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Isolation of Two Lapine Rotaviruses: Characterization of Their Subgroup, Serotype and RNA Electropherotypes*

By

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With 1 Figure

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Summary

Rotaviruses were detected by an ELISA test in stool specimens from diarrheic rabbits in two commercial rabbitries and cultured in MA 104 cells. Their identity was confirmed by electron microscopy and indirect immunofluorescence. They were found to belong to subgroup I by testing with monoclonal antibodies and to serotype 3 by neutralization with homologous and heterologous antisera. Although both viruses were neutralized by antiserum to human serotype 3 the ALA rabbit rotavirus was minimally neutralized by antiserum to the C11 rabbit rotavirus. Electrophoresis of viral RNA revealed 11 segments characteristic of rotavirus, however both rabbit rotaviruses had unusual electropherotypes. They differed from each other with greatly reduced mobility of the tenth segment in one virus and the eleventh segment in the other virus.

Introduction

Rotavirus derived from rabbits was described by Bryden *et al.* in 1976 (2), Petric *et al.* in 1978 (16), Peeters *et al.* in 1982 (15) and Castrucci *et al.* in 1985 (3). Sato *et al.* (18) have isolated a rotavirus from rabbits with diarrhea. Serological surveys of commercial rabbitries in Canada (16), the U.S.A. (4) and Japan (19) showed most young adult and adult rabbits

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had evidence of prior infection with rotavirus indicating widespread occurrence of rotavirus in rabbits.

Thouless *et al.* (21) using a fluorescent foci neutralization test showed that rotavirus from a diarrheic rabbit was serologically distinct from several rotaviruses isolated from humans, calves, piglets, foals, lambs and mice. The rabbit rotavirus of Sato *et al.* (18) differed from the Lincoln strain of calf rotavirus. Since these earlier studies, rotaviruses have been classified into several groups which are serologically unrelated (13, 14). Group A rotaviruses are further divided into subgroups. The subgrouping antigen is located on the the major inner capsid protein which is coded for by the sixth genome segment (12). There are three, possibly more subgroups (10). Rotaviruses are also classified into serotypes. A glycosylated polypeptide, VP7, coded for by the eighth or ninth segment depending, on the strain, and VP3, both on the outer capsid protein, are the antigenic determinants of the serotype (5, 12). Four human serotypes have so far been described and three additional animal and avian ones delineated (7, 10). A number of animal rotaviruses including two simian, one canine and one equine isolate belong to subgroup I and serotype 3 while the human serotype 3 viruses belong to subgroup II.

In the course of our investigations on rotavirus infections in rabbits, we isolated two rotaviruses, from diarrheic rabbits in two separate colonies. We determined the subgroup and serotype of the viruses and examined their RNA electrophoretically.

Materials and Methods

Cell Culture

MA 104 cells were used to isolate and propagate rotaviruses.

Viruses

The following viruses were used in this study: lapine rotaviruses (L:C11:83) and (L: ALA:84); porcine rotavirus (OSU strain P:U.S.A.77:1) obtained from G. N. Woode, Iowa State University, simian rotavirus (S:U.S.A.:79:2) obtained from N. Schmidt, California State Health Dept., Berkeley; canine rotavirus (LSU 79C-36) obtained from G. N. Woode; human rotavirus serotype 3 (H:1463:82) and serotype 1 (H:750:82) were isolated by M. E. Thouless from children with diarrhea in Seattle.

Diagnostic ELISA Test

A capture assay was used for detection of rotavirus. One half of a Costar (Costar, 205 Broadway, Cambridge, MA) microelisa plate was coated with rabbit hyperimmune antirotavirus serum (#720) while the other half was coated with preimmune serum as a control for non specific binding. Ten percent suspensions of stool samples were made in tissue culture medium without calf serum. Seventy five µl of diluent (PBS/Tween 20 0.1 percent, gelatin 0.1 mg/ml) were added to each well. Twenty five µl of stool suspension were added to 2 wells coated with preimmune serum and to 2 wells coated with anti-rotavirus serum. The antigen coated plates were incubated for 1 hour at

37° C. Horseradish peroxidase conjugated antibody to rotavirus (DAKO Corporation, 22F North Milpas St. Santa Barbara CA 93103.) was added and incubated for 1 hour at 37° C. The orthophenylenediamine substrate was added for 30 minutes, then stopped with 8 M H_2SO_4 and the optical density (OD) was read on a Flow Multiscan spectrophotometer at 492 nm. OD readings of >0.1 with control OD values of <0.05 were regarded as positive based on results with terminal dilution of tissue culture virus.

