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Development of diagnostic methods for rotavirus from pigs using monoclonal antibody

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단크론 항체를 이용한 돼지 로타바이러스의 진단법 개발

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초록 : Group A 로타바이러스 VP6에 특이적으로 반응하는 단크론 항체를 이용하여 로타바이러스 감염이 의심되는 돼지 분변으로부터 로타 바이러스를 검색할 수 있는 효소면역측정법을 개발하였다. 이 효소면역측정법에서는 capture antibody로서 protein A-sepharose를 이용하여 단크론 항체로부터 순수 분리한 immunoglobulin을 사용하였으며 detecting antibody는 토끼 면역혈청으로부터 순수 분리한 immunoglobulin에 biotin을 label하여 사용하였다. 개발된 효소면역측정법의 민감도와 특이성을 전자현미경법 및 형광항체법의 것과 비교하여 보았을 때 서로 유사하였으며 분변재료로부터 로타바이러스를 검색하는데 유용한 것으로 나타났다. 개발된 효소면역측정법은 야외로부터 로타바이러스 검색을 위하여 수집된 많은 양의 분변재료를 실험실내에서 screen하는데 유용하게 사용될 것으로 생각된다.

Key words : porcine rotaviruses, enzyme-linked immunosorbent assay (ELISA), monoclonal antibody, biotin.

Introduction

Rotavirus, a member of the Reoviridae family, is a major cause of gastroenteritis in humans, as well as in other mammalian and avian species.[1~3] At least four VP7 serotypes of group A rotavirus have been identified among strains isolated from pigs.[4~6] The porcine rotavirus, Gottfried strain is antigenically related to serotype 4 human rotaviruses[4,6] and serotype 5, OSU strain is antigenically distinct from known human rotavirus serotypes.[4] Other porcine rotavirus isolates have been classified as serotype 3, 11 and mixed serotype of 3 and 5.[7,8] Recently, new candidate porcine rotavirus serotypes have been described but not fully characterized.[9]

The rapid and accurate detection of rotavirus infections is very important not only for diagnosis of gastroenteritis but also for the effective control of this disease. Originally electron microscopy (EM)[10] was used, but recently many different techniques, including radioimmunoassay[11], enzyme immunoassay[12], immune adherence hemagglutination[13], staphylococcal agglutination[14], latex agglutination[15], immunofluorescence in cell culture[16], and polyacrylamide gels to detect rotaviral RNA[17], have been developed for the detection of rotaviruses in fecal specimens. Detection by EM is highly specific, but its sensitivity is low and expensive equipment

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is required. A rapid and more sensitive method is needed to diagnose rotavirus infections.

Recently, various immunoassays such as enzyme-linked immunosorbent assay (ELISA) and latex agglutination test are routinely used as an alternatvie to EM for the detection of rotavirus antigens from human stool specimens.[18,19]

The objective of this study was to develope an ELISA using monoclonal antibodies and compare it with commonly used methods such as EM and cell culture immunofluorescence (CCIF) assays for the detection of rotavirus antigens in fecal samples both from experimentally infected pigs and from field samples.

Materials and Methods

Fecal samples : Fecal samples were collected from experimentally infected gnotobiotic pigs with porcine rotaviruses (OSU and Gottfried strain). Fecal samples from gnotobiotic pigs infected with group B or group C rotaviruses or porcine enteric calicivirus were used as a negative control. For ELISA assay, fecal samples were prepared as 10% suspensions in Eagle's minimum essential medium(EMEM). Rectal swabs were vortexed with 5.0㎖ of EMEM. Each of the various preparations was centrifuged at 1,000 x g for 20 min to remove fecal debris. Supernatant fluids were collected and kept at -20C until used.

Viruses : Porcine group A rotaviruses (Gottfried and OSU), bovine group A rotaviruses(NCDV and I-801), avian group A rotavirus (AEQ) and porcine group C rotavirus (Cowden) were propagated onto MA 104 cell line as previously described.[20,21] Transmissible gastroenteritis viruses (TGE) were propagated onto PK15 cell line.

Polyclonal and monoclonal antibodies : Polyclonal antisera were obtained from rabbits and gnotobiotic pigs hyperimmunized with OSU strain of porcine rotavirus. Rabbit antiserum to OSU was prepared by the intramuscular inoculation of rabbits with 1.0㎖ of CsCl-purified virus emulsified in an equal volume of Freund's complete adjuvant. Two weeks later, rabbits were given a booster injection of viral preparations mixed in Freund's incomplete adjuvant. Two weeks later after booster injection, rabbits were bled and sera were collected. Pig antiserum to OSU was prepared by oral inoculation of gnotobiotic pigs with OSU-containing intestinal contents.

