Evidence for Serotypic Variation Among Bovine Rotaviruses

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With 2 Figures

Summary

Eight field strains of calf rotavirus from the U.K. were compared by neutralisation tests, using convalescent and hyperimmune antisera. Seven of these strains cross-reacted and were considered to be of one serotype, while the 8th was distinguished by a greater than 20-fold two-way difference in neutralisation titre suggesting a second serotype. Three widely-distributed reference strains (U.K., Northern Ireland and Lincoln) cross-reacted with the strains in the dominant serotype, as did 33 of 42 other field calf rotavirus strains. Nine field strains failed to cross-react with either serotype, suggesting the existence of other potential serotypes in the calf population.

Introduction

Rotaviruses are a major cause of diarrhoea in young animals and children (9, 18). Calf rotavirus is of worldwide distribution, and in our experience is the single most important infectious cause of diarrhoea in calves (31).

The antigenic relationships between rotaviruses from all species are complex and there are at least 3 groups of rotaviruses which share no common antigens (5, 20, 25, 27). Recent results obtained with reassortant viruses (12) and monoclonal antibodies (11) have helped clarify the situation within the most common antigenic group. It has been suggested that the term subgroup be used to describe antigens detected by broad serological reactions involving the major structural protein of the core, using assays such as complement fixation, enzyme-linked immunosorbent assay, and immune adherence haemagglutination (15, 39, 40). To date, two subgroups of human rotavirus have been demonstrated (15). The term serotype defines antigens involved in neutralisation reactions, at least one of which is associated with the surface

glycoprotein of the outer shell (15). At least 4 and perhaps 5 human rotavirus serotypes exist (3, 36, 38).

Electrophoretic analysis of the eleven double-stranded RNA (ds RNA) viral genome segments has shown substantial variation in the pattern of segment mobilities (the electrophoretype) both between and within virus from different species (7, 14, 26, 29). Recent hybridisation and nuclease 'finger-printing' studies have shown considerable sequence diversity even within dsRNA segments from strains of virus infecting one host species (27, 34). The non-cross-reactive groups of rotaviruses can be distinguished by electro-phoretic analysis (5, 20, 25, 27), and the subgroups of human rotavirus can also be distinguished by their segment patterns (14).

Two serotypes of calf rotavirus distinguishable by neutralisation test have been reported from Japan (22), and some strains have been found distinct by haemagglutination inhibition test (33). Clearly serotype diversity is of great importance for potential vaccine development. The genome dsRNA pattern of bovine rotaviruses shows extensive variation (26). In this paper we describe investigations into the relationships by neutralisation assay and dsRNA electrophoresis of strains of rotavirus in calves in the United Kingdom.

Materials and Methods

Viruses

Faeces from twelve 1—4 week old calves which were shown by ELISA (8) and by electron microscopy (EM) (32) to contain rotavirus were initially selected for study. The basis for selection was the widely separate geographical origins within the U.K. of the faecal samples. Subsequently 42 other strains from calf faeces submitted to this laboratory for diagnostic examination were studied. Reference tissue culture adapted rotavirus strains used were U.K. (Compton) (6) (cloned by Dr. R. G. Wyatt), Northern Ireland (19) and the Lincoln Nebraska Calf Diarrhoea Virus (21).

Infection of Gnotobiotic Lambs

Gnotobiotic lambs reared singly in plastic isolators were used for the multiplication of the different isolates as well as for the production of specific convalescent antisera.

A 10 per cent suspension of each of the original faeces in 20 mM Tris HCl buffer pH 7.5 (Tris buffer) was blended with an equal volume of fluorocarbon (Arcton 113, ICI), centrifuged at $2000 \times g$ for 30 minutes, and the aqueous phase filtered through a 0.45 μm membrane. Three ml of filtrate was administered orally to each lamb 24 hours after delivery. Faeces were collected and examined daily for rotavirus excretion by ELISA and EM. Twenty-one days after inoculation the lambs were bled for antisera.

Tissue Culture

MA 104 cells used throughout the study were grown in Eagles 59 medium con-taining 10 per cent foetal bovine serum and maintained after inoculation in medium 199 containing 0.5 per cent BSA and 2 μg/ml trypsin (Sigma Chemicals Co. U.K.). All cultures were rolled after inoculation.