Subgrouping ELISA Test

A microelisa plate was coated with hyperimmune antibody to rotavirus (#720). Twenty five µl of stool suspension as described above were added to two wells on the top half of the plate and to two wells on the bottom half of the plate. After incubation and washing the subgrouping monoclonal antibodies (kindly provided by H. B. Greenberg, Stanford University), diluted 1:100,000 were added (subgroup I to the top of the plate and subgroup II to the bottom of the plate). The test was completed as above. OD readings of <0.1 were obtained with the heterologous subgrouping antibody while the OD with the homologous antibody was proportional to the amount of antigen present.

Tissue Culture Adaptation of Lapine Rotaviruses

Diarrheic stools from rabbits in commercial conventional and specific-pathogen-free (SPF) rabbitries were submitted for rotavirus diagnosis. Ten percent suspensions of specimens positive for rotavirus in the diagnostic ELISA test were made in tissue culture medium without calf serum. They were treated with trypsin at 20 µg/ml for 1 hour, then added to two tubes containing confluent monolayers of MA 104 cells. After incubation for an additional hour more serum-free tissue culture medium was added, reducing the trypsin concentration to 2 µg/ml. Tubes were placed in a revolving unit at 37° C. Cytopathic effect consistent with rotavirus infection appeared in 24 hours. The virus was passed in tubes twice before being transferred to small tissue culture flasks.

Antisera Production

Hyperimmune antisera were prepared by injecting rabbits with purified rotavirus. Approximately 40 16 oz bottles of rotavirus were grown for immunization of each rabbit. The infected cells were frozen and thawed twice and the virus precipitated by stirring the clarified tissue culture medium with 8 percent polyethylene glycol and 0.5 M NaCl overnight at 4° C. The precipitate was centrifuged at 10,000 g for 1 hour and resuspended in 0.002 M Tris-HCl with 1.5 mM $CaCl_2$ at pH 7.2. The virus was centrifuged through 45 percent wt/vol sucrose for 90 minutes at 140,000 g into a CsCl cushion. This was diluted in Tris buffer and the virus pelleted at 180,000 g for 60 minutes and resuspended in saline. Between 10^8 and 10^9 fluorescent cell forming units were mixed with Freund's incomplete adjuvant and injected intramuscularly into a rotavirus-seronegative rabbit. After 3 weeks the rabbit was injected intravenously with small doses of the partially purified virus daily for 4 days and serum collected one week later.

Detection of Rotavirus Infected Cells by Immunofluorescence

This technique has been described in detail by BRYDEN *et al.* (1). Briefly, MA 104 cell monolayers in microtiter plates were infected with a suspension of rotavirus, without the addition of trypsin. The microtiter plates were centrifuged at 2000 g for one hour and incubated overnight at 37° C. The medium was removed and the monolayers fixed with methanol. Monolayers were incubated with a 1:500 dilution of rabbit antirotavirus serum (#720). After washing, the infected monolayers were incubated with fluorescein conjugated sheep anti-rabbit gamma globulin diluted 1:300

(Nordic Immunologicals Ltd. Box 1390, El Toro, Ca 92630). They were viewed under blue light on a Zeiss microscope with a vertical illuminator. Specific fluorescence appeared in the cytoplasm of the cells.