Monoclonal antibodies with specificity of rotavirus VP6 were described previously.[21,22] Two monoclonal antibodies, RG25A10 and RG25A11, were prepared using porcine rotavirus Gottfried strain as an immunogen. Monoclonal antibodies, 3H10 and 4B12, were prepared using avian rotavirus AEQ strain as an immunogen. All monoclonal antibodies were VP6-specific by radioimmunoprecipitation test and cross-reacted with different strains of rotaviruses by CCIF test.

Purification and biotinylation of immunoglobulins : Immunoglobulins were purified from ascitic fluids and polyclonal antiserum using protein A-sepharose CL4B (Pharmacia, Sweden) by modifications of previously described method.[23] In brief, ascitic fluids or antisera diluted 1 : 1 with 5 X TBS(20mM Tris-HCl containing 3 M NaCl, pH 8.9) were applied to protein A-sepharose column. The column was then washed with 10㎖ of TBS buffer containing 0.1% Tween 20(TBS-T). Immunoglobulins were eluted with 0.1M glycine buffer, pH 3.0 and 0.5㎖ fractions were collected. Fractions containing detectable immunoglobulins were combined and stored at 4C. Purified immunoglobulins were prepared for biotinylation by dialysis overnight against 1,000 volumes of 0.1M $NaHCO_3$, pH 8.6. Biotinylation reagent was prepared by dissolving the hydroxysuccinimide biotin ester (Enzotin ; Enzobiochemicals, NY) in dimethyl sulfoxide (DMSO) to an equal concentration of protein(1～5mg/㎖) in the sample. To every 1㎖ of purified immunoglobulin, 150 ㎕ of biotinylation reagent was added and allowed to react for 4 hr at room temperature. After the reaction, the biotin-labeled immunoglobulins were dialyzed against PBS overnight and stored at 4C.

ELISA : (ⅰ) *Capture antibody*-Two different capture systems, one utilizing a polyclonal antibody and the other a monoclonal antibody, were used. For this, immunoglobulins were purified from polyclonal antiserum and ascitic fluid using protein A-sepharose column.

(ⅱ) *Detecting antibody*-Three different sera were evaluated as a detecting antibody : (a) rabbit anti-OSU rotavirus antiserum, (b) pig anti-OSU antiserum similar in titer, (c) VP6-specific monoclonal antibody.

In ELISA, biotin-labeled antibody was used as the

detection systems. Optimal concentrations of capture and detecting antibodies and conjugate dilutions were determined by checkboard titrations. In the ELISA, alternate wells of 96-well flat-bottom microtitration plate (MaxiSorp F96 ; Nunc, Denmark) were coated with 100 $\mu\ell$ /well of capture antibody (2μg/mℓ) diluted in the same coating buffer. The plates were incubated overnight at 4C and then were washed 3 times with a washing buffer (0.01 M PBS containing 0.05% Tween 20, pH 7.4). The plates were added with 100 $\mu\ell$ /well of PBS containing 1% bovine serum albumin (BSA ; Sigma) and incubated at 37 C for 2 hr to block nonspecific reactions. After blocking, plates were washed 3 times, and 100 $\mu\ell$ /well of test sample was added and incubated at 37 C for 2 hr or at 4 C overnight. Plates were then washed 3 times as described above, and 100 $\mu\ell$ /well of biotin-labeled detecting antibody diluted in sample diluent was added. After 1 hr incubation at 37 C, the plates were washed 3 times with washing buffer, and 100 $\mu\ell$ /well of peroxidase-labeled streptavidin (Boehringer Mannheim Biochemicals, USA) diluted 1 : 10,000 in sample diluent was added. After 30 min incubation at 37 C and 3 times washing, 100 $\mu\ell$ of substrate solution consisting of 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) and hydrogen peroxide was added to each well and incubated at room temperature for 30 min. The color development was stopped with 1 M H_2SO_4, and absorbance in each well was measured at a wavelength of 414nm on a microplate reader. A sample was considered positive if the absorbance of the test well minus the absorbance of the control well was greater than 0.1 or the absorbance of the test well divided by the absorbance of the control well was greater than 6.

