

## Serotypes of Bovine Rotaviruses Distinguished by Serum Neutralization

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Fourteen cytopathic bovine rotaviruses isolated from naturally affected calves were characterized serologically by serum neutralization. Antisera were prepared in guinea pigs against the three isolates and the Lincoln strain of bovine rotavirus. These four rotaviruses were segregated into at least two distinct serotypes (designated types 1 and 2) by serum neutralization. The remaining 11 isolates were also placed into two groups according to neutralization specificity by using the antisera against types 1 and 2 bovine rotavirus. When colostrum-deprived calves were experimentally inoculated with type 1 or 2 bovine rotavirus, respectively, the calves produced neutralizing antibodies against the inoculated serotype but not against the other serotype. Calf sera obtained from six herds with naturally occurring rotavirus infections possessed neutralizing antibodies against type 1 or 2 or both. Thus, distinct serotypes of bovine rotavirus were demonstrated by serum neutralization. The possible role of serotypes in the epidemiology of rotavirus infection among bovine species is discussed.

Rotavirus has been shown to be a major cause of acute viral diarrhea in a number of mammalian and avian species (6, 13, 14). Several serotypes of human and avian rotaviruses have been distinguished by serum neutralization (SN), enzyme-linked immunosorbent assay, complement fixation (CF), and immune electron microscopy (1, 5, 14, 23, 30-33); in addition, two subgroups of animal rotaviruses have been detected by immune adherence hemagglutination, specific CF, and enzyme-linked immunosorbent assay (10). However, it has been suggested by Kapi-kian et al. that the term serotype be defined by using conventional SN, excluding fluorescent focus neutralization, in cell cultures (10). As a result, human rotavirus neutralization serotypes recently have been successfully determined by plaque reduction and qualitative cytopathic effect (CPE) neutralization assays, employing cultivatable or non-cultivatable human rotaviruses (or both) that had been previously rescued by coinfection with a cultivatable bovine rotavirus (8, 9, 25, 29). In the present study, we also attempted to use conventional SN for serotyping bovine rotaviruses on the basis of CPE in the cell culture.

Although a number of reports have described certain differences among rotavirus strains, for example, histopathological virulence, plaque morphology, pattern of neutralizing kinetics, hemagglutination inhibition property, and electrophoretic mobility of the corresponding genome segments (2-4, 18, 19, 22, 26), no dis-

tinct serotypes have been determined by SN.

In a preliminary report, we provided evidence that antiserum against the Lincoln strain of bovine rotavirus showed different neutralizing behaviors toward the rotaviruses isolated from calves with acute diarrhea, and we suggested the presence of different types of bovine rotavirus which can be discriminated by SN (17).

In the present study, we attempted to confirm the existence of distinct serotypes of bovine rotavirus by SN by using guinea pig immune sera and experimentally infected calf sera. Furthermore, we surveyed the neutralizing antibodies in calf sera obtained from naturally affected herds.

### MATERIALS AND METHODS

**Cells and viruses.** An established fetal rhesus monkey kidney cell line (MA104) was used throughout this study. Propagation of MA104 cells was performed as previously described (12, 17). The maintenance medium adopted for the cells after virus inoculation was of the same composition as the growth medium (12, 17) except that it contained 0.11% bovine serum albumin instead of fetal calf serum.

Fourteen cytopathic bovine rotavirus isolates, including three viruses used for immunization, were isolated from the feces of calves from seven herds affected by acute diarrhea in Japan. Procedures for isolation and identification of the isolates have been previously described (17). All the isolates were passaged more than three times by means of the terminal dilution method and used for this study at passages six to eight on MA104 cells in the presence of 1 µg of trypsin per ml (type 1, Sigma Chemical Co., St. Louis,

Mo.). The Lincoln strain of bovine rotavirus (15) was also grown on MA104 cells in the presence of trypsin.

**Assay for viruses.** Confluent tube cultures of MA104 cells were inoculated with a serial 10-fold dilution of virus, 0.1 ml per tube and four tubes per dilution. After adsorption for 60 min at 37°C, the inocula were removed, and 0.5 ml of maintenance medium containing 1 µg of trypsin per ml was added to each tube. Incubation was continued for 7 days at 37°C in a roller apparatus. The infectivity titer was expressed as the median tissue culture infective dose on the basis of CPE.

**Preparation of antisera.** Antisera were prepared in guinea pigs which had been shown to be free of rotavirus antibody by CF. These guinea pigs were purchased from the Nippon Institute for Biological Science, Tokyo, Japan.