Neutralization of Fluorescent Foci

This procedure was performed as described by THOULESS *et al.* (21). Briefly, 50 µl volumes of virus diluted to give 200—300 fluorescent cells per well were mixed with 50 µl of serial dilutions of the antisera to be tested. Fourfold dilutions were made of homologous serum. Mixtures were incubated for 1 hour at 37° C and added to a microtiter plate with confluent monolayers of MA 104 cells. The plates were centrifuged, incubated, fixed, stained and viewed as described above. The procedure has been modified from that previously reported, in that, the number of fluorescent foci has been increased. This makes reading the end point, where over 50 percent of the fluorescent cells have been eliminated, a more rapid procedure rather than the two hours per plate required to count every cell. It is equally reproducible.

Electropherotyping of Rotavirus RNA

The technique used is an adaptation of the method reported by RODGER and HOLMES (17).

RNA Extraction

0.5 ml of 0.1 M sodium acetate containing 1 percent SDS and 0.5 ml of a phenol chloroform mixture (Phenol, m-cresol, 8-OH quinoline, chloroform and isoamyl alcohol) were added to either 50—100 mg of feces or 1 ml of rotavirus infected tissue culture medium. The mixture was shaken on a vortex mixer and centrifuged in an Eppendorf centrifuge for 5 minutes. The supernatant was reextracted with chloroform to remove traces of phenol. If this double stranded RNA preparation failed to give strong enough bands on electrophoresis, it was concentrated by salting out with 3 M sodium acetate and precipitated with cold absolute alcohol.

Polyacrylamide Gel Electrohporesis

A Laemmli discontinuous system was used with SDS omitted from all the buffers. A 10 percent polyacrylamide separating gel and a 3.5 percent stacking gel were used. Forty µl of RNA extract mixed with 20 µl of 0.5 M Tris buffer, glycerol and bromophenol blue were added to the gel and electrophoresed at 10 mA/gel for 16 hours in a water cooled slab gel apparatus (Hoffer Scientific Products).

Silverstain

The method used was that of HERRING *et al.* (9). The gel was placed in 200 ml of 10 percent ethanol and 0.5 percent acetic acid for 60 minutes. This was decanted and 0.011 M silver nitrate added. This was gently rocked for 30 minutes and washed 4 times with distilled water. The developing solution (NaOH in formaldehyde solution) was added for 5—10 minutes until the bands were stained. The gel was washed well in water and stored in clear plastic wrap at 4° C in a damp box until photographed.

Results

Tissue Culture Adaptation of Lapine Rotaviruses

Diarrheic stool samples from weanling rabbits (4—8 weeks old) in a commercial rabbitry that supplied the University of Washington were found to be positive for rotavirus by the diagnostic ELISA test. Specimens

were preincubated with trypsin and inoculated onto monolayers of MA 104 cells in rolling tubes. Cytopathic effect compatible with rotavirus infection appeared in the first inoculated cultures and the viruses were transferred to tissue culture flasks after only two passages in tubes. Only one isolate (referred to as C11) was further characterized as it was found that virus in all the stools inoculated onto tissue culture had the same electropherotype. Rotavirus (referred to as ALA) was also isolated from intestinal contents (submitted by T. R. Shoeb of the University of Alabama) of 4 preweanling rabbits which died of diarrheal disease in a specific-pathogen-free colony in Tennessee.

Subgrouping of Lapine Rotaviruses

Tissue culture adapted rabbit rotaviruses were tested in a subgrouping ELISA test using monoclonal antibodies. The results (Table 1) clearly showed that both rabbit rotaviruses belonged to subgroup I.

Table 1. *Subgrouping of lapine rotaviruses by ELISA using monoclonal antibodies*

	Monoclonal antibody	
Rotavirus	SGI	SGII
C11	1.413	0.062
ALA	1.891	0.050

Optical densities at 492 nm. The values are the mean of three sets of experimental results

