韓家衛誌 第22卷 第4號
Korean J Vet Serv(1999) 22(4) : 363-370

Isolation and identification of porcine reproductive and respiratory syndrome virus from serum samples collected from swine farms

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돼지 농장으로부터 수집한 혈청가검물에서 돼지생식기 호흡기증 바이러스의 분리 및 동정

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요 약

돼지 호흡기 생식기증 바이러스(porcine reproductive and respiratory syndrome virus: PRRSV) 감염이 의심되는 농장으로부터 수집된 혈청가검물 646개로부터 MARC-145 cell을 이용하여 PRRSV 분리를 시도한 바 MARC-145 세포단층상에 세포변성효과(cytopathic effects : CPE)를 나타내는 바이러스 36주를 분리하였다. 분리된 36주가 PRRSV인지 여부를 확인하기 위하여 PRRSV를 실험적으로 접종한 혈청을 이용하여 간접형광항체시험과 혈청중화시험을 실시한 결과 36주 모두가 PRRSV로 동정되었다. 혈청학적인 동정법과 더불어 reverse-transcription polymerase chain reaction을 이용하여 PRRSV open reading frame 5(ORF5)의 유전자를 증폭한 결과 선발된 6주 모두에서 80bp의 flanking sequencing를 포함하여 약 680bp의 ORF5의 유전자를 증폭할 수 있었다.

Key words : PRRSV, Isolation, Identification

Introduction

Porcine reproductive and respiratory syndrome(PRRS) has emerged as an important disease of swine through the world[1~11]. PRRS is manifested by severe reproductive failure in sows and gilts, high mortality in preweaning pigs and respiratory disease in growing pigs.

This work was supported by Korea Research Foundation Grant(KRF-1997-001-G 00162)

Reproductive failure characterized by late-term abortion, premature farrowings, increased numbers of stillborn and mummified fetuses, and to a lesser extent, decreased farrowing rates. Concurrent infection with bacteria has been commonly observed and led to decreased average daily gain, decreased feed efficiency and increased mortality[12,13]. Economic loss due to PRRV infection has been evaluated[14,15].

The PRRSV is classified as a member of the genus *Arterivirus*, family *Arteriviridae*. This family *Arteriviridae* includes equine arteritis virus(EAV), lactase dehydrogenase-elevating virus(LDV), and Simian hemorrhagic fever virus (SHFV)[16~20]. The genome of the virus, a positive-strand RNA, is 15kb in length and contains 8 open reading frames that encode viral proteins. ORFs 1a and 1b encode the viral RNA polymerase, and ORFs 2, 3 and 4 encode structural proteins of the virion[21]. ORF5 which encodes a 25-kDa *N*-glycosylated envelope glycoprotein is the most variable and ORF6 encoding the 18-kDa non *N*-glycosylated protein is the most conserved[22]. ORF7 encodes the 15kDa nucleocapsid protein. Recently, a neutralization domain in the protein encoded by ORF4 of Lelystad virus(LV) was identified and thus the region is considered to be antigenically involved[18]. Strain variation is a characteristic of this group of viruses. There are substantial antigenic and molecular variations among the PRRS virus strains[23~27].

The PRRS has been diagnosed by the serological methods. These methods include indirect fluorescent antibody(IFA) test[28,29], enzyme linked immunosorbent assay(ELISA)[30,31], and serum neutralization test[32]. Isolation of PRRSV has often been attempted on the purpose of examining virus circulation in a certain swine herd. The circulation of PRRSV may influence vaccine efficacy and eradication program in infected swine herds.

This study reports the isolation of the cytopathic virus from swine serum and identification of the isolates as a PRRSV by using the IFA test and virus neutralization (VN) test. In addition to serological identification, ORF5 of PRRSV genome was amplified by reverse-transcription polymerase chain reaction(RT-PCR).

Materials and Methods

Cell and reference virus culture

The MARC-145 cells, permissive to PRRSV, were maintained in Eagle's minimum essential media(MEM) supplemented with sodium bicarbonate and fetal calf serum. The procedures for propagation of reference PRRSV CNV-1 strain on the MARC-145 cell monolayers have been previously described in detail[28,33].