Blocking assay : Eight rotavirus-positive and two rotavirus-negative fecal samples were used in a blocking assay to test the specificity of ELISA. Fecal samples were confirmed as rotavirus-positive by CCIF assay, EM and virus isolation in cell culture. The antibodies used for blocking were rabbit preimmune and anti-OSU hyperimmune serum. The procedure of blocking assay was the same as in ELISA except that there was one more blocking step before the addition of conjugate. The OD values were compared between the hyperimmune serum blocked and preimmune serum blocked wells of each fecal sample. Positive and negative rotaviral antigen controls were included in the blocking assay.

CCIF assay : The presence of group A rotavirsues or rotavirus antigens in fecal samples was determined by CCIF assay as previously described.[21]

EM : Fecal samples were first diluted and then clarified by centrifugation at 1,000 × g for 30 min. The supernatant was centrifuged at 1,000,000 × g for 2hr. The pellet was resuspended with 50 $\mu\ell$ of distilled water. One drop of the resuspended pellet was mixed with one drop of 2% phosphotungstic acid and transferred one drop to a 400-mesh collodion-carbon coated copper grid. After 1min, samples were removed by blotting the edge of the grid, dried and examined with a Zeiss EM 109 transmission electron microscope at 80 KV.

Results

Standardization of ELISA : Sensitivity between a polyclonal and monoconal antibody or protein A-purified and nonpurified antibody was compared as a capture antibody. However, there were no differences in this comparison (data not shown). There were high backgrounds in ELISA system where monoclonal antibody was used as both capture and detecting antibody. However, backgrounds were reduced when monoclonal antibody was used as a capture antibody and polyclonal antibody was used as a detecting antibody or vice versa (data not shown). Backgrounds were also reduced when monoclonal antibody was used as a capture antibody and detecting monoclonal antibody was biotin-labeled. Thereafter, protein A-purified monoclonal antibody and biotin-labeled polyclonal antibody were used as a capture and detecting antibody, respectively.

Fig 1 shows the comparison results of efficiency of two different polyclonal antisera as a detecting antibody. In this experiment, the capture antibody was protein A-purified monoclonal antibody (RG25A10 : 2 μg/mℓ). Two different antisera, one being rabbit anti-OSU antiserum and the other gnotobiotic pig anti-OSU antiserum, were biotin-labeled and compared their efficiency as a detecting antibody. Biotin-labeled detecting antibody from rabbit anti-OSU antiserum was more sensitive than that from pig anti-OSU anti serum. The OD values of TCF (negative control) was similar at the same concentration of detecting antibody (0.625 μg/mℓ). However, high backgrounds were observed at higher concentration

Table 1. Comparison of enzyme-linked immunosorbent assay (ELISA) with cell culture immunofluorescence (CCIF) and electron microscopy (EM) tests for detection of rotaviruses from experimentally infected fecal samples

Samples No.	Source of Feces*	DPI** (Days)	Infected*** Viruses	ELISA	CCIF	EM
T 1444	LIC	5	Gottfried	−	−	+
U 217	LIC	4	Gottfried	+	+	+
U 218	SIC	4	Gottfried	+	+	+
U 277	RS	2	Gottfried	+	+	NT****
U 636	RS	1	Gottfried	+	+	NT
U 643	RS	2	Gottfried	+	+	NT
U 1095	LIC	2	Gottfried	+	−	+
U 1098	LIC	2	Gottfried	+	+	+
U 1256	RS	2	Gottfried	+	+	NT
U 229	Feces	1	OSU	+	+	+
U 232	SIC	1	OSU	+	+	+
U 246	LIC	2	OSU	+	+	+
U 1257	Feces	2	OSU	+	+	+
U 1258	Feces	2	OSU	+	+	+
U 1258-1	RS	2	OSU	+	+	NT
U 1258-2	RS	2	OSU	+	+	NT
U 1259	RS	2	OSU	−	+	NT
T 1487	LIC	2	Cowden	−	−	+
T 1497	LIC	3	PEC	−	−	NT
T 1514	LIC	13	RVLV	−	−	−
T 1515	SIC	13	RVLV	−	−	−
U 265	LIC	3	RVLV	−	−	+
U 501	LIC	2	Cowden	−	−	+
U 551	LIC	2	RVLV	−	−	+
U 552	SIC	2	RVLV	−	−	+

* : LIC=large intestinal contents, SIC=small intestinal contents, RS=rectal swab.

** : Days postinfection.

*** : Gottfried, OSU=group A porcine rotavirus strains, Cowden=group C porcine rotavirus strain, RVLV= porcine rotavirus-like virus, PEC=porcine enteric calicivirus.