Serotyping of Rabbit Rotavirus

Hyperimmune antisera was prepared against lapine rotavirus (C11), which had been grown in tissue culture and purified by centrifugation through sucrose, in a rabbit which was seronegative for rotavirus. Hyperimmune antisera to porcine (OSU), canine (K9) and human serotype 1 (750) and 3 (1463) rotaviruses had previously been prepared (unpublished). The human isolates 750 and 1463 were designated serotype 1 and 3 respectively on the basis of neutralization by sera (#720 and #285) prepared previously by one of the authors (7). Cross neutralization of the isolates with antisera by the fluorescent focus neutralization test revealed that the two lapine rotaviruses belonged to serotype 3 (Table 2). The antiserum to human serotype 3 virus neutralized rotavirus C11 to high titer. This titer was 64-fold greater than with the antiserum to porcine rotavirus and 256-fold greater than with the antiserum to human serotype 1 virus. The antiserum to canine rotavirus (serotype 3), showed only a 4-fold difference. However, there was a major one way cross reaction, as antibody to the rabbit rotavirus C11 neutralized itself to very high titer but neutralized the human serotype 3 128-fold less and the canine virus 32-fold less. Rabbit rotavirus ALA was

also neutralized to a very high dilution with antiserum to the human serotype 3 virus but by a 64-fold less dilution of sera to C11 or K9 rotaviruses. Hence both these rabbit rotaviruses show strong one way cross reactions within serotype 3 and are quite distinct serologically from one another. These tests were repeated several times and were highly reproducible, never differing by more than one dilution.

Table 2. *Neutralization of fluorescent foci produced in MA 104 cells by hyperimmune sera*

	Antisera				
Rotavirus	Lapine (C11)	Canine (K9)	Human 3 (1463)	Human 1 (750)	Porcine (OSU)
Lapine (C11)	327,680	81,920	>327,680	1,280	5,120
Lapine (ALA)	5,120	5,120	>327,680	320	1,280
Canine (K9)	10,240	163,840	>327,680	320	5,120
Human 3 (1463)	2,560	5,120	>327,680	160	5,120
Simian (MMU)	2,560	20,480	>327,680	320	2,560
Human 1 (750)	640	5,120	5,120	81,920	640
Porcine (OSU)	<80	2,560	5,120	<80	163,840

The numbers are the reciprocals of the serum dilutions giving a 50 percent or greater reduction in the numbers of fluorescent foci

Rabbit Rotavirus RNA Electropherotype

RNA was extracted from rabbit rotavirus C11, electrophoresed in polyacrylamide gel and silver stained. It was compared with rotaviruses used in the cross neutralization experiments (Fig. 1). The pattern is different from the more typical pattern of the other viruses. KALICA *et al.* (11) described the division of rotavirus RNA segments into four size classes. The sequence of bands they showed and that shown for non rabbit rotaviruses in Fig. 1 could be described as 4, 2, 3, 2 whereas C11's could be described as 4, 3, 3, 1. One of the smaller segments, the tenth has greatly reduced mobility. The ALA rotavirus also has an unusual RNA electropherotype. It could be described as 4, 2, 4, 1 with greatly reduced mobility of the eleventh segment.

Discussion

A strain of rotavirus from rabbits was isolated in tissue culture by SATO *et al.* (18). It cross reacted with the Lincoln strain of calf rotavirus by immunofluorescence but not by neutralization indicating that it was a different serotype than the bovine virus. The strain isolated by CASTRUCCI *et al.* (3) did cross react with a different strain of bovine rotavirus. THOULESS *et al.* (21) previously showed that a strain of lapine rotavirus was serotypically different from 6 rotaviruses from other species. The two strains of lapine

