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Biological and Physicochemical Properties of Porcine Epidemic Diarrhea Virus Chinju99 Strain Isolated in Korea

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Abstract : A disease with severe diarrhea occurred in a herd of one thousand, 1-week-old piglets in Chinju, Korea, and was diagnosed as porcine epidemic diarrhea by the detection of N gene of porcine epidemic diarrhea virus (PEDV) from small intestines. A PEDV, named as Chinju99, was also isolated from the intestines after two blind-passages in Vero cells supplemented with trypsin (10 μg/ml), and the biological and physicochemical properties of the isolate were characterized. The virion was roughly spherical in shape and had spike peplomers on its outer surface. The virus exhibited cytopathic effects such as rounding degeneration at initiation of infection and syncytia formation later in Vero cells. The virus was labile to 20% ether and 5% chloroform but stable in acid with pH 4-7 at 4°C. The infectivity of the virus was maintained at 50°C for 180 min, and the buoyant density of the virus in sucrose was 1.180 g/ml. All biological and physicochemical properties of the virus were typical features of coronaviruses.

Key words **:** PEDV, Isolation, N gene.

Introduction

Since the first outbreak of porcine epidemic diarrhea (PED), the etiological agent was reported in Belgium[15] and the UK[3]. The disease has also been recognized in Canada[19], Hungary[9], Germany[16], Japan[17] and Korea[11]. Pigs of all age groups are susceptible to PED, and the mortality in suckling piglets of 1-week-old is as high as 90%[14]. Clinical signs and pathological findings in the PED are indistinguishable from those in transmissible gastroenteritis. The etiological agent, porcine epidemic diarrhea virus (PEDV), belongs to the same family *Coronaviridae*[5,6,13,20] with transmissible gastroenteritis virus (TGEV).

Viral disease is routinely diagnosed by the isolation of etiological virus and neutralization test using specific antiserum. However, primary isolation of PEDV in routine cell cultures was difficult until propagation of PEDV was successful by adaptation to Vero cells using medium containing trypsin[7,10]. PED is one of the devastating diseases in suckling piglets and occurs frequently in Korea. Kwon *et al* (1993)[11] isolated a PEDV and described antigenic peptides of the isolate in Korea. Kwon *et al* [12] also reported that 45% of tested pigs were sero-positive to the virus by ELISA. However, no further studies on the isolation of the virus have been made, and to our knowledge, biological and physicochemical properties of the Korean isolate of PEDV have not been fully characterized.

In the present study, a strain of PEDV was isolated from piglets suffering from diarrhea in Chinju, Korea, and the biological and physicochemical properties of the isolate were characterized.

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Materials and Methods

Samples for virus isolation

A severe diarrhea occurred in a herd of one thousand, 1-week-old suckling piglets in a farm located in Chinju, Korea in 1999. The piglets suffered from diarrhea, vomiting and dehydration, and the disease was presumptively diagnosed as PED based on the clinical signs. Intestinal tissues and feces of these piglets were collected and transported to the laboratory. The samples were emulsified with 10 volumes of minimal essential medium (MEM) (Invitrogen, USA) and used for the detection of viral gene and isolation of PEDV. The tissue emulsion, which was used for the viral isolation, was treated with streptomycin (500 μg/ml) and penicillin (500 U/ml) at 37°C for 1 h, and clarified by centrifugation at 5000 × g for 30 min. The supernatant was further passed through a syringe filter of nitrocellulose membrane with pores of 0.45 μm to remove bacterial contamination.

Diagnosis of PED by reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was done to detect PEDV gene from the intestinal tissues and feces using sense primer (5'TAAGCAACAGCAGAAGCCTA3') and antisense primer (5'TTAATTTCCTGTATCGAAGAT3'). These primers were designed and aligned based on nucleotide sequence of nucleocapsid (N) gene of PEDV CV777 and Br1/87 strains[1] from GenBank data base (accession No. Z14976) to amplify DNA of 601 bases.