Purification and immunization of the rotavirus were performed as previously described (17). Briefly, rotavirus propagated on MA104 cells was concentrated by polyethylene glycol (molecular weight, 7,500; Showa Chemicals Co., Tokyo, Japan) and then treated twice with fluorocarbon. The virus was then purified by density gradient centrifugation with cesium chloride solution. The purified virus suspended in phosphate-buffered saline (pH 7.2) was mixed with an equal volume of Freund incomplete adjuvant and injected into the footpads of guinea pigs three times at 2-week intervals. The animals were bled 1 week after the last injection. Antisera were inactivated at 56°C for 30 min before testing.

SN. SN was performed by mixing equal volumes of a serial twofold dilution of the sera with the virus suspension diluted to give 200 median tissue culture infective doses per 0.1 ml. The diluent employed for the serum and virus dilution was maintenance medium. The mixture was incubated at 37°C for 60 min, followed by 4°C overnight, and inoculated into four tube cultures of confluent monolayers of MA104 cells. After incubation at 37°C for 7 days in the roller apparatus, the infectivity was estimated, and the neutralizing antibody titer was expressed as the reciprocal of the serum dilution which inhibited 50% of infection. In the serological survey, the neutralizing antibody titer was expressed as the reciprocal of the highest serum dilution that completely inhibited CPE when the two tube cultures of MA104 cells were used for each dilution.

CF. CF was conducted by the microtiter method with two 100% hemolytic units of complement as previously described (12, 17). The CF antigen titer was determined by checkerboard titration with the antiserum.

**Animals.** For experimental infection with the bovine rotaviruses, three colostrum-deprived newborn calves which had been shown to be free of neutralizing antibody to any serotypes of the bovine rotaviruses were used within 24 h after their birth; they were designated 1, 2, and 3. The calves were kept separate from each other and fed substituted milk which had been inactivated by heating. They were inoculated orally with 10 ml of infected culture fluid of one of the serotypes. Clinical signs were observed, and blood samples were collected weekly for 4 weeks.

**Sera from field cases.** A total of 66 serum samples were obtained from 2- to 3-month-old calves from six herds that had been affected naturally by rotavirus.

The rotaviruses could be demonstrated by electron microscopy or isolation on MA104 cells from fecal samples of these herds at the relevant time. All sera were inactivated at 56°C for 30 min before examination. Subsequent serial twofold dilutions were performed on sera initially diluted 1:10.

## RESULTS

### Serological relationship among bovine rotaviruses by SN and CF with guinea pig immune sera.

Antisera were prepared against three viruses, designated II-2, KK-3, and KN-4, isolated from different herds and the Lincoln strain of bovine rotavirus. The results of SN and CF are shown in Table 1. No antisera revealed more than twofold variation in the CF antibody titers against these four prototype viruses (1:2,560 to 1:5,120). Homologous titers of the antisera in SN were 1:1,810, 1:752, 1:1,613, and 1:1,039 against the Lincoln, II-2, KK-3, and KN-4 viruses, respectively. Cross-reaction in SN was observed between the Lincoln and II-2 viruses but not between these two viruses and KK-3 virus. Antisera against the Lincoln and II-2 viruses neutralized the KN-4 virus, but antiserum against the KN-4 virus failed to neutralize these two viruses. Partial cross-reactivity was observed between the KK-3 and KN-4 viruses by SN. Table 1 includes a proposal of designated serotypes of the bovine rotaviruses: type 1 is represented by the Lincoln and II-2 viruses, type 2 by the KK-3 virus, and, tentatively, type 3 by the KN-4 virus.

**Comparison of bovine rotavirus isolates by SN with guinea pig immune sera.** An attempt was made to compare the 11 bovine rotavirus isolates according to the neutralizing specificity of bovine rotavirus type 1 and 2 antisera (Table 2).

TABLE 1. Serological relationship among bovine rotaviruses by SN and CF tests<sup>a</sup>

Virus	Test employed	Reciprocal antibody titer to antiserum against:				Proposed sero-type by SN
		Lincoln	II-2	KK-3	KN-4	
Lincoln <sup>b</sup>	SN	<b>1,810</b>	135	<20	<20	1
	CF	<b>2,560</b>	2,560	2,560	5,120	
II-2	SN	1,839	<b>752</b>	<20	<20	1
	CF	5,120	<b>2,560</b>	5,120	5,120	
KK-3	SN	<20	<20	<b>1,613</b>	40	2
	CF	5,120	5,120	<b>5,120</b>	5,120	
KN-4	SN	4,150	1,754	469	<b>1,039</b>	3 <sup>c</sup>
	CF	5,120	5,120	5,120	<b>5,120</b>	

<sup>a</sup> Antiserum was prepared in guinea pigs. Homologous values are in boldface type.

<sup>b</sup> Reference strain (15).

<sup>c</sup> Tentative designation.