**** : Not tested

of biotin-labeled pig antiserum ($> 1\ \mu g/m\ell$) compared to the same concentration of biotin-labeled rabbit antiserum. The OD values of Gottfried/OSU strain were 0.86/0.81 (rabbit anti-OSU) and 0.62/0.36(pig anti-OSU) at the concentration of $0.625\ \mu g/m\ell$.

Different kinds of monoclonal antibodies were compared for their efficiency as a capture antibody(Fig 2). Plates were coated with same concentrations of four different protein A-purified monoclonal antibodies. After reacting with serially diluted viruses(OSU, Gottfried, NCDV and AEQ), biotin-labeled detecting antibody was added. Two monoclonal antibodies, RG25A10 and RG25A11, could detect OSU, Gottfried and NCDV strains but not detect AEQ strain. The OD values of Gottfried and NCDV strains from monoclonal antibody 3H10 were low compared to those from monoclonal antibodies RG25A10 and RG25A11. None of the rotavirus strains could be detected using monoclonal antibody 4B12.

Different strains of rotavirus were used to examine the capability of developed ELISA test for rotavirus detection. Plates were coated with $2\ \mu g/m\ell$ of protein A-purified monoclonal antibody RG25A10. After reacting with serially diluted different viruses(OSU, Gottfried, NCDV, I-801, AEQ, Cowden, and TGEV), biotin-labeled detecting antibody was added. As we expected, Cowden strain(group C rotavirus) and TGE virus were not detected in this ELISA system. However, avian rotavirus, AEQ strain, showed very low OD values(Fig 3).

Comparison of different methods for detection of rotavirus : Sensitivities of developed ELISA were compared with those of CCIF and EM test. Table 1 shows the comparison results of fecal samples from experiment-

Table 2. Comparison of enzyme-linked immunosorbent assay (ELISA) with cell culture immunofluorescence (CCIF) and electron microscopy (EM) tests for detection of rotaviruses from field fecal samples

Samples No.	ELISA	CCIF	EM
T 1327	+	−	+
U 683	+	+	+
U 684	+	+	+
K 1	+	+	+
K 2	+	+	+
K 3	+	+	+
K 4	+	+	+
K 5	+	NT*	+
K 6	+	NT	+
K 7	+	NT	+
K 8	+	NT	+
K 9	+	NT	+
K 10	−	−	−
K 11	−	−	−
K 12	−	−	−

* : Not tested

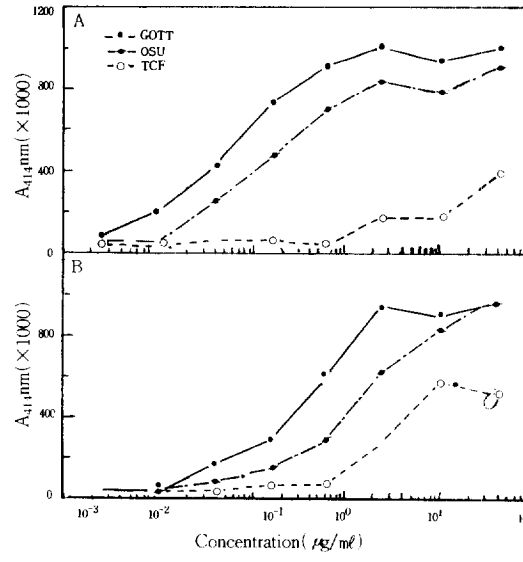

Fig 1. Comparison of the efficiency of biotin-labeled rabbit and pig anti-OSU antibody as a detecting antibody. The plates were coated with protein A-purified monoclonal antibody (2 μg/mℓ) and different concentrations of biotin-labeled rabbit anti OSU(A) or pgi anti OSU (B) antibody was added as a detecting antibody

ally rotavirus infected gnotobiotic pigs. Different results were observed from three fecal samples (T1444, U1095 and U1259). EM was not done from rectal swab samples because of small volumes. However, other samples had a good agreement in results with CCIF and EM. Viral particles were observed by EM from fecal samples infected with group C rotavirus (Cowden) and rotavirus-like virus (RVLV). However, they were all negative by ELISA and CCIF tests. Table 2 shows the comparison results of field fecal samples.

