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Serologic and electropherotypic characterization of the bovine rotaviruses isolated in Korea

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국내분리 소 로타바이러스의 혈청학적 특성

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초 록 : 국내분리 소 로타바이러스의 genomic RNA 형태, 분리주간의 교차면역반응 그리고 단클론 항체를 이용한 중화시험에 의해 혈청형을 조사한 바 다음과 같은 결과를 얻었다. 국내분리주들의 genomic RNA 형태는 크게 NCDV 형태(7/11 시료)와 non-NCDV 형태(4/11 시료)의 두가지로 나타났다. 교차면역시험에서 표준주인 NCDV주에 대한 양성혈청은 국내분리주들에 대해서는 비교적 낮은 중화력을 나타내었으나 국내분리주에 대한 양성혈청들은 타 국내분리주 뿐만 아니라 NCDV주에 대해 서로 높은 중화력을 나타내었는데, 분리주 중 678, P44, M4에 대한 양성혈청은 NCDV를 포함한 대부분의 분리주에 대해 100%의 중화력을 나타내었다. 또한 288주에 대한 양성혈청은 288, 678,P44, M4주에 대해서는 높은 중화력을 나타내었으나 다른 분리주들에 대해서는 비교적 낮은 중화력을 나타내었다.

국내분리주의 단크론 항체를 이용한 G혈청형 감별결과는 G6 유사형이 45.8%(11/24주), G10 유사형이 54.2%(13/24주)로서 두가지 G형이 존재하였다. 한편 G6형에 반응한 것들의 P형은 표준주인 NCDV주(P1)와는 다른 것으로 확인되었으며, G10 유사형에 속하는 14주는 모두 P11 혈청형으로 판명되었다.

Key words : bovine rotavirus, electropherotype, serotype.

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Introduction

The relationship between rotavirus and calf diarrhoea was first revealed by Mebus *et al*[1] in 1969. The infection in calf is dependent upon age, by which results in the incubation period of 15 hours to 3 or 4 days. Clinical symptoms are depression, anorexia, diarrhoea and dehydration[2].

Serotypic characterization of group A rotaviruses is based on the neutralization determinants located on two outer capsid proteins, VP4(a protease-sensitive protein that determines the P-serotype specificity) and VP7(a glycoprotein that determines the G-serotype specificity)[3]. A total of 14G serotypes has been described to date, and serotypes G6 and G10 have been reported as the most common types found in cattle[4].

Kim *et al*[5] reported that some field isolates were serologically different, representing at least two serogroups[5] in Korea.

While Lyoo *et al*[6] reported that serotype and electropherotype of field isolates were comparable to NCDV strain, the prototype bovine rotavirus.

In this study, the authors tried to test and characterize the serological distinction and electropherotype of rotavirus isolates from the clinical specimens.

Materials and Methods

Cells and Viruses : Bovine rotavirus(BRV) was isolated from diarrheic fecal sample or intestinal contents of calf in MA-104(Monkey kidney cell line) cells as described previously[6]. Briefly, 10% suspensions of samples were prepared in minimum essential medium(α-MEM, Gibco), clarified by centrifugation at 1,000g for 30 minutes, and filtered through 0.4μm syringe filters(Acrodisc, Gelman). The inoculum was pretreated with 10μg/ml crystal trypsin(Type IX, Sigma), adsorbed onto monolayered MA-104 cell for 1 hour at 37℃ and the monolayers were rinsed twice with MEM. Fresh serum-free maintenance medium supplemented with trypsin(1~2μg/ml) and antibiotics was replaced into the culture clusters. The cultures were observed for cytopathic effects through several passages. *Bovine rotaviruses NCDV*, I-801, B223 and VMRI strains were used as reference viruses.

Electropherotyping of RNA : Bovine rotavirus genomic viral RNA was extracted from the diarrheic fecal samples of calves or virus infected cells by a modification of the procedure described by Squire *et al*[7]. The sample RNA was electrophoresed in 10% polyacrylamide gel with a constant current of 13mA for 16 hours. The gel was stained with silver nitrate.

Electron microscopy(EM) and Immunofluorescent antibody assay : BRV was partially purified from the supernatant of the virus infected cells by ultracentrifugation at 75,000g for 3 hours through 30%(w/w) sucrose cushion. The purified virus was washed with distilled water, pelleted by ultracentrifugation at 100,000g, placed on carbon-coated copper grids(#300 mesh, Agar) negatively stained with 4% uranyl acetate, and examined for viral particles using electron microscope(Model 7100FA, Hitachi, Japan).

Indirect fluorescent antibody test was carried out using BRV MAb(VP6 specific) by the procedure described by Murakami *et al*[8] and examined by fluorescent microscope (Model BH-2, Olympus, Japan).

