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RT-PCR and nested PCR amplification of the PRRSV genes from boar semen for the rapid and sensitive differential diagnosis

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Nested PCR 및 RT-PCR을 이용한 PRRSV의 정액내 신속 감별진단법

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초 록 : 돼지 생식기호흡기증후군(porcine reproductive and respiratory syndrome, PRRS) 바이러스가 웅돈에 감염되었을 경우에는 정충의 기형 등 정액의 질 저하와 더불어 정액에 바이러스가 함유되어 있어 종부시 모돈에 바이러스를 전파하는 것으로 알려져 있다. 감염된 웅돈의 정액으로 인공수정을 실시할 경우 농장의 모돈 전체에 순식간에 바이러스를 전파하여 막대한 피해를 유발할 가능성이 높다. 따라서 감염웅돈을 신속히 검색하여 격리함으로써 피해를 사전에 방지해야 하며, 이를 위해서 웅돈의 감염여부를 신속히 확진하는 진단법의 개발이 필요한 실정이다. PRRS의 진단을 위해서는 바이러스학적인 진단법으로는 바이러스 분리동정, 혈청학적인 방법으로 바이러스 분리동정이 필수적이나 검사시간이 많이 소요되고, 분리동정 자체가 까다로운 단점이 있다.

본 연구에서는 웅돈의 정액내에서 PRRSV 바이러스에 대한 유전자를 RT-PCR법으로 증폭하는 방법을 개발하였으며, 진단의 민감도를 높이기 위하여 Nested PCR법으로 재확인 할 경우, 바이러스의 역가가 1 $TCID_{50}$만 함유되어 있어도 진단이 가능한 조건을 확립하였다. 이 방법을 이용할 경우 웅돈의 정액시료에 대한 PRRS 바이러스 감염여부를 신속, 정확하게 검색하여 감염웅돈을 통한 PRRS 바이러스의 전파를 미리 차단할 수 있으므로 PRRS 방제에 효과적으로 이용될 것으로 사료된다.

Key words : PRRSV, RT-PCR, nested PCR, boar, semem.

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Introduction

A new emerging PRRSV belongs to classified *Arteriviridae* with positive sense viral genomic RNA approximately 15kb in size[1]. The virus has similar genomic structure and replication strategy with equine arterivirus(EAV). lactate dehydrogenase virus(LDV) in rodent and simian hemorrhagic fever virus(SHV) in monkey[2]. The virus cause reproductive failure in late gestation of pregnant sows and respiratory distress with type Ⅱ pneumocyte proliferation in all age of pigs. This widely disseminated swine pathogen causing reproductive and respiratory disease as well as considered to be an effector to potentiate other bacterial respiratory disease[3]. But infection of the PRRSV does not always result in clinical symptom. Complexity of the pathogenesis and factors may be associated with the virus and mechanisms involved in various form of the clinical symptom were not understand clearly. There is still controversial debate remained behind the pathogenesis of the virus but it's clear that the virus infected pigs and herd have more susceptibility than naive animals and more economic loses than uninfected farms[3,4]. There is a clear evidence has been demonstrated that the PRRSV infected boar shed virus via semen whether boar has antibody in the blood stream or no antibodies present[5,6]. In stallion semen EAV was detected by nested PCR assay[7]. We developed RT-PCR and nested PCR method for the detection of the PRRSV genome in boar semen and cell culture materials, and also successfully applied for the field diagnosis of the PRRSV infection in boars. Even PCR is one of a most sensitive technique for the detection of the target gene from various source of the samples but there is still certain limitation such as sensitivity and specificity. Low titer of the viral contamination in the samples won't be able to be detected by PCR even correct primer set has been used for the test because of the PCR product amplified by PCR couln't be visualized by ethidium bromide agarose gel staining. A nested PCR for the PRRSV genome detection from boar semen was very sensitive and effectively utilized for the routine diagnostic method. We believe that this method will provide effective control measure for the PRRSV infection in boars as well as reproductive failures dirived from PRRSV infected semen either natural and/or artificial insemination. Recently sero-diagnostics such as indirect immunofluorescent assay(IFA) and ELISA for the PRRSV infection have been developed and widely used in the laboratories[8]. But these method only can detect antibodies less than 6 month after the virus infection. In boar semen the PRRSV shed for 3 month regardless of antibody presence in the circulatin blood. There is sequence diversity exist among field isolate of PRRSV and two main stream have been published. North American and European strain have distinct sequence variance. Serological test also indicate that there is heterogeneity in amino acid level by sequence analysis and indirect immunofluorescent test[9, 10]. One of the aim for the this experiment was to deterimine similarity of the Korean field isolate with other known strains of PRRSV by RT-PCR.