Specificity of the ELISA : Table 3 shows the results of the blocking assay. An OD value reduction took place on the all the positive samples in the wells blocked with

Table 3. Mean absorbance values of rotavirus-positive and negative samples determined by a blocking enzyme-linked immunosorbant assay(ELISA)

Sample No.	Mean OD values at 414 nm			
	Blocked with preimmune serum		Blocked with hyperimmune serum	
	1 : 25*	1 : 100*	1 : 25	1 : 100
1	0.328	0.574	0.076	0.060
2	0.726	0.611	0.083	0.081
3	0.819	0.877	0.136	0.189
4	0.371	0.422	0.035	0.041
5	0.906	0.844	0.183	0.151
6	0.351	0.584	0.060	0.051
7	0.583	0.631	0.055	0.095
8	0.255	0.196	0.076	0.073
9	0.177	0.071	0.070	0.044
10	0.043	0.017	0.020	0.030
Gottfried**	0.898	0.863	0.191	0.155
TCF ***	0.052	0.026	0.050	0.035

* : Fecal samples were diluted 1 : 25 and 1 : 100 with PBS and tested.
** : Porcine rotavirus Gottfried strain infected tissue culture fluids were used as positive control
*** : Virus non-infected tissue culture fluids were used as negative control

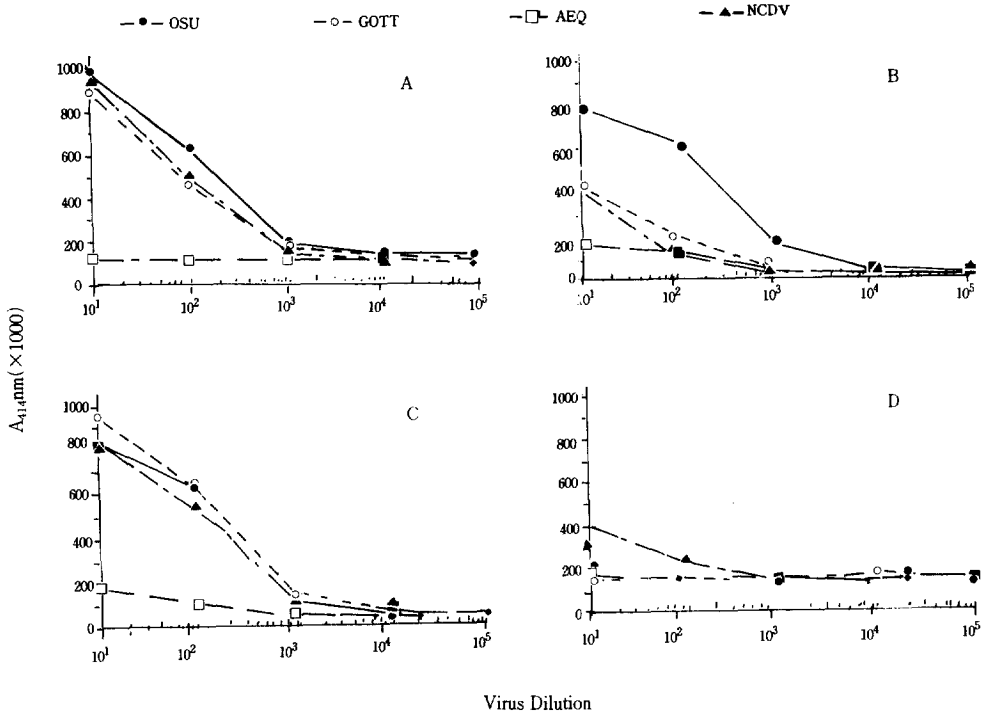

Fig 2. Comparison of the efficiency of four different monoclonal antibodies as a capture antibody. The plates were coated with protein A-purified monoclonal antibody RG25A10(A), 3H10(B), RG25A11(C), and 4B12(D) as a capture antibody and tested using various strains of rotavirus.

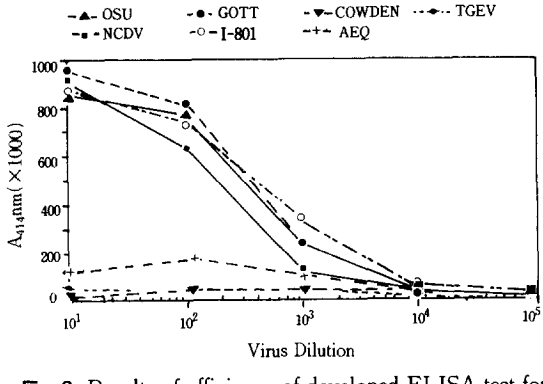

Fig 3. Results of efficiency of developed ELISA test for the detection of rotaviruses. The plates were coated with protein A-purified monoclonal antibody (2 μg/mℓ) as a capture antibody and tested using various strains of rotavirus.

rabbit anti-OSU antiserum as compared with wells blocked with preimmune serum. There was no OD value reduction in the negative samples.