Production of antisera and virus neutralization(VN) test : Guinea pigs(Hartly) were used for the production of antisera to four field isolates and NCDV strain. The animal was immunized with the mixtures of purified antigen(2mg/ml) and complete Freund's adjuvant(1/1, V/V). After 4 weeks, boost immunization using the antigen emulsified with incomplete Freund's adjuvant were administered to the animal. The animals were bled 2 weeks later and then sera were collected. The produced sera were inactivated for 30 min at 56℃ and antibody titers were measured by VN.

VN test was performed in MA104 cells grown in 96-well flat-form microtest plates. For neutralization, serial 10 fold dilutions of viruses with serum free culture medium were mixed with an equal volume of antisera containing VN antibody titer of 8, and incubated for 60 min at 37℃ in separate microplate. And then, neutralized mixture of virus and serum was transfered to monolayer cultured plate which was washed with PBS to remove of the serum component

and incubated for 37℃ for 3 days. Neutralized virus titers after treatment with various antisera were compared to the cross neutralization activities of sera against the other viruses tested.

Serotyping by fluorescence focus neutralization(FFN) test : FFN test with MAbs specific to G6 and G10 types of BRV was performed to determine the serotype specificity of isolates as described previously[5].

Results

Virus isolation and RNA electropherotyping : Seventeen strains of bovine rotaviruses were isolated from the calves with diarrhea.

These were further adapted in MA104 cells and then examined for elecrtopherotypes. The samples passaged about 5 to 10 times in the cell cultures developed a typical cytopathic effects in 2 or 3 day after inoculation.

In the results of electropherotyping of genomic RNA, the RNA electropherotypes of isolates were divided into two groups roughly which were NCDV type and non-NCDV type(Table 1, Fig 1). Three isolates within NCDV type had extra band between segment 4 and segment 5.

Table 1. Electropherotypes of bovine rotavirus isolates

NCDV Type	Non-NCDV type
6-4	M4
6-5	32
P44*	678
P45	0221-7
2128*	
598	
288*	

*The strain had extra band between segment 4 and segment 5.

Immunofluorescence and Electron microscope(EM) of isolates : The MA104 cells infected with bovine rotaviruses isolates showed the viral antigens localized in the cytoplasm of cells as shown in Fig 2. In EM observation, the virus particles were observed as a typical rota-shape with 60~70nm in size as shown in Fig 3.

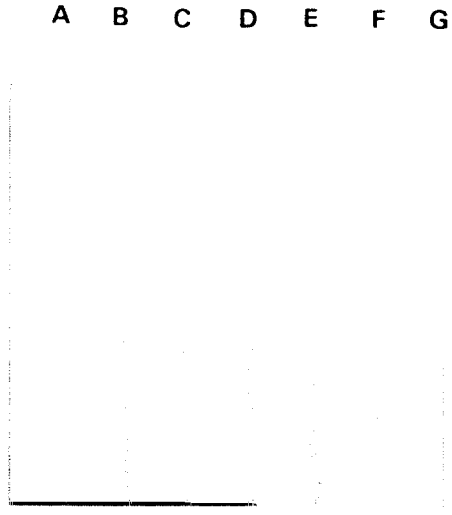

Fig 1. Electropherotypes of bovine rotavirus isolates.
A. M4 B. P45 C. P44 D. 6-4 E. 6-5 F. 2128 G. 91141

Fig 2. Immunofluorescent pattern of bovine rotavirus isolate M4 in MA104 cells using monoclonal antibody specific to VP6 of BRV.

Cross reactivity among bovine rotavirus isolates : In cross neutralization test, antiserum to NCDV strain had very low neutralizing activity against BRV field isolates but antisera to isolates 288, 678, P44 and M4 had very high cross neutralizing activities against NCDV strains(Table 3).

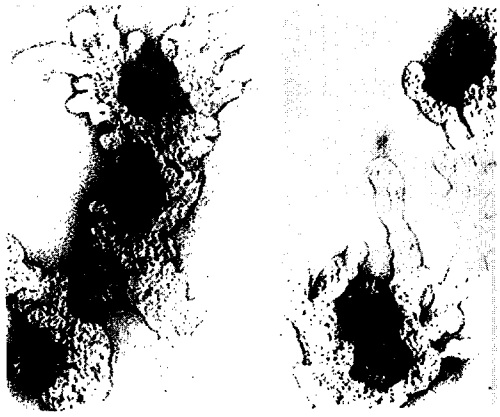

Fig 3. Electron micrograph of bovine rotavirus isolates M4(100, 000×)

Table 3. Cross reactvity of bovine rotavirus field isolates

Virus	Antisera to					
	Negative	NCDV	288	678	P44	M4
NCDV	4.0*	0.0	0.0	0.5	0.0	0.0
288	6.0	4.0	0.0	0.0	0.0	0.0
678	5.0	3.5	0.0	0.0	0.0	0.0
P44	6.0	3.5	0.0	1.0	0.0	0.0
M4	5.5	4.5	1.5	0.0	0.0	0.0
VMRI	4.0	4.0	2.5	0.0	0.0	0.0
0221-7	4.5	4.5	3.5	0.0	0.0	0.0
0221-1	5.0	5.0	4.0	1.0	0.0	0.0
2128	6.0	6.0	3.0	1.0	0.0	0.0

* Virus titers after treatment with various antisera(Log $TCID_{50}$/ml).