Detection of TGEV N gene from the same samples was also done using sense primer (5'CTTGGAAGAGAACTGCAG3') and antisense primer (5'TTAGTTCGTTACCTCATC3') designed to amplify DNA of 442 bases based on the nucleotide sequence of British FS772/70 strain[2] by alignment on

GenBank data base (accession No. Y00542). Emulsion of intestinal tissues and feces was mixed with Trizol® (Invitrogen) following the manufacturer's indication. After centrifugation of the mixtures at 10,000× g for 15 min, total RNA was extracted from the supernatant by alcohol precipitation. In a tube, 25 μl of the RNA and 1 μl of antisense primer were mixed, and the first-strand cDNA was made by RT at 37°C for 90 min using kit reagents including AMV reverse transcriptase (Promega, USA). To make double-stranded cDNA by PCR, 5 μl of the first-strand cDNA was added with 30.5 μl of distilled water, 5 μl of 10X PCR buffer, 1 μl of 10 mM dNTPs, 2.5 μl each of 20 μM sense and antisense primers, and 3 μl of 25 mM $MgCl_2$ (Perkin-Elmer PCR kit, USA). The mixture was preheated at 94°C for 5 min and 0.5 μl of *Taq* DNA polymerase (5 U/μl) was added, and overlayed with 100 μl of mineral oil. The PCR was done for 30 cycles of 1 in each at 94°C for denaturation, 50°C for annealing and 72°C for polymerization, and a cycle of final extension at 72°C for 7 min using thermocycler (Hybaid Omn-E, England). The amplified DNAs were observed by resolving on 1% agarose gel.

Observation of virus particles

The emulsion of intestinal tissues was clarified by centrifugation at 5,000× g for 30 min, and the supernatant was passed through a syringe filter of nitrocellulose membrane with pores of 0.45 μm. An aliquot of the supernatant was stained with equal volume of 2% phosphotungstic acid, and the mixture was loaded on the carbon Formvar-coated copper grid. Morphology of the viral particles was observed by electron microscope (Hitachi H600, Japan).

Isolation of virus

Isolation of PEDV in Vero cell cultures was done following the methods of Hofmann and Wyler[7]. Vero cells were cultured in MEM containing 5% fetal calf serum (FCS) (Invitrogen), streptomycin (100 μg/ml) and penicillin (100 U/ml) in a 5% CO_2 incubator at 37°C for maintenance. After subculture for 2 days, Vero cells were inoculated with 1 ml of the intestinal tissue emulsion, and the inoculum was removed after attachment for 2 h. The cells were cultured with MEM containing streptomycin (100 μg/ml), penicillin (100 U/ml) and trypsin (10 μg/ml) (MEM-T) without FCS in a 5% CO_2 incubator at 37°C. Appearance of cytopathic effect (CPE) was observed daily, and blind passages were done with culture supernatant after repetitive freezing and thawing of the cells if there was no CPE observed. The culture supernatant was also used for the confirmation of viral propagation by RT-PCR for N gene. The virus showing CPE and positive to N gene was isolated after plaque purification. For plaque purification, Vero cells cultured in 6-well plate were inoculated with virus and overlayed with 1% Seaplaque agarose (FMC, USA). For observation of the viral plaques, the plaques were stained with 0.01% neutral red.

Viral stability in organic chemicals

Following the method of Hofmann and Wyler[8], stability of the virus with ethyl ether and chloroform was determined. Titer of the virus was adjusted to 10^4 PFU/ml after propagation in Vero cells with MEM-T. The virus was treated with 20% ethyl ether and 5% chloroform at room temperature for 1 h, respectively. After treatment, propagation ability of the virus in Vero cells cultured with MEM-T was observed by plaque assay.

Viral resistance to heat

The virus of 10^4 PFU/ml was heated at 50, 60, 70 and 80°C for 30 min, respectively, and cooled on ice bath following the method of Hofmnann and Wyler[8]. Then, the propagation ability of the virus in Vero cells cultured with MEM-T was observed by plaque assay. The resistance of the virus to heat at 50°C was also determined by time courses of 0, 5, 10, 15, 30, 60, 120 and 180 min, respectively.

Viral resistance to acid

Following the method of Hofmann and Wyler[8], the virus of 10^4 PFU/ml was diluted to 1:10 in MEM-T and pH of the viral fluid dilutions was adjusted to 2, 3, 4, 5, 6, and 7, respectively, with 2N HCl. After acidic treatment at 4°C for 6 h, pH of the viral fluid was readjusted to 7.4 with 2N NaOH. Propagation ability of the virus in Vero cells cultured with MEM-T was observed by plaque assay.