TABLE 2. Comparison of 11 bovine rotavirus isolates by SN with guinea pig immune sera<sup>a</sup>

Isolate	Reciprocal neutralizing antibody titer to the following antisera:			
	Lincoln <sup>b</sup> (type 1)	II-2 (type 1)	KK-3 (type 2)	KN-4 (type 3)
AS-14	2,106	10,240	<20	<20
OY-3	4,276	507	<20	<20
II-1	1,016	1,014	<20	<20
II-3	160	68	<20	<20
II-4	2,560	640	<20	<20
II-5	1,164	NT <sup>c</sup>	<20	<20
KA-10	<20	<20	1,023	<20
OY-5	<20	<20	1,280	<20
OY-7	<20	<20	508	<20
OY-8	<20	<20	288	<20
OY-9	<20	<20	281	<20

<sup>a</sup> Homologous antibody titers of antisera against II-2, KK-3, and KN-4 viruses are shown in Table 1. The type designations in parentheses are those proposed in Table 1.

<sup>b</sup> Data reported previously (17). The homologous antibody titer of antiserum against Lincoln virus was 1:5,953. Lincoln is the reference strain (15).

<sup>c</sup> NT, Not tested.

The 11 isolates were clearly placed into two groups; six isolates were neutralized by type 1 rotavirus antisera but not by type 2 rotavirus antiserum, and the remaining five isolates were inversely neutralized or not by these antisera. However, no isolates were neutralized by the tentative type 3 rotavirus antiserum.

Of the 11 isolates, isolates OY-3, 5, 7, 8, and 9 were derived from a single outbreak of diarrhea in the same herd. One of the isolates, OY-3, was shown to be serologically different from the other four isolates, in so far as these isolates

were examined, by antisera to type 1 and 2 rotavirus.

**Neutralizing antibody in experimentally infected calves with type 1 or 2 bovine rotavirus.** Development of neutralizing antibody was examined by SN in colostrum-deprived newborn calves which had been inoculated with type 1 or 2 bovine rotavirus (Fig. 1). Calves 1 and 2 were orally inoculated with 10 ml of infected culture fluid of type 2 (KK-3) rotavirus at a titer of 10<sup>6</sup> median tissue culture infective doses per ml. Calf 3 was orally inoculated with type 1 (AS-14) rotavirus at the same volume and titer. The AS-14 rotavirus was used as one of the type 1 bovine rotaviruses according to neutralization specificity by SN, although the results of cross-SN for this virus are not shown here. All the calves manifested clinical signs of diarrhea within 1 to 2 days after inoculation, and symptoms continued for 4 to 6 days. Calves 1 and 2 produced antibodies against type 2 (KK-3) rotavirus at the respective titers of 1:452 and 1:47 at 4 weeks after inoculation but not against type 1 (II-2) rotavirus at a titer of <1:10 throughout this experiment. On the other hand, calf 3 produced antibodies against type 1 (II-2 and AS-14) rotaviruses at the respective titers of 1:180 and 1:640 at 4 weeks after inoculation, although it did not produce antibodies against type 2 (KK-3) rotavirus throughout this experiment.

**Survey of neutralizing antibody to types 1 and 2 bovine rotavirus in calf sera from field cases.** A total of 66 sera obtained from 2- to 3-month-old calves were examined for neutralizing antibody response to types 1 (II-2) and 2 (KK-3) bovine rotavirus (Fig. 2). Four sera which possessed antibodies against type 2 rotavirus at titers of 1:80 and  $\geq$ 1:320 failed to neutralize type 1

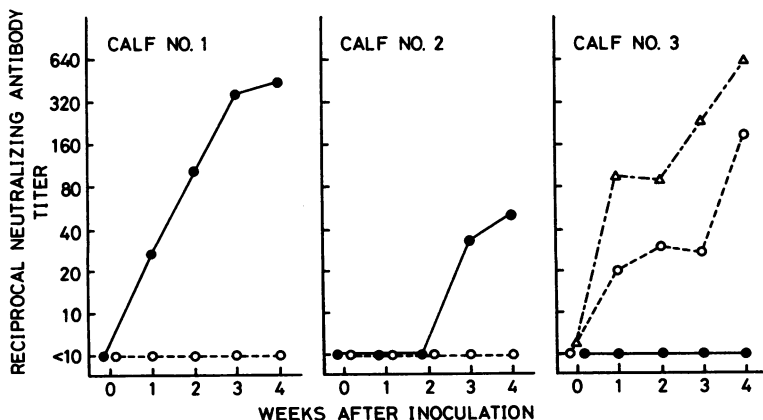


FIG. 1. Development of neutralizing antibodies in newborn calves inoculated with type 1 or 2 bovine rotavirus. Calves 1 and 2 were inoculated orally with type 2 (KK-3), and calf 3 was inoculated orally with type 1 (AS-14) virus. The type designations are proposed in Table 1. Symbols indicate neutralizing antibody titers against type 1 virus (○, II-2; △, AS-14) and type 2 virus (●, KK-3).