Tissue culture adapted strains of rotavirus were treated with 5 μg/ml of trypsin at 37° C for 30 minutes before inoculation. Virus stocks were produced by harvesting

infected cultures by 3 cycles of rapid freezing and thawing after 2—3 days or at maximum cytopathic effect (CPE).

To isolate rotavirus from field samples 0.5 ml of trypsin treated (10 µg/ml) faecal filtrate was inoculated to cell monolayers. Cultures were passaged at 3-day intervals and examined daily for CPE and at each passage by immunofluorescence for rotavirus. Isolates were cloned by passaging thrice at terminal dilution from the 6th or 7th passage level.

Rotavirus Purification

To prepare virus from the twelve gnotobiotic lamb faeces, samples were diluted in 3 volumes of Tris buffer, extracted with Arcton, and centrifuged at $2000 \times g$ for 30 minutes. Ten per cent sodium dodecyl sulphate was added to the supernate to a final 1 per cent v/v and incubated at room temperature for 5 minutes. This initial step was omitted when tissue culture grown rotavirus was used. Preparations were then pelleted at $71,000 \times g$ for 45 minutes. The pellets were homogenised in 1—2 ml Tris buffer, layered onto a discontinuous gradient consisting of 2 ml of a solution containing 1.31 M CsCl and 1.58 M sucrose, overlaid by 2 ml 1.58 M sucrose in Tris buffer and centrifuged at $154,400 \times g$ for 60 minutes at 5° C. The opalescent band which appeared just below the interface was harvested, diluted four-fold and pelleted. Pellets were resuspended in 1—2 ml of Tris buffer and layered onto a 5-step CsCl/ sucrose gradient to which 1.0 µg/ml ethidium bromide had been added and then centrifuged at $50,400 \times g$ for 18 hours at 5° C. The gradient consisted of 1.66 M sucrose/ 1.49 M CsCl and 1.56 M sucrose/1.49 M CsCl at the extremities. An intermediate density was achieved by mixing equal volumes of the two extremes, and two further steps were achieved by mixing the intermediate solution with the two extremes. The virus band was located by fluorescence under ultraviolet light, harvested with a syringe, diluted in Tris buffer, and pelleted. The pellets were examined by EM using negative staining with 1 per cent ammonium molybdate (pH 6.0) and the proportion of complete virions estamined.

When tissue culture grown rotavirus was the starting material, virus was pelleted from the cleared supernate, and the resuspended pellet layered directly onto the 5-step CsCl gradient.

Hyperimmunisation of Rabbits

Purified virus pellets containing greater than 99 per cent complete virions were diluted to 1 ml in Tris buffer with 2 per cent Tween 80 added, and emulsified in incomplete Freund's adjuvant. Each rabbit (previously shown to be free of neutralising antibody to U.K. calf rotavirus) received a deep intramuscular injection of 1.0 ml of the emulsion at two different sites. The injections were repeated 14 days later and the rabbits were bled by cardiac puncture 7—10 days after the second injection.

Neutralisation Test

With both tissue culture adapted rotavirus strains and faecal rotavirus, neutralisation of fluorescent focus production in MA 104 cells was used, essentially as described by BEARDS et al. (3, 35). Titres (NT) are expressed as the reciprocal of the serum dilution reducing fluorescent foci by 50 per cent.

In the serotyping of field strains of rotavirus in faeces with standard rabbit serotyping antisera, a constant serum-varying virus assay was used. Faeces were extracted with fluorocarbon, mixed with antibiotics and centrifuged at $200 \times g$ for 30 minutes. Half $\log_{10}$ dilutions of the supernatant fluids were treated with trypsin at 10 µg/ml for 1 hour at 37° C, then incubated with 4 antibody units of typing antiserum for 1 hour at 37° C (1 antibody unit was the amount of antibody neutralised

by 100 TCID$_{50}$ of homologous rotavirus), and thereafter assayed for fluorescent foci on MA 104 cells.

Polyacrylamide Gel Analysis (PAGE) of the dsRNA

Double stranded RNA was prepared from purified virions by phenol-chloroform extraction (13) and precipitation with alcohol and was fractionated in 7.5 per cent polyacrylamide gels with no stacking gel. The Laemmli buffer system (16) was used and electrophoresis was for 12 hours at a constant current of 0.1 mA/cm^2; each gel track was loaded with 100—200 ng of dsRNA. After electrophoresis the gels were washed for 3—4 hours by gentle agitation in 5 changes of 1 per cent acetic acid/ 10 per cent ethanol and were then stained with silver as already described (13).

