingerprints in the nucleotide 10 to 40 region. This indicated that the five groups of rotaviruses differ from each other not only in those RNA segments encoding the group antigen, but in all of their RNA segments. It would be helpful if these homology studies could be broadened in a series of cross-hybridization analyses between the RNAs of the various rotavirus groups as has been done, for example, in the case of the mammalian reoviruses (Gaillard & Joklik, 1982). However, the absence of a high-yielding tissue culture cultivation system of the atypical virus groups makes such studies impractical at present. Work is currently in progress to clone the genomes of type members of the various rotavirus groups and this should facilitate such cross-group hybridization studies. In this context the use of cloned probes for the group A rotaviruses (McCrae & McCorquodale, 1982) has shown that there is less than 50% sequence homology between group A genomes and those of the other rotavirus groups defined to date (S. Pedley & M. A. McCrae, unpublished observations). Despite these reservations the nucleic acid-based results obtained in this study complement the serological analysis and provide additional

evidence for the definition of two new rotavirus groups, D and E, with D/132 and E/DC-9 being the type members respectively.

Additional atypical rotavirus isolates continue to be reported; for example two atypical human viruses have been shown to belong to group C (Bridger *et al.*, 1984). However, the classification of many of these isolates has yet to be determined (Hung *et al.*, 1983; Vonderfecht *et al.*, 1984). Although serological surveys have already indicated that some of the atypical groups are widespread in the field (McNulty *et al.*, 1984; Bridger & Brown, 1985; Chasey *et al.*, 1985), their importance in the disease problem remains to be established. Nevertheless, the size and severity of the recent outbreak of human gastroenteritis in China (Hung *et al.*, 1983, 1984) associated with a non-group A rotavirus indicates that at least some atypical rotaviruses cause serious disease.

REFERENCES

BOHL, E. H., SAIF, L. J., THIEL, K. W., ARDEN, A. D. & CROSS, R. F. (1982). Porcine pararotavirus: detection, differentiation from rotavirus and pathogenesis in gnotobiotic pigs. *Journal of Clinical Microbiology* **15**, 312–319.

BRIDGER, J. C. (1980). Detection by electron microscopy of caliciviruses, astroviruses and rotavirus-like particles in faeces of piglets with diarrhoea. *Veterinary Record* **107**, 532–533.

BRIDGER, J. C. & BROWN, J. F. (1984). Antigenic and pathogenic relationships of three bovine rotaviruses and a porcine rotavirus. *Journal of General Virology* **65**, 1151–1158.

BRIDGER, J. C. & BROWN, J. F. (1985). Prevalence of antibody to typical and atypical rotavirus in pigs. *Veterinary Record* **116**, 50.

BRIDGER, J. C., CLARKE, I. N. & McCRAE, M. A. (1982). Characterisation of an antigenically distinct porcine rotavirus. *Infection and Immunity* **35**, 1058–1062.

BRIDGER, J. C., PEREIRA, H. G. & McCRAE, M. A. (1984). *Abstracts of the 6th International Congress of Virology, Sendai, Japan*, p. 216.

CHASEY, D., BRIDGER, J. C. & McCRAE, M. A. (1985). A new type of atypical rotavirus in pigs. *Archives of Virology* (in press).

CLARKE, I. N. & McCRAE, M. A. (1981). A rapid and sensitive method for analysing the genome profiles of isolates of rotavirus. *Journal of Virological Methods* **2**, 203–209.

CLARKE, I. N. & McCRAE, M. A. (1982). Structural analysis of electrophoretic variation in the genome profiles of rotavirus field isolates. *Infection and Immunity* **36**, 492–497.

CLARKE, I. N. & McCRAE, M. A. (1983). The molecular biology of rotaviruses. VI. RNA species-specific terminal conservation in rotaviruses. *Journal of General Virology* **64**, 1877–1884.

CUBITT, W. D. & HOLZEL, H. (1980). An outbreak of rotavirus infection in a long stay ward of a geriatric hospital. *Journal of Clinical Pathology* **33**, 306–308.

DIMITROV, D. H., ESTES, M. K., RANGELOVA, S. M., SHINDAROV, L. M., MELNICK, J. L. & GRAHAM, D. Y. (1983). Detection of antigenically distinct rotaviruses from infants. *Infection and Immunity* **41**, 523–526.

DU-PONT, H. L. (1984). Rotaviral gastroenteritis, some recent developments. *Journal of Infectious Diseases* **149**, 663–666.

ECHEVERRIA, P., BLACKLOW, N. R., CUKOR, G. G., VIBULANDHITKIT, S., CHANGCHOWALIT, S. & BBOONTHAI, P. (1983). Rotaviruses as a cause of severe gastroenteritis in adults. *Journal of Clinical Microbiology* **18**, 663–667.