Discussion

Many ELISA tests were developed for the detection of enteric viruses such as human rotaviruses, adenoviruses and bovine coronaviruses from fecal specimens.[24~27] Under optimal conditions, these ELISA tests were very sensitive, specific and convenient for the detection of viral antigens. In this study, an ELISA test was developed using monoclonal antibody for the porcine rotavirus detection. There are several advantages for using monoclonal antibody against polyclonal antiserum in ELISA test. Antibodies with same specificity can be obtain indefinitely from hybridoma cells. Also there are no cross-reactions with different viruses.

It was reported that completely monoclonal antibody-based EIA is superior in specificity to the monoclonal and polyclonal antibody-based EIA for the detection of rotaviruses.[28] High backgrounds were observed in this study where monoclonal antibodies were used as a capture and detecting antibody. However, backgrounds were

reduced when monoclonal antibody was used as a capture antibody and detecting monoclonal antibody was biotin-labeled. Backgrounds were also reduced when monoclonal antibody was used as a capture antibody and polyclonal antibody was used as a detecting antibody or vice versa(data not shown). It may be due to the fact that the monoclonal antibodies used as a capture and detecting antibody cross-reacted nonspecifically each other. Rabbit antiserum appeared to be better than pig antiserum as a detecting antibody. It may be explained that convalescent sera from naturally acquired infections or gnotobiotic animals were not satisfactory as regards to specificity.

Biotin-avidin system was generally used as amplification of immunological tests. In this study, biotin-labeled detecting antibody was very effective for the detection of rotaviruses as low as concentration of 0.625 ㎍/㎖. Also it is very easy to label antibody with biotin. For these reasons, biotin-labeled antibody can be used widely in ELISA test.

It was surprising that avian group A rotaviruses were not detected using this standardized ELISA. Monoclonal antibodies, RG25A10 and RG25A11 were suitable as a capture antidody for the developed ELISA for the detection of porcine rotaviruses from fecal samples but not suitable for the detection of avian rotaviruses. When monoclonal antibodies, 3H10 and 4B12, were used as a capture antibody, it could not detect avian group A rotaviruses, either. These two monoclonal antibodies were prepared against avian group A rotavirus and reacted with different animal rotaviruses by CCIF test.[22] Furthermore, monoclonal antibody 4B12 was not suitable as a capture antibody because it could not detect porcine and bovine rotaviruses. It may be explained that monoclonal antibody 4B12 has a low binding activity to solid phase.

It is usually known that the ELISA test is superior to EM and FA in sensitivity and specificity.[28] In this study, it can not be compared directly, but developed ELISA is as sensitive as EM and CCIF. However, considering the other factors such as time, equipment and labor for tests, ELISA test is much better than EM and CCIF. For EM, it requires a expensive equipment and many samples can not be handled per day. For CCIF test, it takes several days to get results and needs a fluorescent microscopy. Although ELISA test can be automated using some expensive equipments such as washer and reader for ELISA, it can be performed without problems at laboratories which did not have a washer and a reader. Moreover, it has a great advantage of handling many samples at one time.

Non-group A rotavirus infections in human and other animals are increasing now.[29~31] Non-group A rotaviruses did not cross-react with group A rotaviruses through common antigen. Therefore, non-group A rotaviruses could not be detected by developed ELISA test in this study. Monoclonal antibodies against porcine group C rotavirus were produced and characterized.[32]

Development of a new ELISA test for the detection of porcine group C rotaviruses is in progress using monoclonal antibody.

Conclusion

An enzyme-liked immunosorbent assay (ELISA) suitable for the detection of rotaviruses from fecal samples was developed using VP6-specific monoclonal antibody. It involves the use of protein A-purified monoclonal antibody as a capture antibody. Detecting antibody was prepared by purifying the immunoglobulins from rabbit antiserum using protein A-sepharose and labeling with biotin. The sensitivity and specificity of this test were compared with those of EM and FA. The ELISA reported in this study was as sensitive as EM and FA and useful to detect rotaviruses from fecal samples. This test appears to be very suitable for routine laboratory work and may prove useful for large-scale screening in the field sample.

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