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Cell adaptation of KPEDV-9 and serological survey on porcine epidemic diarrhea virus(PEDV) infection in Korea

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돼지 유행성설사바이러스 국내 분리주(KPED-9)의 세포증식성 및 혈청학적 역학조사

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초 록 : 돼지 유행성 설사 바이러스(KPEDV-9)주를 이용하여 세포내 증식성을 비롯한 혈청학적 역학조사를 수행하였던 바 다음의 결과를 얻었다.

돼지 유행성 설사바이러스 국내 분리주는 Vero 세포에 연속계대시 증식성이 증가되었으며 90대 계대시 $10^{5.5}$ $TCID_{50}$/ml의 역가를 나타내었다. 조직배양 순화주를 이용하여 감염세포내에서 20Kb 이상의 RNA 가 존재함을 확인할 수 있었으며 전자현미경 검사시 5~10nm 의 외피항원 및 80~300nm크기의 coronavirus 특징을 나타내었다.

설사증상을 나타내는 돼지의 장가검재료를 이용하여 유행성 설사 바이러스의 감염실태를 조사하였던 바 18%에 상당하는 감염 양성율을 확인하였으며 ELISA법에 의한 항체검사결과 전국적으로 약 45%의 항체 양성율을 나타내었다.

Key words: porcine epidemic diarrhea virus, KPEDV-9, cell adaptation, RNA profile, electron microscopy, etiological surveillance ELISA, serological survey

Introduction

Porcine epidemic diarrhea virus(PEDV) as a coronavirus is the etiological agent of enteropathogenic diarrhea in swine.[1-3] The overall clinical symptoms of PEDV are very similar to transmissible gastroenteritis (TGE) virus infection in field, except that PEDV shows more wide range of clinical signs in fattening and weaning pigs. Previously, we reported the isolation and derivation of korean isolate (KPEDV-9 strain) in Vero (African green monkey kidney) cells.[4] However, the epidemiological survey on PEDV infection is not properly conducted in this country partly because of difficulties in serological diagnostic system.

The present study was carried out to further characterize KPEDV-9 strain. In addition, the serological method was developed and the results

were described in this paper.

Materials and Methods

Virus and cell : Vero cell from American type culture collection (ATCC) was regularly maintained in alpha-MEM(Gibco) with 5% fetal bovine serum and was used for propagation of KPEDV-9. The virus was serially passaged in roller culture. The virus was inoculated on 80-90% monolayered Vero cells at 37℃ for 1 hour, washed once with phosphate buffered saline (PBS, pH7.4)and propagated in alpha-MEM with 0.02% yeast extract, 0.3% tryptose phosphate broth(TPB)and trypsin at the concentration of 2-3ug/ml as described previously.[4] The supernatant was usually harvested 4-5 days postinfection and used for next passage.

Identification of viral RNA and electron microscopy of KPEDV-9 : For identification of viral RNA, cytoplasmic RNA were extracted both from control and virus inoculated cells by the procedures of perbal.[5] The extracted RNA was then analyzed in 0.8% denaturing agarose gel according to the methods of glyoxal and dimethylsulfoxide.[6] For purification of virus, the virus-inoculated cells and supernatant was freezed and thawed once. The cell culture debris was then removed after centrifugation at 3000 rpm for 10 minutes and the supernatant was treated with polyethylene glycol 6000(MW) according to the procedures of Chu et al.[7] The viral solution was precipitated and resuspended at 1/10th of original volume with TEN buffer (0.01M Tris, 0.001M EDTA, 0.1M NaCl, pH 7.4) The resuspended solution was further centrifuged at 60,000xg for 2 hours on 15% (W/V) potassium tartarate cushion in TEN buffer. The pellet was suspended in TEN buffer to 1/700 of the initial volume and used for electron microscopy examination. This viral sample was stained with 2% uranyl acetate and examined on the electron microscopy (Hitachi).