Results

Infection of Gnotobiotic Lambs

Rotavirus multiplication occurred in all 12 lambs, virus being excreted in faeces for at least 5 days. Coincident with virus excretion the faeces became loose and yellowish. A transient anorexia was noted in one lamb (678).
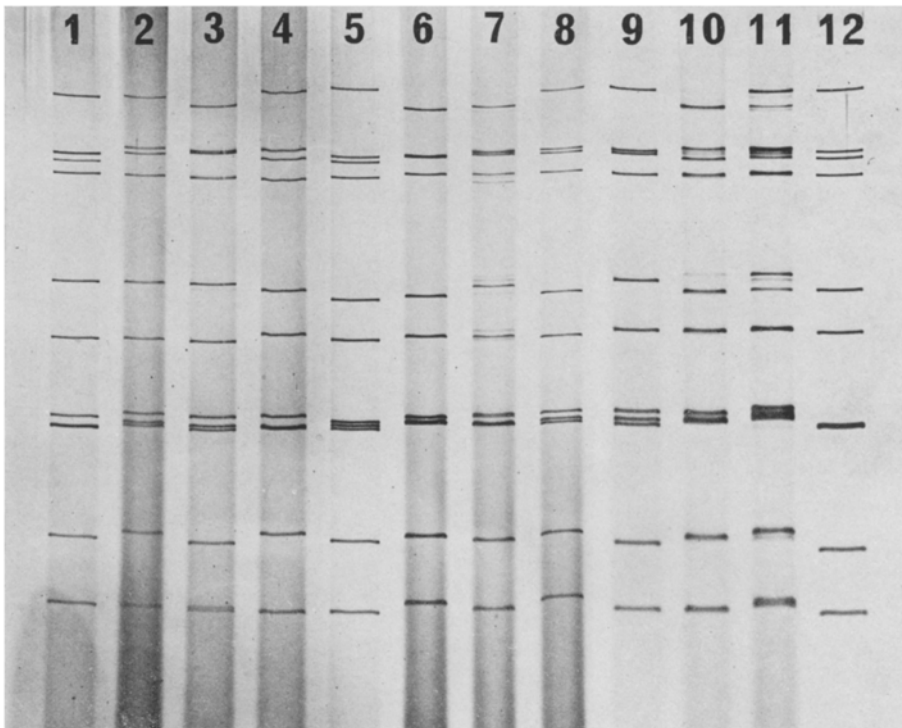

Fig. 1. Polyacrylamide-gel electrophoresis of dsRNA from 12 isolates. Tracks *1* 637; *2* 639; *3* 646; *4* 641; *5* 642; *6* 651; *7* 657; *8* 669; *9* 649; *10* 663; *11* 666; *12* 678. Note the existence of more than 11 segments in tracks 3, 7, 10 and 11

Examination of viral dsRNA by PAGE revealed that all 12 samples had different electrophoretic patterns. However, 4 of the samples (tracks 3, 7, 10 and 11) had more than 11 segments (Fig. 1). This was considered to reflect the presence of more than one strain of rotavirus in the original calf faeces, so subsequent studies were performed on the remaining 8 strains only.

Cell Culture Adaptation of Faecal Rotavirus

Two of the eight lamb-passaged calf rotavirus strains were selected for adaption to cell culture, 639 as typical serologically of 7 of the strains, and 678 as a distinct strain (see results below).

By the 4th passage level both strains produced slight CPE after 2 days incubation, and by the 6th passage this had developed to a CPE involving complete destruction of the monolayers after 3 days incubation.

