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RT-PCR for detection of group A, B and C porcine rotaviruses

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RT-PCR 기법을 이용한 돼지 로타바이러스 주요 혈청형 감별진단

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초 록 : 본 연구에서는 돼지 로타바이러스의 혈청형 A, B 및 C를 동시에 진단할 수 있는 RT-PCR 기법을 개발하였다. 각각의 혈청형에 특이적인 primer를 이용하여 RT-PCR 기법으로 분변시료에서 직접 바이러스 동정을 실시한 결과, 23건의 로타바이러스 감염 분변시료에서 13건이 혈청형 A, 3건이 혈청형 B, 2건이 혈청형 C로 나타나 국내에서도 A, B 및 C형의 돼지 로타바이러스가 공히 발생하고 있음을 확인하였으며, 발생분포는 외국의 예와 유사하였다. 이 RT-PCR기법은 돼지 로타바이러스의 주요 혈청형인 A, B 및 C형의 동시감별진단법으로 이용될 수 있을 것으로 판단된다.

Key words : porcine rotavirus, RT-PCR, serogroup.

Introduction

Rotaviruses are double-stranded RNA viruses with 11 segments which are one of a most common and important viral gastroenteritis pathogen in neonates of animals and human. In swine as in other animal species, rotaviruses cause intestinal malabsorption resulting in gastroenteritis and some death. Four(A, B, C and E) out of the seven identified serogroups(A to G) of rotaviruses are known to infect pigs[1].

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Table 1. List of primers used in RT-PCR and nested PCR for detection of group A, B and C porcine rotaviruses

Group of virus	Name of primer	Sequence(5'-3')	Position of sequence
A	VP4F	GGCTATAAAATGGCTTCGCTC	1-21
	VP4R	AATGCTTGTGAATCATCCCAG	1094-1074
	SP6	ACCATGTGTTAGAAAGCCAGC	519-499
	SP7	GCCCTCTACAGTTGGCGCAAG	279-259
B	RB1	CTATTCAGTGTGTCGTGAGAGG	18-39
	RB3	CGAAGCGGGCTAGCTTGTCTGC	451-430
	RB4	CGTGGCTTTGGAAAATTCTTG	506-486
C	RC1	CTCGATGCTACTACAGAATCAG	994-1015
	RC3	GGGATCATCCACGTCATGCG	1320-1301
	RC4	AGCCACATAGTTCACATTTCATCC	1349-1326

Several methods have been reported for the diagnosis of rotaviral infection in fecal sample. Electron microscopy and immunoelectron microscopy for the detection of viral particles, latex agglutination and ELISA for the detection of viral protein and electropherotyping for viral RNA were developed for diagnosis of rotaviral infection. Recently, RT-PCR and hybridization assay for the detection of viral RNA are the most commonly used methods[1-3]. This study described an RT-PCR method for the detection of porcine group A, B and C rotaviral RNA in fecal samples.

Materials and Methods

Fecal Samples : Twenty-three porcine fecal specimens submitted to diagnostic laboratory of NVRI from nation wide were used for the study. All the samples were proven to contain rotaviruses by one or other techniques including electron microscopy, latex agglutination and/or polyacrylamide gel electrophoresis of rotavirus RNA.

Viral RNA extraction : Rotavirus dsRNA was extracted from infected cell culture or fecal specimens according to previously described techniques with some modification[4,5]. A 200mg of fecal specimen suspended with an equal volume of 0.1M sodium acetate buffer and clarified by centrifugation at 12,000 X g(microcentrifuge, Heraus) for 5 min. The supernatant was transferred into a new tube and added 1/10 volume of 10% SDS and 1/200 volume of proteinase K(30mg/ml) into the tube, mixed and incubated at 37℃ for 1 hour. And then the solution was extracted with equal volume of phenol : chloroform(pH 6.6) and centrifuged at 12000 X g for 10 minutes. The dsRNA was purified from the supernatant by using a RNaid kit(Bio 101, INC. La Jolla CA, USA) according to the manufacturer's instructions.