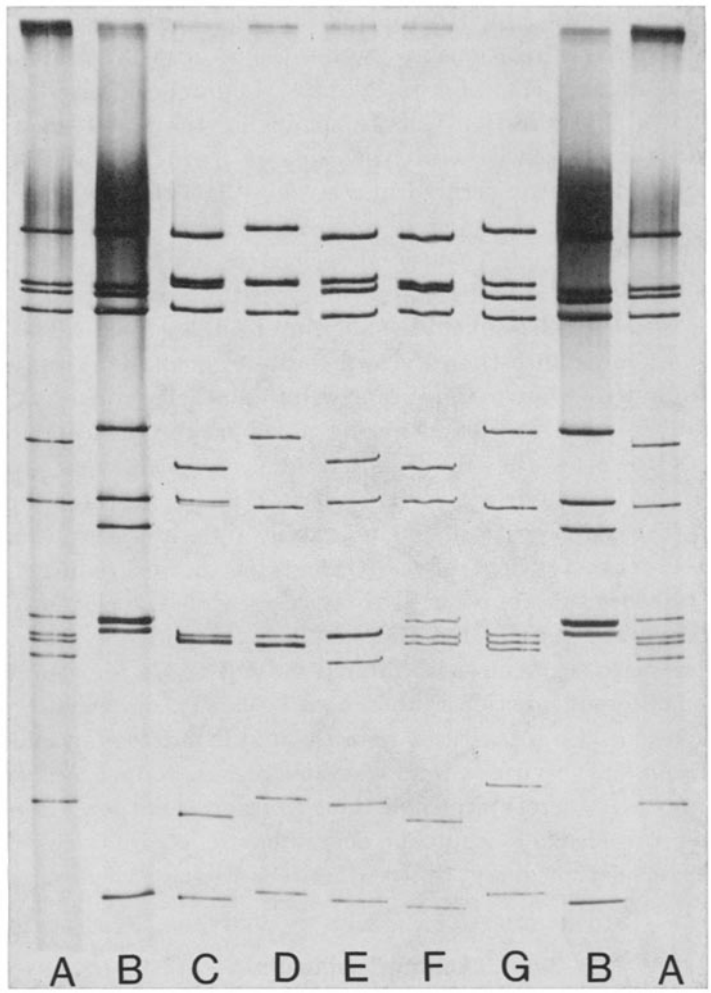

Fig. 1. Comparison of RNA electropherotypes of two strains of lapine rotavirus with rotavirus RNA from other species. Rotavirus RNA was extracted from infected tissue culture media and electrophoresed in a 10 percent polyacrylamide gel and silver stained. *A* lapine rotavirus ALA; *B* lapine rotavirus C 11; *C* human rotavirus (serotype 3); *D* simian rotavirus MMU; *E* human rotavirus (serotype 1) 750; *F* porcine rotavirus OSU; *G* canine rotavirus K 9

rotavirus isolated and described in this report are both subgroup I and serotype 3. However the serum to one virus neutralized the other to a 64-fold lower titer than itself. In the absence of antiserum to human serotype 3 these two lapine rotaviruses would have been considered separate serotypes as there is a greater than 20-fold difference between the homologous and heterologous titers. This type of one way cross reaction was also observed between these same strains of simian and canine rotavirus (8). It

has also been described by Hoshino *et al.* (10). They present a diagram attemping to map the relationships within the serotype 3 group of viruses. It is unclear where the rabbit rotaviruses should be inserted. With the fluorescent foci neutralization test we got higher titers and greater differentials than they observed with their plaque reduction assays. This is contrary to what was conjectured in a recent WHO report (23) on nomenclature.

Subgroup I viruses have been described as having a "short" RNA pattern and subgroup II as having a "long" pattern as a result of reduced mobility of the eleventh segment in subgroup I viruses (6, 20). This segment moves a shorter distance than its own tenth segment. Pararotaviruses or Group B rotaviruses have significantly different electropherotypes (22). The two rabbit rotavirus isolates belong to subgroup I hence are Group A rotaviruses. However, they have different electropherotypes from other subgroup I and II rotaviruses so far described and from the rat pararotavirus (22). The RNA pattern of the rabbit rotavirus strains are quite distinct. The tenth segment of the C11 strain showed reduced mobility compared to other subgroup I or II rotaviruses while the eleventh segment of the ALA strain showed reduced mobility. We do not yet not know whether these two segments with altered mobility code for a protein with the same or different function in these two viruses.

In conclusion, two rotaviruses were isolated from stool specimens from diarrheic rabbits. The viruses were classified as subgroup I and serotype 3 although they were serotypically distinct from one another. RNA electropherotyping revealed 11 segments characteristic of rotavirus, however, the pattern was a typical and differed between the two viruses.

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