The two isolates were cloned by three passages at terminal dilution. Examination of virus genome by PAGE at stages from faeces to cloned virus showed a consistent migration pattern within each isolate (Fig. 2) making the possibility of strain cross contamination unlikely.
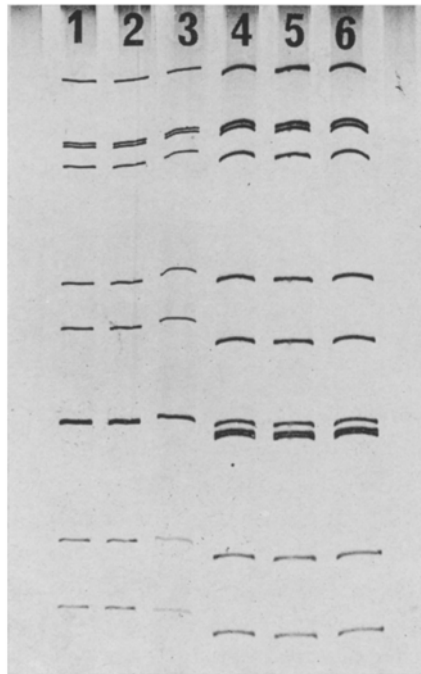

Fig. 2. Comparison of the dsRNA of 639 and 678 after adapation to tissue culture with dsRNA from the original faeces. Tracks *1* and *4* dsRNA of faecal 678 and 639 respectively; tracks *3* and *6* dsRNA of tissue culture adapted 678 and 639 respectively; tracks *2* and *5* a mixture of the dsRNA from faecal and tissue culture adapted virus

Neutralisation Tests

Convalescent Lamb Antisera Reacted with Faecal Virus

All the lamb antisera possessed NT antibody to the homologous rotavirus strains, and also showed varying cross-neutralisation with other strains (Table 1). Within 6 of the strains 637, 639, 641, 649, 651 and 669 there was a high degree of cross-reactivity, with not more than an 8-fold variation between homologous and heterologous titres.

Table 1. *Neutralising titres of antisera from convalescent gnotobiotic lambs, to 8 strains of calf rotavirus in faeces*

Rotavirus	Antiserum to rotavirus							
	637	639	641	642	649	651	669	678
637	*128*	128	32	128	128	128	64	16
639	64	*128*	512	128	64	32	64	8
641	64	64	*64*	64	64	64	64	32
642	128	128	512	*1024*	128	128	128	32
649	128	128	32	ND	*128*	128	64	16
651	128	64	256	256	128	*64*	64	16
669	128	64	64	64	64	64	*128*	16
678	32	16	32	16	32	32	16	*256*

Homologous titres are in italics

ND   not done

Antiserum to strain 678 showed 4 to 32 fold lower titres against all heterologous antigens, and 678 antigen was poorly neutralised by all other antisera. Strain 642 showed one-way cross-reaction with the group of 6 strains; 642 antigen was efficiently neutralised by other antisera, while 642 antiserum neutralised heterologous strains less efficiently.

Hyperimmune Antisera to Faecal Virus Reacted with Faecal Virus

The 6 strains considered similar in their reactions with convalescent lamb antisera were also similar in their reactions with hyperimmune antisera, with not more than a 4-fold variation in titre between homologous and heterologous strains (Table 2). Strain 642 appeared to be more closely related to the 6 strains in these tests, with efficient neutralisation of 642 virus by other antisera, and up to a 16-fold variation in titre of 642 antiserum with heterologous virus strains. Once again strain 678 showed significant distinction from all 7 other strains, with greater than 20-fold differences of titre in both directions.

This evidence suggested that seven strains including 642 were of one serotype, with less than 20-fold difference in titre, and that strain 678 was a distinct serotype.

Table 2. *Neutralising titres of hyperimmune rabbit antisera to faecal rotavirus strains, to 8 strains of calf rotavirus in faeces*

Rota-virus	Antiserum to rotavirus							
	637	639	641	642	649	651	669	678
637	*204,800*	102,400	51,200	102,400	409,600	51,200	25,600	6,400
639	204,800	*102,400*	102,400	204,800	819,200	102,400	25,600	3,200
641	102,400	51,200	*51,200*	51,200	204,800	102,400	25,600	6,400
642	409,600	102,400	204,800	*819,200*	12,800	204,800	51,200	6,400
649	204,800	51,200	204,800	819,200	*204,800*	51,200	51,200	1,600
651	51,200	51,200	204,800	409,600	819,200	*102,400*	51,200	3,200
669	102,400	51,200	51,200	204,800	204,800	N D	*51,200*	3,200
678	3,200	6,400	6,400	3,200	6,400	3,200	3,200	*102,400*

Homologous titres are in italics

N D Not done

Hyperimmune Antisera to Tissue Culture Virus Reacted with Tissue Culture Virus

The cell culture adapted U.K., Northern Ireland, and Lincoln strains were compared with 639 and 678 viruses isolated in cell cultures. The U.K., Northern Ireland and 639 strains appeared identical by cross neutralisation tests (Table 3). The Lincoln strain was less efficiently neutralised by antisera to these 3 strains, and thus showed slight one-way variation. However, the differences were never greater than 16-fold, so the Lincoln strain should still be considered as the same serotype.