Materials and Methods

Cells and viruses : A PRRSV was isolated and identified from 6 weeks old pig with severe respiratory symptoms. Blood from pigs with respiratory sign was collected and serum preparation was obtained right after blood coagulation and used for virus isolation. Briefly, monolayered MA-104 cels were washed twice with serum free minimal essential medium(MEM) and infected with serum preparation. MA-104 cells inoculated with serum was placed in CO_2 incubator for 60 minutes then inoculum was discarded and fresh MEM with 5% fetal calf serum was added. Virus isolation and identification was follwed by method described elsewhere[3]. Field isolates, VR2332 and North American strain of the PRRSV was used as test and control standard viruses.

RT-PCR : Reverse transcription and polymerase chain reaction(RT-PCR) was performed to standardize DNA amplification from boar semen samples. Boar semen was clarified by low speed centrifugation and sperm ell fraction with other somatic cells was collected and lysed with lysis buffer. First strand cDNA was synthesized using reverse transcriptase (M-MLV, Gibco-BRL USA) and used and tem-

plate DNA for the PCR. PCR condition used in the test was 3 min. at 94℃ for denaturation and continued 30 cycles of amplification at 94℃ for 20 sec. and annealed at 47℃ for 30 seconds, extended at 72℃ for 30 seconds then lasted extension step at 72℃ 15 min. Several different primer sets were used in the RT-PCR and chose the most sensitive primer set for the next experiment. Oligomers used in the PCR for ORF6 include 5'-GGG GAT CCA GAG TTT CAG CGG-3' for forward primer and 5'-GGG AAT TCT GGC ACA GCT GAT TGAC-3' for reverse primer with expected size of DNA product of 617bp in length. ORF 7 primer set consists of 5'-GGG AAT GGC CAG CCA GTC AAT CAA CTG T-3' for the forward primer and 5'-TGT AGA AGT CAC GCG AAT CAG GCG CAC T-3' for reverse primer with expected size of the PCR product of 311 bp in length. PCR products were electrophoresed and visualized by ethidium bromide staining.

Specificity of the RT-PCR : To determine specificity of the RT-PCR using primer set designed for the PRRSV gemone amplification, common swine viral pathogens such as hog cholera virus(HC), Aujeszky's virus(ADV), porcine parvovirus(PPV), trasmissible gastroenteritis virus(TGEV) porcine epidemic diarrhea virus(PEDV), swine influenza virus(SIV), Japanese encephalitis virus(JEV) and encephalomyocarditis virus(EMCV) were employed. PCR amplification of the DNA viruses and RT-PCR amplification methods were followed by previous publications described elsewhere.

Nested PCR : Even PCR is one of a most sensitivie methods for the detection of the pathogens by amplifying their genome but there is a limitation of the PCR amplification. To enhance sensitivity of PCR for the detection of the PRRSV genome in boar semen internal primers corresponding to ORF7 were designed. Oligonucleotides used for the nested PCR were 5'-CCA GAT GCT GGG TAA GAT CAT C-3' for forward primer(and reverse primer sequence was 5'-CAG TGT AAC TTA TCC TCC TGA-3' with expected PCR product DNA of 236bp in size.