ESPEJO, R. T., PUERTO, F., SOLER, C. & GONZALEZ, N. (1984). Characterisation of a human pararotavirus. *Infection and Immunity* **44**, 112–116.

FLEWETT, T. H. & WOODE, G. N. (1978). The rotaviruses. *Archives of Virology* **57**, 1–25.

GAILLARD, R. K. & JOKLIK, W. K. (1982). Quantitation of the relatedness of reovirus serotypes 1, 2 and 3 at the gene level. *Virology* **123**, 152–163.

HUNG, T., CHEN, G., WANG, C., CHOU, Z., CHAO, T., YE, W., YAO, H. & MENG, K. (1983). Rotavirus like agent in adult non-bacterial diarrhoea in China. *Lancet* ii, 1078.

HUNG, T., CHEN, G., WANG, C., YAO, H., FANG, Z., CHAO, T., CHOU, Z., YE, W., CHANG, X., DEN, S., LIONG, X. & CHANG, W. (1984). Waterborne outbreak of rotavirus diarrhoea in adults in China caused by a novel rotavirus. *Lancet* i, 1139–1142.

McCRAE, M. A. & FAULKNER-VALLE, G. P. (1981). Molecular biology of rotaviruses. I. Characterisation of basic growth parameters and pattern of macromolecular synthesis. *Journal of Virology* **39**, 490–496.

McCRAE, M. A. & McCORQUODALE, J. G. (1982). Molecular biology of rotaviruses. IV. Molecular cloning of the bovine rotavirus genome. *Journal of Virology* **44**, 1076–1079.

McCRAE, M. A. & McCORQUODALE, J. G. (1983). The molecular biology of rotaviruses. V. Terminal structure of viral RNA species. *Virology* **126**, 204–212.

McNULTY, M. S., ALLEN, G. M., TODD, D., McFERRAN, J. B. & McCRACKEN, R. M. (1981). Isolation from chickens of a rotavirus lacking the rotavirus group antigen. *Journal of General Virology* **55**, 405–413.

McNULTY, M. S., TODD, D., ALLEN, G. M., McFERRAN, J. B. & GREENE, J. A. (1984). Epidemiology of rotavirus infection in broiler chickens: recognition of four serogroups. *Archives of Virology* **81**, 113–121.

MATA, L. (1983). Epidemiology of acute diarrhea in children, an overview. In *Acute Diarrhea: The Nutritional Consequences in Children*, pp. 3–22. Edited by J. A. Bellanti. New York: Vevey/Raven Press.

PEDLEY, S., BRIDGER, J. C., BROWN, J. F. & McCRAE, M. A. (1983). Molecular characterization of rotaviruses with distinct group antigens. *Journal of General Virology* **64**, 2093–2101.

RODGER, S. M., BISHOP, R. F. & HOLMES, I. H. (1982). Detection of a rotavirus-like agent associated with diarrhea in an infant. *Journal of Clinical Microbiology* **16**, 724–726.

SAIF, L. J., BOHL, E. H., THIEL, K. W., CROSS, R. F. & HOUSE, J. A. (1980). Rotavirus-like, calicivirus-like and 23 nm virus-like particles associated with diarrhea in young pigs. *Journal of Clinical Microbiology* **12**, 105–111.

SHERIDAN, J. F., EYDELLOTH, R. S., VONDERFECHT, S. L. & AURELIAN, L. (1983). Virus specific immunity in neonatal and adult mouse rotavirus infection. *Infection and Immunity* **39**, 917–927.

SNODGRASS, D. R. & WELLS, P. W. (1976). Rotavirus infection in lambs: studies on passive protection. *Archives of Virology* **52**, 201–205.

SNODGRASS, D. R., HERRING, A. J., CAMPBELL, I., INGLIS, J. M. & HARGREAVES, F. D. (1984). Comparison of atypical rotaviruses from calves, piglets, lambs and man. *Journal of General Virology* **65**, 909–914.

TODD, D., McNULTY, M. S. & ALLEN, G. M. (1980). Polyacrylamide gel electrophoresis of avian rotavirus RNA. *Archives of Virology* **63**, 87–97.

VESIKARI, T., ISOLAURI, E., D'HONDT, E., DELEM, A. & ANDRE, F. L. (1984). Protection of infants against rotavirus diarrhea by RIT 4237 attenuated bovine rotavirus strain vaccine. *Lancet* i, 977.

VONDERFECHT, S. L., HUBER, A. C., EIDEN, J., MADER, L. C. & YOLKEN, R. H. (1984). Infectious diarrhea of infant rats produced by a rotavirus-like agent. *Journal of Virology* **52**, 94–98.

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 relationships between viruses (rotaviruses) from acute gastroenteritis of children, calves, piglets, mice and foals. *Infection and Immunity* **14**, 804–810.