Plaque assay for the determination of viral stability

Plaque assay was done for the determination of viral titers and stability after treatment with organic chemicals, heat and acid as described in the method of Hofmann and Wyler[8]. Viral fluid was tenfold-diluted, and 200 μl of the fluid was inoculated into Vero cells which were cultured at a seeding rate of 2×10^5 cells/ml in each well of 24-well plates. After attachment for 2 h, the inoculum was removed and the cells were washed twice with phosphate buffered saline (pH 7.4). The cells were cultured with 0.5 ml of MEM-T at 37°C, 5% CO_2 incubator for 4 h and added with 0.5 ml of MEM containing 4% FCS, streptomycin (100 μg/ml) and penicillin (100 U/ml) to neutralize trypsin in MEM-T. After culture for 48 h, the medium was removed and the cells were fixed with 1 ml of 4% formaldehyde for 1 h. Continuously, the formaldehyde solution was removed, and the cells were stained with 0.5% crystal violet for 10 min and washed with distilled water. After drying, the cells were observed for plaques by inverted microscope.

Buoyant density of the virus

Viral fluid from Vero cell cultures was clarified by centrifugation at 5,000× g for 30 min, and the supernatant containing virus was ultracentrifuged at 50,000× g (Beckman SW 41Ti rotor, USA) for 15 h at 4°C. The pellet was resuspended to a concentration of 1/100 in TNE buffer (0.05 M tris-HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4). The viral fluid was lay-

ered on top of a discontinuous, 10-50% sucrose cushion in TNE buffer and ultracentrifuged at 50,000× g (Beckman SW 41Ti rotor) for 17 h at 4°C. The virus-containing fraction was collected and buoyant density of the virus was measured.

Results

Diagnosis of PED by the detection of viral gene

In RT-PCR for the detection of viral gene from intestinal tissues and feces of piglets suffered from diarrhea, DNAs with predicted size of approximately 600 bases were amplified from jejunum and colon using specific primers to N gene of PEDV. On the other hand, no DNAs were amplified from the same samples using specific primers to TGEV N gene (Fig 1). The disease in piglets was, therefore, confirmed as PED by the results in PCR.

Isolation and biological properties of the virus

The viral particles in the emulsion of intestinal tissues appeared roughly spherical in shape and more than 100 nm in size by electron microscope. The virus also harbored club-shaped spike peplomers on the outer surface of the virions (Fig 2). In primary isolation of the virus from tissue emulsion in Vero cell cultures with MEM-T for 5 days, neither viral growth nor CPE was shown. However, the virus was propagated with CPE after two blind-passages of the culture supernatant. The PEDV N gene was also detected from the supernatant of the cell cultures by RT-PCR. The virus was, therefore, isolated by plaque purification and named as Chinju99.

When CPE revealed in Vero cell cultures by the Chinju99 was closely observed, the cells began to aggregate at the early stage of viral infection. Rounding degeneration and syncytia formation were obvious at 15 h post infection. At 20 h, syncytia were prominent and more than 100 nuclei were harbored in a syncytium, and 80-90% of the cells showed rounding degeneration (Fig 3). The cells were completely degenerated and detached from culture vessel afterward.

Physicochemical properties of the virus

The Chinju99 was labile to 20% ether and 5% chloroform as the initial viral titers of 10^4 PFU/ml decreased as low as $10^{0.5}$ PFU/ml after treatment with these organic chemicals. On the other hand, the virus was stable in acid with pH 4 to 7 at 4°C and resistant to heat at 50°C for 180 min but inactivated after heating at 60-80°C for 30 min (Table 1). The buoyant density of the virus in sucrose was 1.180 g/ml.