Differentiation of the field isolate by PCR using specific primer sets : To determine similarity of the field isolate with other known PRRSV strain differential RT-PCR procedure has been applied. Primer sets dierived from ORF 1b was used in the test and sequencs were 5'-GTA TGA ACT TGC AGG ATG-3' for forward primer and 5'-GCC GAC AAT ACC ATG TGC TG-3' for reverse primer with expected size of PCR amplified DNA product 186bp in length for specific to European strain of PRRSV. Other primer set was 5'-GGC GCA GTG ACT AAG AGA-3' for forward primer and 5'-GTAACTGAACACCATATGCTG-3' for reverse primer derived from north Amierican strain with expected PCR product was 107bp. To amplify both European and north American strains conserved sequence derived priv-

Table 1. Primer sets used for the optimization fo the RT-PCR and their expected size of amplified DNA

Primer	Sequences	Size
ORF7F1	5'-TCGTGTTGGGTGGCAGAAAGC-3'	484
ORF7R1	5'-GCCATTCACCACACATTCTTCC-3'	
ORF7nF2	5'-CCAGATCGTGGGTAAGATCATC-3'	236
ORF7nR2	5'-CAGTGTAACTTATCCTCCTGA-3'	
ORF7F3	5'-TAAATATGCCAAATAACAAC-3'	467
ORF7R3	5'-TAGGTGACTTAGAGGCACA-3'	
ORF7F4	5'-ATGGCCAGCCAGTCAATCA-3'	432
ORF7R4	5'-TCGCCCTAATTGAATAGGTG-3'	
ORF7F5	5'-GGGAATGGCCAGCCAGTCAATCAACTGT-3'	311
ORF7R5	5'-TGTAGAAGTCACGCGAATCAGGCGCACT-3'	
ORF6F1	5'-GGGGATCCAGAGTTTCAGCGG-3'	617
ORF6R1	5'-GGGAATTCTGGCACAGCTGATTGAC-3'	
ORF7F6	5'-GGGGATCCTTGTTAAATATGCC-3'	448
ORF7R6	5'-GGGAATTCACCACGCATTC-3'	

er set of 5'-CCT CCT GTA TGA ACT TGC-3'(forward) and 5'-AGG TCC TCG AAC TTG AGC TG-3'(reverse) with 255bp expected PCR product was used.

Results

Optimization of the RT-PCR : Several different primers were disigned for the amplification of the ORF6 or 7 and test to choose most sensitive reaction(Fig 1). Lane 6 for the ORF6 and lane 4 for the ORF7 showed specific and sensitive reaction and used for the next experiment. Lane 2 showed considerable reaction but it was nested PCR reaction from lane 1 product.

Sensitivity of the RT-PCR : RT-PCR method has been

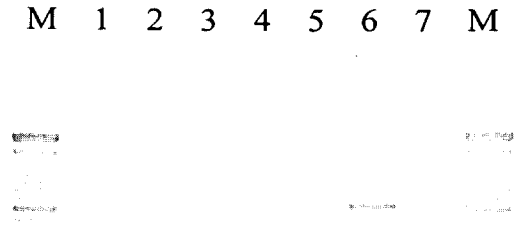

Fig 1. Optimization of the RT-PCR using primer sets listed in Table 1 and lanes indicate DNA product in sequential order. Lane 2 showed 236bp nested PCR product using lane A DNA as a template. Lane M : 100bp ladder.

Fig 2. Sensitivity of the RT-PCR using selected ORF6 and 7 primer sets. The ORF6 showed sensitivity of 10^6 $TICD_{50}$/ml(lane 1) and sensitivity of the ORF 7 was 10^4 $TCID_{50}$/ml(lane 8).