Serotyping of field isolates : The serotypes of field isolates were divided into two groups in G6-like type(11 strains) and G10-like type(13 strains) when determined by FFN using type-specific monoclonal antibodies(Table 4).

Table 4. Reactivity pattern of bovine retavirus isolates with G6 and G10 specific monoclonal antibodies by fluorescence focus neutralization(FFN) test

G6 specific reactive strains	G10 specific reactive strains
NCDV*, 1-801*, 598, A, C, E, 2(9), 1(6), 6(VRI-2), 7(VRI-3), 604, P44	B223*, 0221-7, 288, M4, VMRI*, 678, 32, 02771-1, 91181, 48, 6-5, 28, 90065, 592, 101

* Reference strains.

Nevertheless P type of field isolates grouped in G6-like type was unknown, but was different from that of NCDV(G6P1) strain. However, all 14 field strains grouped in G10-like type were subsequently identified as P11-like type(Table 5).

Table 5. Reactivity pattern of bovine rotavirus isolates with P specific monoclonal antibodies by fluorescence focus neutralization test

Viruses	Monoclonal antibodies				
	Serotype G6				Serotype G10
	16D3(P1)	5E1(P?)	4C2(P?)	MA49(P?)	C11F6(P11)
NCDV*	+	-	-	-	-
I-801*	+	-	-	-	-
598	-	+	+	+	-
A	-	+	+	+	-
C	-	+	+	+	-
E	-	+	+	+	-
2(9)	-	+	+	+	-
1(6)	-	+	+	+	-
6(VRI-2)	-	+	+	+	-
7(VRI-3)	-	+	+	+	-
6-4	-	+	+	+	-
6-5	-	+	+	+	-
P44	-	+	+	+	-
B223*	-	-	-	-	+
0221-7	-	-	-	-	+
288	-	-	-	-	+
M4	-	-	-	-	+
VMRI	-	-	-	-	+
678	-	-	-	-	+
32	-	-	-	-	+
0277-1	-	-	-	-	+
91181	-	-	-	-	+
48	-	-	-	-	+
28	-	-	-	-	+
90065	-	-	-	-	+
592	-	-	-	-	+
101	-	-	-	-	+

* bovine rotavirus standard strain.

Discussion

It was been known that simultaneous use of FFN serotyping and RNA electropherotyping are useful for finding antigenic variants or strains with new serotypes of bovine rotavirus[9~12]. In this study, bovine rotavirus isolated from Kyunggi, Chungnam and Kangwon provinces were investigated to determine the electropherotypes, serotypes, serological cross reactivity. In addition, we compared cross neutralizing activity of the virus by antisera against Korean isolates and NCDV strain.

Most BRV strains have been grouped into serotype G6, which is represented by the prototype strains NCDV and UK[3]. Previously Lyoo *et al* reported that the serotypes and electropherotypes of Korean isolates were comparable to NCDV strain. In this study, we detected fourteen isolates of serotype G10 likes and eleven isolates of serotype G6 likes, and also confirmed that there were more than two kinds of P types. RNA electropherotypes were divided into two groups which were NCDV type and Non-NCDV type. Some of NCDV type of BRV had extra band between segment 4 and segment 5. These might be due to the contamination of different strains or the result of a rearrangement of viral RNA segment. The observation in this study did not agree with the recent report[13] that some specified distinct serotypes possessed a distinct viral RNA patterns in PAGE.

We investigated cross neutralizing reactivity among antisera to four isolates(288, 678, P44, M4) and standard NCDV strain. In this study, we knew that NCDV strain, which is used as vaccine strain, was easily neutralized by antisera against field isolates, but field isolates were almost not neutralized by antiserum against NCDV strain. From this, we confirmed that field isolates have more immunizing potentials to BRV infection than NCDV strain has. Therefore some of these field strains could be used as candidates for vaccine strain.

Further antigenic and molecular analysis such as neucleotide sequence data are needed to confirm our results and apply for selecting efficient vaccine strain.

Conclusion

This study was carried out to investigate electropherotypes, serotypes, and cross reactivities by VN among field isolates.

Electropherotypes were divided into two group based on the differences of migration patterns of second migration group(segment 5, 6). In cross neutralization test, NCDV strain which is used as vaccine strain, were easily neutralized by antisera against field isolates, but field isolates were almost not neutralized by antiserum against NCDV strain. The G serotypes of field isolates were mainly G6 like (11/24 strains) and G10 like(13/24 strains).

Among the G types, all 14 strain grouped in G10 like were identified as P11 like.

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