Primers : Oligonucleotide primers used in RT-PCR and nested primers for detection of group A, B and C porcine rotaviruses were designed based on sequence data, which encodes gene 4(VP4), gene 8 and gene 6, respectively(Table 2).

Table 2. Distribution of porcine rotaviruses by RT-PCR from fecal samples collected from pigs with diarrhea in the field

No. of samples	No. of serogroup			
	A	B	C	ND
23	13	3	2	5

* ND : not determined.

The primer sets for the first round amplification(VP4F and VP4R) and nested PCR(SP6 and SP7) of group A rotaviruses were disigned from the sequence of Goottfried and OSU strain gene 4 sequences(Genebank accession No. M 33516 and X13190)[6,7] using by "Peimer disigner" program (Scientific & Educational Software, USA). The primer sets for group B and C rotaviruses were adapted from previous report[3].

RT-PCR : Reverse transcription and polymerase chain

reactions were performed following the previous report with some modifications[2,3]. The purified dsRNA was denatured by heating at 97℃ for 5 minutes in the presence of 7% DMSO and quickly placed on ice. The solution was added to the reaction mixture consisting of10mM tris(pH 8.3); 40mM KCl; 1.5mM $MgCl_2$; 0.2mM each dNTPs; and 0. 2nM of each correnponding set of oligonucleotide primers. The primer pairs RB1-RB4, RC1-RC4, and VP4F-VP4R were used as a pool of primers in an amplification.

2.5 unit of Taq DNA polymerase(GIBCO, BRL) and 4 unit of AMV reverse transcriptase(Invitrogen, CA, USA) were added, and the mixture was homogenized and overlaid with wax bead(Ampliwax, Perkin Elmer). The tubes were placed in a thermocycler(Perkin Elmer Cetus, Norwark, CT, USA)) for an initial incubation at 42℃ for 45 min, followed by 30 cycles of PCR(94℃ for 1 min, 42℃ for 2 min, 72℃ for 1 min) and a final incubation at 72℃ for 7 min.

Nested PCR : A nested PCR was performed with dsDNA template from the first amplification using primer set corresponding to the target sequence. The primer pairs VP4F-SP6 and SP7, RB1-RB3 and RC1-RC3 were used to confirm the identity of group A, B and C rotaviruses, respectively. The amplification conditions was followed by previous reports[2,3].

Results

The RT-PCR amplification was performed using 23 fecal samples, which known to contain rotaviruses. Thirteen samples were showed 1,094bp DNA fragment which is specific to VP4 of group A rotavirus(Fig 1, lane 1, 2, 3 and 5). Using the primer pair RB1-RB4, specific to group B rotavirus, the expected 489bp DNA fragment(Fig 1, lane 6, 7 and 8) was showed from 3 fecal samples. Similarly using the primer pair RC1-RC4, specific to group C rotavirus, the expected 356bp DNA fragment(Fig 1, lane 9 and 10) was showed from 2 fecal samples. And 6 fecal samples did not reacted with any of group A, B and C specific primers(Fig 1, lane 4).

The nested PCR assay was performed for 18 dsDNA products from first RT-PCR. All samples showing either group A, B or C rotaviruses specific DNA fragment in first RT-PCR were further confirmed by the nested PCR(Fig 2, X and Y). For group A rotavirus, nested PCR were performed using P6 and P7 specific primer sets to improve efficiency (Fig 2, X).