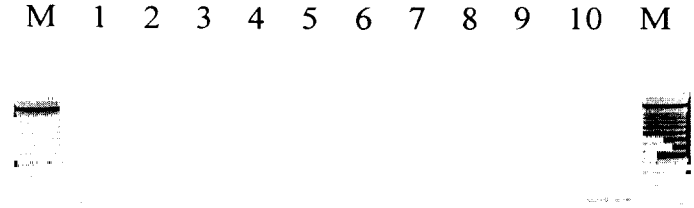

Fig 3. Specificity of the RT-PCR using PRRSV ORF7 primer set with swine viral pathogens include PRRSV(lane 1 and 16 ADV(lane 2), PPV(lane 3), TGEV(lane 4). PEDV(lane 5), SIV(lane 6), JEV(lane 7), HCV(lane 8), EMCV(lane9)
Differentiation of the PRRSV field isolate by RT-PCR using primer sets corresponding to North American and European strains.

established and sensitivity of the test was 10^4 $TCID_{50}$/ml in cell culture as well as experimentally mixed semen with field strain PL96-1. Sensitivity of the ORF6 amplification was iluustraed in Fig 2. Lane 1 containing 10^6 $TCID_{50}$/ml showed 617bp ORF spedivic PCR product but and no visible band was detected virus titers lower than 10^5 $TCID_{50}$/ml.

Specificity of the RT-PCR : The primer set designed for the amplification of the PRRSV ORF7 was specific to PRRSV but to other common swine viral pathogen. Fig 3. lanes 1 and 10 showed PRRSV specific genome amplification but lane 2 to 9 did not show any specific DNA band of the PCR products. This result indicate that the primer set used in the test was specific only to PRRSV and applicable to detect the virus infection in pigs.

Nested PCR : Sensitivity of the RT-PCR was improved by nested set PCR with primers designed based on the internal sequenc of the PRRSV. The nested PCR product of 236bp was detected the virus concentration as low as 1 $TCID_{50}$/ml shown in Fig 4 lane n6. This resulted reflect that the nested PCR used in the experiment was sensitive enought for the detection of the PRRSV nucleic acid in boar semen. Lanes R1 to R7 in Fig 4 represent regular ICR with same condition

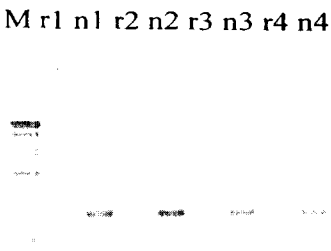

Fig 4. Sensitivity of the RT-PCR and nested PCR detection of PRRSV nucleic acid from boar semen. RT-PCR detect 10^5 $TCID_{50}$/ml(lane r2) and nested PCR enhanced sensitivity to 1 $TCID_{50}$/ml(lane n6). C : Control.

Detection of the PRRSV nucleic acid boar semen from farm suffering reproductive failure by nested PCR : Boar semen samples from farm suffering reproductive failure was submitted and nested PCR was successfully amplified PRRSV nucleic acid. The first round RT-PCR did not show any visible DNA product in agarose gel electrophoresis followed by ethidium bromide staining. In Fig 5 lane 1 to 4 did not show any PCR amplified DNA products at the first round reaction but lane 5 which was PRRSV control showed expected 484bp DNA product in size. Nested PCR using first round RT-PCR products as a template field specimen lane 7 and reference virus lane 10 showed 236 bp distinct PRRSV DNA band.

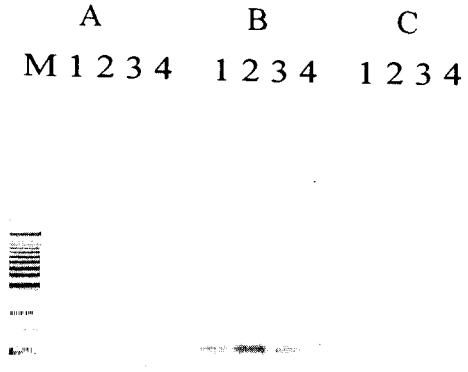

Fig 5. Differential RT-PCR to identify similarity of the field isolate using primers specific to north American and European strains. Lane 1 : PL96-1, lane 2 : North American strain, lane 3 : VR2332. A : Euroean strain specific primer set, B : North American strain specific primer set, C : Conserved sequence derived primer set.