Discussion

A strain of PEDV, named as Chinju99, was isolated from intestinal tissues of piglets, which was presumptively diagnosed as PED by the detection of PEDV N gene based on the nucleotide sequence of PEDV CV777 and Br1/87[1]. The virus in the infected intestines showed particles with roughly

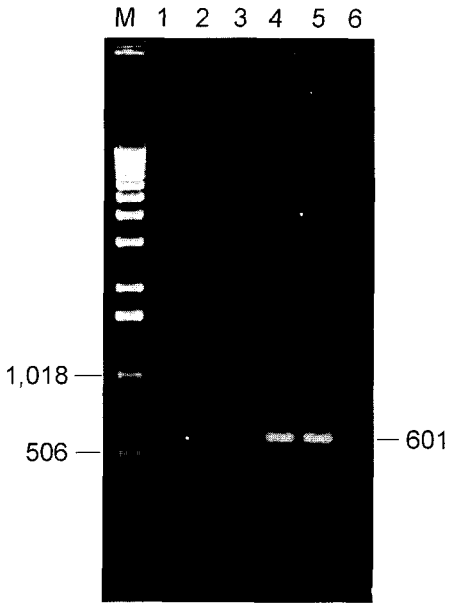

Fig 1. Detection of PEDV N gene in the intestinal tissues of piglets by RT-PCR: 1, 2 and 3, no DNAs amplified from jejunum, colon and feces, respectively, by specific primers to TGEV N gene; 4 and 5, DNAs of 601 bases amplified from jejunum and colon, respectively, by specific primers to PEDV N gene; 6, no DNA of PEDV N gene amplified from feces; M, 1 kb DNA ladder marker (Invitrogen).

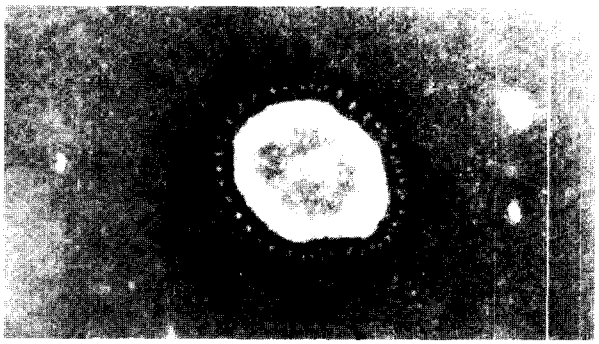

Fig 2. PEDV Chinju99 showing roughly spherical shape and spike peplomers on the virion by electron microscopic observation (bar; 100 nm).

spherical shape and spike peplomers on the surface as the typical morphology of coronaviruses reported previously[7,8,13]. Although no viral growth was recognized in primary culture of the intestinal tissue emulsion, the viral growth was observed by CPE after two blind-passages in Vero cell cultures with MEM-T. It was, therefore, recognized that the virus was adapted to Vero cells, facilitated by trypsin supplemented in MEM-T. Trypsin has been used as an ingredient facilitating the infectivity of PEDV to cell cultures[4,8,18]. The virus exhibited CPE such as rounding degeneration at initial stage of propagation and prominent syncytia formation later, which