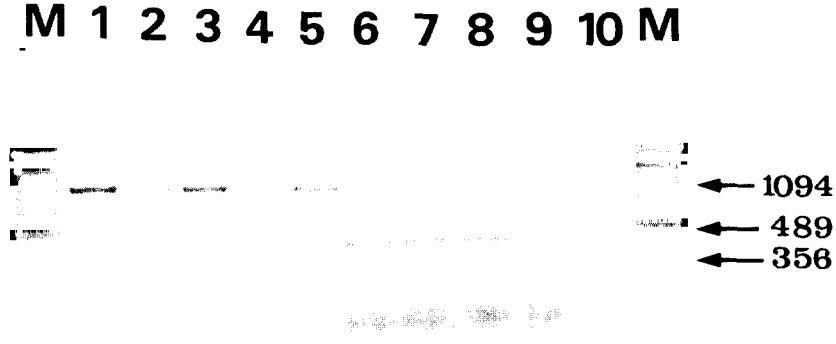

Fig 1. Amplification of porcine group A, B and C rotavirus by RT-PCR from fecal samples. Group A(lane 1, 2, 3 and 5), group B (lane 6, 7 and 8) and group C(lane 9, 10) rotavirus specific DNA fragment was amplified in a tube with three different sets of primers. Identification of rotavirus group was determined by size of PCR product. Lane 4 showed multiple bands with various size which was not able to identified.

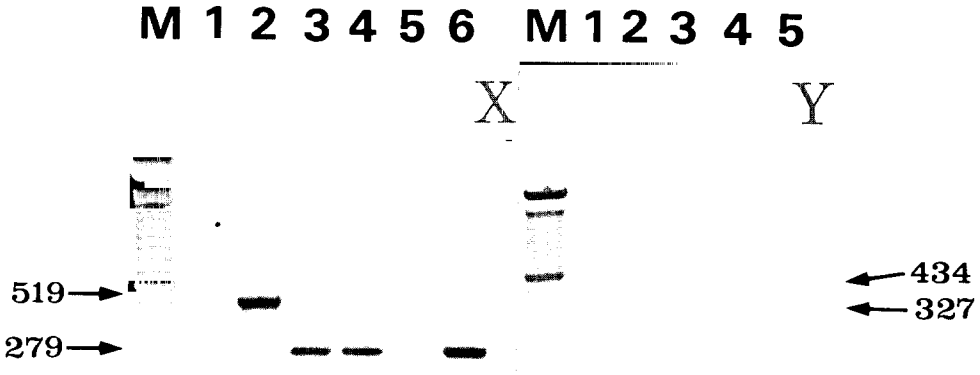

Fig 2. Identification of serogroups by nested PCR as a confirmation assay. A 519(P6) or 279bp(P7) DNA fragement was amplified from the first RT-PCR product(X). Group B(lane 1, 2 and 3) and C rotavirus(lane 4 and 5) specific DNA fragments were showed in Y.

Discussion

A RT-PCR has been developed to detect the group A, B and C porcine rotaviruses. The method was very sensitive and applicable for the differential diagnosis of porcine rotavirus group A, B and C from fecal samples. Since rotaviruses are difficult to propagate in cell culture, this RT-PCR method could provide molecular epidemiology of the rotaviral gastroenteritis in swine. Among the 23 fecal samples, 13 for group A, 3 for B and 2 for C rotaviruses were detected, and confirmed by nested PCR using specific primer set corresponding to the target genes. Five out of 23 rotavirus positive samples were not reacted with any of primer sets used in this experiment. This result indicate that there is a rotavirus with distinct viral genome may exist in the field. Other possible reason is the RNA degradation during experimental process. To identify these unidentified viruses, additional tests such as hybridization and/or gene cloning and sequence analysisneed to be carried out with these samples.

The rotaviral infection was 9% of the porcine enteric disease cases in Iowa, and eighty-nine percent(85/96) of rotavirus cases were group A, 6%(6/96) were group B, and 5% (5/96) were group C infection, respectively[6]. But the epidemiological data relate to rotavirus group in pig diarrhea has not been reported in Korea. In this study, the group A, B and C rotaviruses were detected 72%(13/18), 16%(3/18) and 11%(2/18), respectively. For the more reliable data of rotavirus serotype prevailance in Korea, it would be need to increase sample size more than we tested in this study. To determine genetic characteristics of swine rotavirus in Korea, further work on genotyping need to be performed. Information on the rotavirus genotype will provide a clue to control rotaviral gastroenteritis in piglets.

Conclusively, this RT-PCR method is recommended to detect and direct differentiation of rotavirus groups either in fecal samples and/or cell culture materials.

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