Isolation of PRRSV

The MARC-145 cells were grown in 24- or 48-well tissue culture plates in an atmosphere of 5% CO_2. The serum samples were diluted 10 times in culture media and centrifuged at 10,000g for 10 minutes. 200$\mu\ell$ of diluted serum sample was inoculated onto cell monolayers and incubated at 37℃ for 1 hour to allow virus adsorption. The inoculum was removed and 1 ml of MEM was

added. Cytopathic effects(CPE) specific for PRRSV were observed daily. Once CPE was observed, the virus culture media was harvested and stored at -70℃. Monolayers without CPE were subjected to additional 2 passages to confirm negative virus isolation results.

Indirect fluorescent antibody test

An indirect fluorescent antibody(IFA) test was performed to identify the cytopathic virus isolate as being a PRRSV using reference-positive sera, as previously described[28, 29]. In briefly, the virus isolate was inoculated onto confluent MARC-145 cell monolayers, When initial type of CPE emerged, the cell monolayers were fixed with ice cold, absolute ethanol. After ethanol fixation, each well was washed twice with phosphate buffered saline(PBS) and 30$\mu\ell$ of 1 : 20 diluted PRRS virus antibody positive reference serum was add. The plate was then incubated at 37℃ in a CO_2 incubator for 45 minutes. After incubation, each well was washed 5 times with PBS. 30$\mu\ell$ of 1 : 40 diluted rabbit anti-swine IgG conjugated fluorescein isothiocyanate(FITC) was added. The plate was incubated at 37℃ for 30 minutes. After washing again with PBS, the monolayers were examined under a fluorescence microscope.

Virus neutralization(VN) test.

Virus neutralization test was also performed to confirm cytopathic virus isolates as being a PRRSV. The PRRSV antibody positive reference serum was obtained from piglet infected intranasally with a reference PRRSV CNV-1 strain. Serum was heated at 56℃ for 30 minutes. The culture media of the cytopathic virus isolates were diluted to make a final infectivity of 100 median tissue culture infectious doses(100 $TCID_{50}$). 50$\mu\ell$ of diluted virus culture media was added into 96-well plate and then an equal volume of antibody positive reference serum which was diluted 4 times was added. The serum-virus mixtures were incubated for 1 hour at 37℃ and then 100$\mu\ell$ of MARC-145 cells(5-10$\times 10^5$cells/ml) was placed into each well. The results were read after 2~3 days incubation in an atmosphere of 5% CO_2.

Reverse transcription and polymerase chain reaction(RT-PCR)

Extraction of virus RNA from 200$\mu\ell$ of virus culture media was conducted according to the method reported elsewhere[24,33,34]. The oligonucleotide primers(forward primer ; 5'TT GAA TTC AGC CTG TCT TTT TGC CAT, reverse primer ; 5' TT GGA TCC CCT TTT GTG GAG CCG TGC) specific for ORF 5 gene of PRRSV was designed according to the sequence information of *Lelystad virus* (EMBL/Genbank Data Libraries Accession No. M96262). The cDNA of the PRRS virus ORF5 gene was synthesized by reverse transcription and resulting cDNA was amplified by PCR. The resulting PCR products were confirmed by electrophoresis on 1.2% agarose gel.

Results

Isolation of PRRSV from swine sera

Total 36 cytopathic viruses were isolated from 646 swine sera. The CPE specific for

PRRSV on the MARC-145 cell monolayers was observed after 1 to 3 passages(Fig 1).

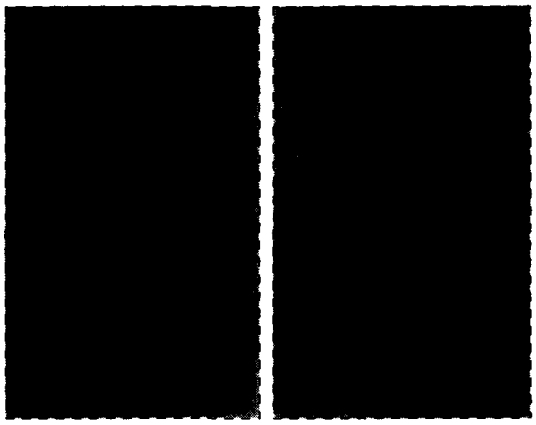

Fig 1. Cytopathic effects observed on the MARC-145 cell monolayer. Noninfected cell monolayer (left). Infected cell monolayer with degenerating cells observed 3 days postinoculation (right).