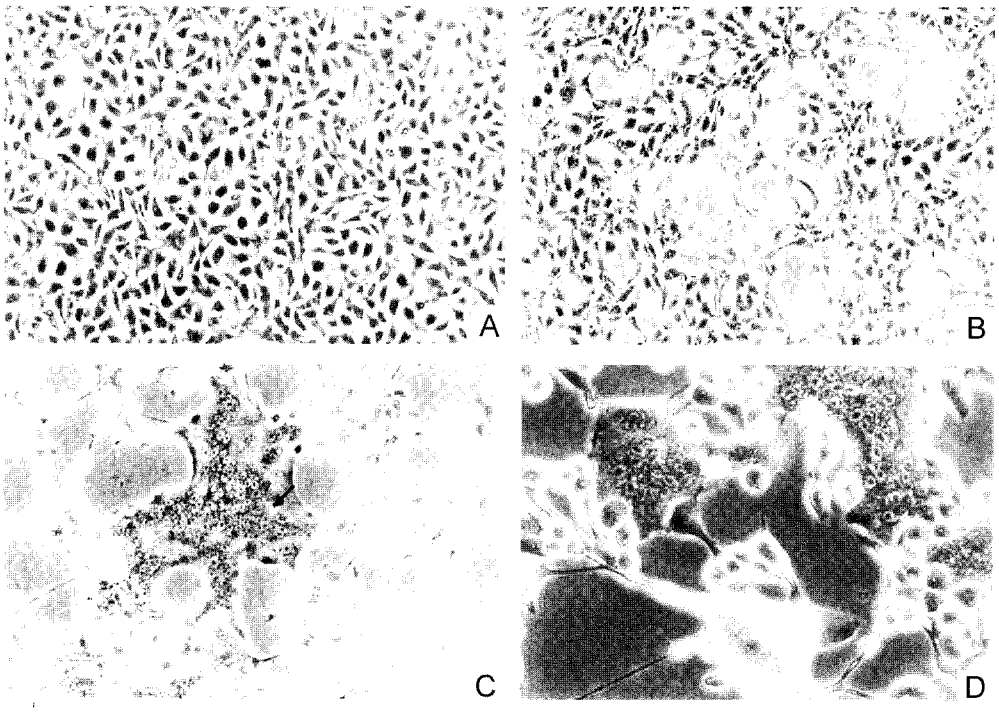

Fig 3. Cytopathic effects in Vero cells infected with PEDV Chinju99: A, mock-infected cells (X100); B, aggregation of the virus-infected cells at 4 h p.i. (× 100); C, rounding degeneration and syncytia formation (arrow) in the virus-infected cells at 15 h p.i. (× 100); D, marked cell rounding and syncytia formation at 20 h p.i. (× 200).

Table 1. Physicochemical properties of PEDV Chinju99[a]

Properties	Stabilities
20% ether	Labile[b]
5% chloroform	Labile[b]
Acid with pH 4-7 at 4°C	Stable
Heat at 50°C	Resistant[c]
60°C	Sensitive
70°C	Sensitive
80°C	Sensitive

[a]Virus in titer of 10^4 PFU/ml was used.
[b]Virus titers decreased to as low as $10^{0.5}$ PFU/ml after treatment for 1 h.
[c]Virus maintained the infectivity after heating at 50°C for 180 min.

were characteristic features of the PEDV after adaptation to Vero cells[7,8,14]. Kusanagi *et al*[10] also reported on the isolation of a PEDV strain in Vero cell cultures supplemented with trypsin and observed cell-fusion and syncytia formation in the infected cells.

The Chinju99 was labile to lipid solvents such as ether and chloroform, which can be the evidence that the virion has lipid-layered envelope[8,13,14]. On the other hand, the virus was stable in acid with pH 4-7 at 4°C and resistant to heat at 50°C for 180 minutes. Hofmann and Wyler[8] also reported the stability of PEDV in acid with pH 5-9 at 4°C and heat at 50°C for 30 min. The buoyant density of the virus in sucrose (1.180 g/ml) was similar to 1.176-1.182 g/ml of German isolate V215/78[8].

In conclusion, all biological and physicochemical properties of the PEDV Chinju99 was characteristic features of coronaviruses[8,13,14]. To our knowledge, the biological and physicochemical properties of a PEDV isolated in Korea were first reported in the present study.

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국내 분리 돼지 유행성설사 바이러스 Chinju99주의 생물학적 및 물리화학적 성상

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초 록 : 진주 지역에서 1000 여두의 생후 1주령 포유 자돈군에 심한 설사증이 발생하였던바 소장조직으로부터 RNA를 추출한 후 reverse transcription-polymerase chain reaction을 실시하였던 결과, porcine epidemic diarrhea (PED) virus (PEDV)의 N 유전자가 검출되어 PED로 진단되었다. Trypsin (10 μg/ml)을 첨가한 배지에서 Vero 세포를 배양하면서 장조직으로부터 바이러스 분리배양을 시도하였던 결과 2회의 blind passage 후에 PEDV를 분리할 수 있었다. 따라서 이 분리주를 Chinju99로 명명하였으며, Chinju99주의 생물학적 및 물리화학적 성상을 조사하였던 결론은 다음과 같다. Chinju99주는 전자현미경 소견에서 비정형의 타원형 입자로서 표면에 spike 구조를 가지고 있었으며, Vero 세포내 배양시에 점진적으로 원형변성, 합포체형성 등의 세포변성 소견을 나타내었다. 또한 20% ether, 5% chloroform에서 불안정하였으나 pH 4-7의 산성조건에서는 안정성을 나타내었다. 동시에 50°C에서 180분간 처리 후에도 감염성이 유지되었고 sucrose 용액에서의 부유밀도가 1.180 g/ml으로서, coronavirus의 전형적인 생물학적 및 물리화학적 성상을 나타내었다.

주요어 : PEDV, Isolation, N gene