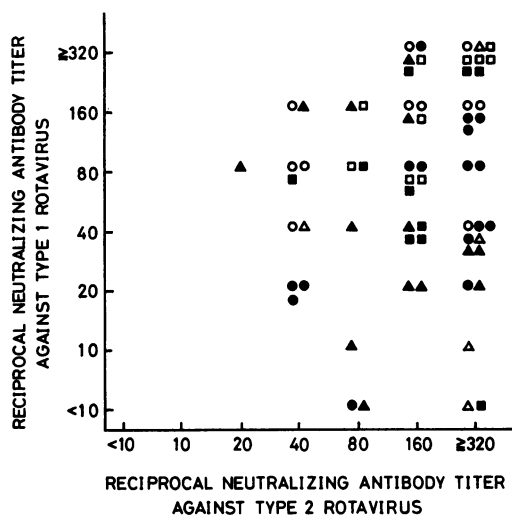


FIG. 2. Relationship between neutralizing antibody titers against bovine rotavirus types 1 and 2 in calf sera collected from six herds naturally affected by rotavirus. The type designations are proposed in Table 1. Symbols indicate sera derived from the same herd.

rotavirus at titers of  $<1:10$ . The remaining sera, however, possessed antibodies against both types 1 and 2. In addition, such reactivity of sera against both types 1 and 2 was observed even in the same herds.

### DISCUSSION

Although many reports have described different properties among bovine rotavirus strains (2-4, 18, 19, 22, 26), no report has been published about the distinct serotypes of bovine rotavirus by SN except our preliminary report, which suggested the presence of different types of bovine rotavirus (17). In the present investigation with bovine rotaviruses obtained after serial passages by means of the terminal dilution method, we successfully distinguished at least two distinct serotypes of bovine rotavirus by cross-SN with guinea pig immune sera. Similar results were also obtained with these immune sera and plaque-purified bovine rotaviruses (unpublished data). These serotypes were not segregated by conventional CF, which reacts with a common group-specific antigen of the rotavirus (24, 28), but were segregated by SN. The 11 bovine rotavirus isolates were also clearly placed into two groups by using guinea pig immune sera against bovine rotavirus types 1 and 2.

Calves experimentally inoculated with one of the two distinct bovine serotypes (types 1 and 2) produced neutralizing antibodies against only the homologous type of the rotavirus. However, no antibody response was observed against the heterologous type of rotavirus. This result sug-

gests that postinfection sera in the natural host do not allow cross-immunization among the bovine rotavirus serotypes. Although several trials to prevent bovine rotavirus infection by vaccine in calves have been attempted (11, 16, 21), the existence of bovine rotavirus serotypes presents a new problem in the immunoprophylaxis of the infection.

The results of a survey for neutralizing antibodies against rotavirus types 1 and 2 in calf sera revealed that some calves possessed antibodies against a single type of bovine rotavirus. These cases may result in the occurrence of a single infection caused by one of the serotypes in the field. Almost all the calves, however, possessed antibodies against both types 1 and 2. Since no cross-reaction was observed between types 1 and 2 from the results of experimental infection in the present study, it was considered that the calves might have been independently infected with either type 1 or 2.

Rodger and Holmes reported that different bovine rotaviruses were obtained in one place during a single outbreak of diarrhea according to the electrophoretic migration patterns of genome segments (19). The same observation was presented in another report (26). Although we did not elucidate the relationship between the serotypes and the electropherotypes of genome segments, we were able to show the coexistence of distinct types of bovine rotavirus during a single outbreak of the disease. In the present study, one of the isolates (OY-3) proved to be serologically different from the other isolates of the same herd by SN with antisera against rotavirus types 1 and 2. Early epidemiological findings suggested a possible role of distinct serotypes in recurrent or sequential infections caused by rotavirus in human cases (7, 20). It was also found in the case of bovine disease that recurrent outbreaks of rotaviral diarrhea are common in affected herds (27). Consequently, we think that the coexistence of different types of rotavirus may be responsible for recurrent outbreaks of the disease in bovine species as well as in humans (7, 20).

One of the prototype viruses, KN-4, was neutralized by antisera against bovine rotavirus types 1 and 2, although the antiserum of the KN-4 virus failed to neutralize all the bovine rotaviruses used in this study except KK-3 virus, which was neutralized at a low titer. Based on this result, we conjectured that the KN-4 virus may be a different serotype from type 1 and 2 rotaviruses; therefore, we tentatively designated the KN-4 virus type 3 bovine rotavirus.

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