Identification of the field isolate of PRRSV : PRRSV field isolate was identified as a strain similar to that of north American strain by RT-PCR. VR2332, north American strain and Korean field isolate were used in the test. Primer set which designed from conserved sequence of the PRRSV was successfully amplified DNA from VR2332, North American strain and Korean field isolate PL96-1 as depicted in Fig 6. Field isolate PL96-1 reacted with primers corresponding to north American strain of the PRRSV but did not with European strain dirived primer set. Lane 1 to 3 showed no PCR products with expected amplified product of 186bp is size. Lane 5 to 7 showed PCR amplified products of 107bp in size with PL96-1, north American and

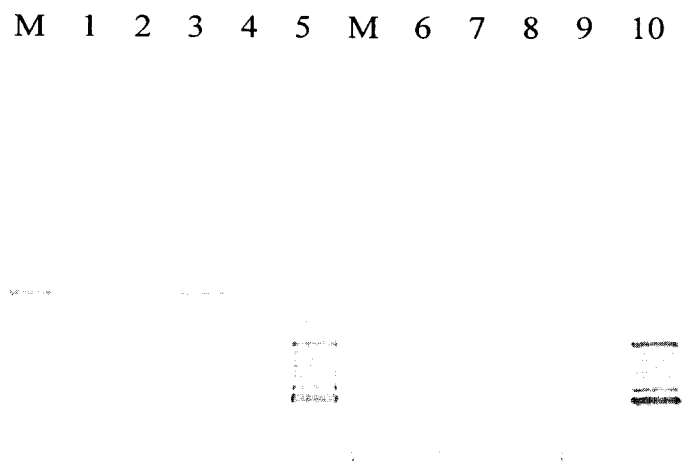

Fig 6. Nested PCR to detect PRRSV specific nucleic acid from boar semen collected from pig farm suffering reproductive failure. RT-PCR : lane 1-4(field semen samples), lane 5 : PL96-1. Nested PCR lane 6-9(field samples), lane 10 : PL96-1.

VR2332 strains of PRRSV. Lanes 9 to 11 showed 255bp PCR products with primer set derived from conserved sequence of PRRSV. Lanes 4, 8 and 12 were MA-104 mock infected control did not react with any primer sets used in the experiment.

Discussion

PRRSV causes respiratory and reproductive failure in swine. Reproductive disease in sows has been characterized by late gestation abortion storm in infected farm. Because of its nature of the pathogenic mechanism in sow a diagnosis is not easy task in pigs. Even sensitive and rapid diagnosis do not provide effective control measure in infected animals. One of a most effective way to control PRRSV in swine is blocking introduction of the virus into niave farm by reliable test procedure in animals. Especially, farmers favor artificial insemination and sometimes they have misconception on the AI. Some farmers have misconception that AI is safe and free from disease trasmission. But semen from unknown farm may have a risk for health statue of the farm receiving services. Viral transmission modes are various such as fecal-oral route, mucosal surface, sharing syringes, sexual transmission etc. A PCR amplification of the PRRSV in specimen from various source may be the most sensitive test until this moment. Appropriate procedures could reduce non-specific reaction and produce a result with great confidence. The RT-PCR we applied in this experiment was specific to PRRSV but did not react with other swine viral pathogens such as ADV, PPV, TGEV, PEDV, SIV, JEV, HCV, and EMCV. The RT-PCR sensitivity for the amplification of PRRSV viral genome was $10^4 TCID_{50}$/ml which may not be sensitive enough to detect the virus contaminated in boar semen. To enhance the sensitivity of the test internal primer set was applied and this nested PCR was sensitive enough to detect 1 $TCID_{50}$/ml of the PRRSV in boar semen. In conclusion, detection of the PRRSV nucleic acid from boar semen is important to control the virus transmission via natural or artificial insemination. Nested PCR is one of the most sensitive diagnostic technique to detect PRRSV infection in boars. We believe that this test procedure will be successfully applied for the PRRSV diagnosis in boars as well as other specimens form various sources. Field isolate was identified as a similar strain to that of north American strain. This data indicates that field strain isolated from pigs might have a origin of pigs imported from north America. This simple technique also provides molecular epidemiology of the PRRSV in Korea and further investigation with wide

variety of the isolates needs to be completed to establish nation wide epidemiology of the PRRSV infection

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