Table 3. *Neutralising titres of hyperimmune rabbit antisera to rotavirus strains grown in tissue culture, to 5 strains of calf rotavirus in tissue culture*

Rotavirus	Antiserum to rotavirus				
	U.K.	Northern Ireland	Lincoln	639	678
U.K.	*5,120*	102,400	409,600	102,400	3,200
Northern Ireland	5,120	*102,400*	409,600	51,200	3,200
Lincoln	320	51,200	*409,600*	6,400	400
639	5,120	102,400	409,600	*102,400*	3,200
678	160	3,200	51,200	25,600	*204,800*

Homologous titres are in italics

Antiserum to 678 virus had heterologous titres in the range 64-512-fold less than the homologous titre although 678 virus was clearly distinguished by a greater than 20-fold titre difference by only 2 of the other antisera.

Serotyping Faecal Rotavirus Strains

Due to the consistent distinction of 678 virus, it was considered to represent a separate serotype. Thus typing of field rotavirus strains was carried out using hyperimmune antisera to cloned 639 and 678 viruses. Allocation to serotype was on the basis of at least a 20-fold reduction in virus titre after incubation with the appropriate antiserum, compared with both the virus control titre and the titre with the other antiserum.

Sixty additional calf faeces samples containing rotavirus were examined. No typing of 18 strains was possible due to their failure to produce fluorescing foci in MA 104 cells. Antiserum to virus 639 neutralised the virus in 33 samples, while virus in 9 samples was not neutralised by either antiserum.

dsRNA Segment Pattern

The PAGE analysis of the viral dsRNA (Fig. 1) showed that all strains examined varied in their migration patterns, with no clear relationship to serotype.

Discussion

This study demonstrates the existence of two serotypes of rotavirus in calves. Whether the field viruses not neutralised by either antiserum were mixtures of more than one strain or represent potential new serotypes is currently under investigation. These two serotypes were defined by a neutralisation assay using fluorescent focus reduction with a 20-fold or greater two-way difference in titre as criterion for distinction (10, 38). The fact that the majority of our field calf rotavirus strains as well as three widely-used reference strains, all shared the same serotype, indicates that this serotype is at present the most common in the United Kingdom. This serotype, which we designated serotype 1, is probably similar to the serotype 1 proposed by Murakami *et al.* (22), as both were found similar to the Lincoln strain. The reference strain for serotype 1 could be either Lincoln, or the cloned U. K. strain used in our studies.

The *in vivo* active and passive immune relationships between the two serotypes are as yet unknown. Passive immunisation by dam vaccination is currently the most favoured method of prophylaxis in cattle (23, 30, 37). However, as passive immunity has been shown to be heterogenous between species by the protection of piglets against pig rotavirus infections using bovine colostrum (4, 17), the same broad passive protection may occur among serotypes of bovine rotavirus. In any case, the predominant occurrence of a single serotype suggests that in most instances a monovalent vaccine may be effective.

The techniques for cell culture isolation of rotaviruses from faeces used for calf rotavirus (1, 2) and for human rotavirus (28) proved successful in

this study. In addition to the two strains 639 and 678, four other strains that were not neutralised by antisera to either of the two serotypes have now been isolated and cloned. However, there was a degree of selection of suitable strains for culture, as 18 faecal samples containing rotavirus detected by EM and ELISA did not produce any fluorescent foci in MA 104 cells. The serotyping of such viruses presents a difficult problem.

Examination of the rotavirus genome dsRNA segments by PAGE provided a means of quality control by isolate identification throughout the isolation and cloning of field strains. Identical dsRNA migration patterns in original faeces and cloned virus make laboratory contamination very unlikely due to the great diversity of patterns seen in rotavirus surveys (24, 26). PAGE examination also detected mixed strain rotavirus infections in 4 of our original 12 faeces samples.

Acknowledgements

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