Indirect fluorescent antibody response

Thirty six cytopathic virus isolates were inoculated onto MARC-145 cell monolayers and the infected cell monolayers were fixed with cold ethanol when the early CPEs were observed. The IFA test was conducted to identify cytopathic virus isolates as a PRRSV. The IFA results showed that MARC-145 cell monolayers infected with cytopathic virus isolates were stained with fluorescence color (Fig 2). The fluorescence color was mainly located at the cytoplasm of infected MARC-145 cells. 36 virus isolates were identified as a PRRSV.

Virus neutralization response

In the VN test, 36 virus isolates were also identified as a PRRSV. All cytopathic virus isolates were neutralized by the reference serum.

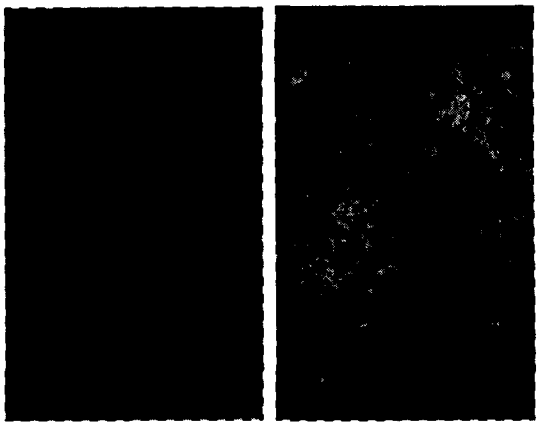

Fig 2. Indirect immunofluorescence staining of the cytopathic virus isolate-infected MARC-145 cell monolayer. Non-infected cell monolayer (left). Infected cells with cytoplasmic fluorescence observed 3 days postinoculation (right).

RT-PCR

Genomic viral RNAs of the selected cytopathic virus isolates were extracted from culture media, and the cDNA of the ORF5 was synthesized by reverse transcription and amplified by PCR. The PCR products was examined by electrophoresis on 1.2% agarose gel. An appropriate band of about 680bp including 80bp flanking sequence was seen on agarose gel stained with ethidium bromide(Fig 3).

Discussion

Isolation of PRRSV from serum samples of the diagnostic submission is not practical

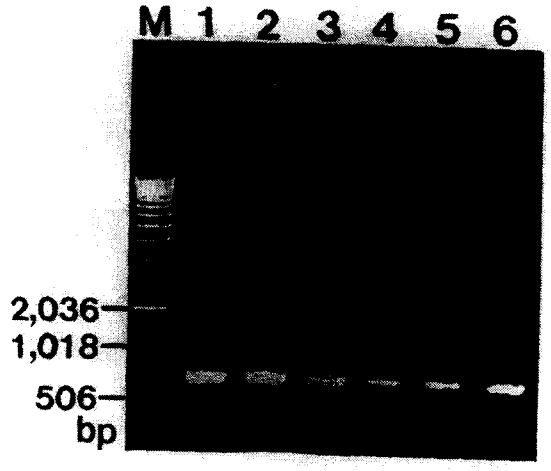

Fig 3. Agarose gel electrophoresis of PCR products. Lane M : 1kb DNA ladder, lane 1 to 6 represent 6 cytopathic virus isolates.

for the diagnosis of PRRS. However, virus isolation is useful to examine virus circulation in a certain swine herd using a permissive cell line. In this study, most of virus isolates showed their specific CPE after 1 to 3 passages. Total 36 isolates were identified as PRRSV in both IFA and VN test. Amplification of ORF5 gene of PRRSV may be an additional confirmation of the isolates as being a PRRSV. Low rate of isolation(36/646 ; 5.6%) appeared to be inhibition of virus replication by PRRSV antibody in serum samples. It was also thought to be that the interval between infection and sampling of serum is long enough to eliminate virus from blood circulation.

Summary

Isolation of PRRSV was attempted from 646 swine sera collected from swine farms. The MARC-145 cell, which is highly permissive to PRRSV, was used for virus isolation, propagation, IFA test, and VN test. Total 36 cytopathic viruses to MARC-145 cells were isolated. The virus isolates were identified as a PRRSV by the IFA test and VN test using the reference sera prepared by experimental infection of reference PRRSV CNV-1 into 30 day-old pig. In addition to serological conformation, ORF5 of genomic RNA of 6 selected cytopathic viruses were amplified by the RT-PCR. The resulting PCR products were examined by electrophoresis on 1.2% agarose gel. An appropriate bands of about 680bp including the flanking sequence of total 80bp were seen on agarose gel.

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