Etiological survey on PEDV infection : The cryostat-microtome sectioned intestines of pigs showing acute diarrhea were examined for the presence of infection using biotinylated rabbit sera against PEDV as described before.[4] Briefly, the samples were reacted with biotinylated rabbit sera against PEDV for 45 minutes at 37℃ and washed in PBS. The washed samples were further incubated with streptoavidin FITC conjugate at same condition. The incubated tissues were then washed again with PBS and examined on fluorescent microscopy.

Animal inoculation and serum samples : Five pigs were tested for the detection of PEDV antibody. Four pigs were inoculated with KPEDV-9 at the passage level of 30, 50 and 90, respectively. One pig was remained as control. After two weeks of first inoculation, second inoculation at same passages were followed. Thereafter, sera were collected at the weeks of 1, 2, 3 and 4 weeks later, respectively. The sera from control and inoculated pig were tested for the presence of antibodies against PEDV by ELISA and TGE by serum neutralization assay. A total of 469 sera of slaughtered pigs from local province were collected and tested for the presence of PEDV antibodies by ELISA. At each test, standard negative and infected positive serum were tested together to monitor the sensitivity and specificity of the test.

ELISA procedure : The resuspended viral pellet was treated with 0.5% TritonX-100(Sigma) for 30 minutes at room temperature at shaking rotator and detergent was then removed through extra gel colume(Pierce) according to procedures of manufacturer. The collected antigen was then used for ELISA based on the standard protocol.[8]

The dilution of antigen and second antibody were adjusted to optical density(OD) around 0.2(A 490) using negative porcine sera. Usually 1-2 μg of protein per well in 96 well microplate (Costar) was coated in 50 mM carbonate buffer (pH 9.6) at 5℃ overnight, followed by blocking with 1% skim milk at 37℃. 1/200 diluted porcine sera in PBS with 0.5% Tween 20(PBST) were reacted at 37℃ for 30 minutes and then washed extensively with PBST for three times at 5 minutes interval. The reacted plate was washed again at same condition and incubated with 2000 fold diluted horseradish peroxidase (HRP) labelled anti-porcine lg G (KPL) for 1 hour at 37℃. The plate was developed in OPD(o-phenylenediamine) at room temperature for 30 minutes. The reaction was

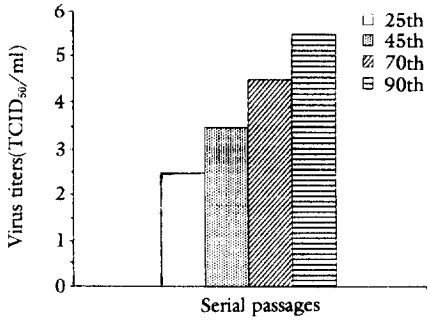

Fig 1. Adaptation of KPEDV-9 in vero cells. Viral titers are expressed as Log_{10}.

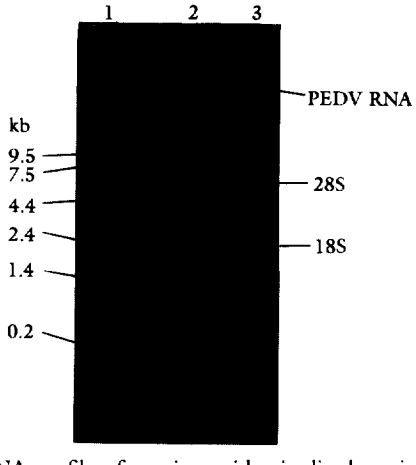

Fig 2. RNA profile of porcine epidemic diarrhea virus. Standard RNA size marker (lane 1), RNA extracted from uninfected control cells (lane 2) and RNA extracted from virus infected cells (lane 3), respectively. RNA sizes in Kb are indicated.

stopped with H_2SO_4 before measuring OD at 490nm.

Results

Cell adaption of KPEDV-9 strain : The viral titers were about $10^{2.5}TCID_{50}$/ml at the passage 25th. However, the viral yields have increased in vero cell culture after serial passages. As shown in Fig 1, the infective titers of KPEDV-9 reached up to $10^{5.5}TCID_{50}$/ml at the passasge level of 90th.

Identification of viral RNA and electron microscopy : For the identification of PED viral RNA,

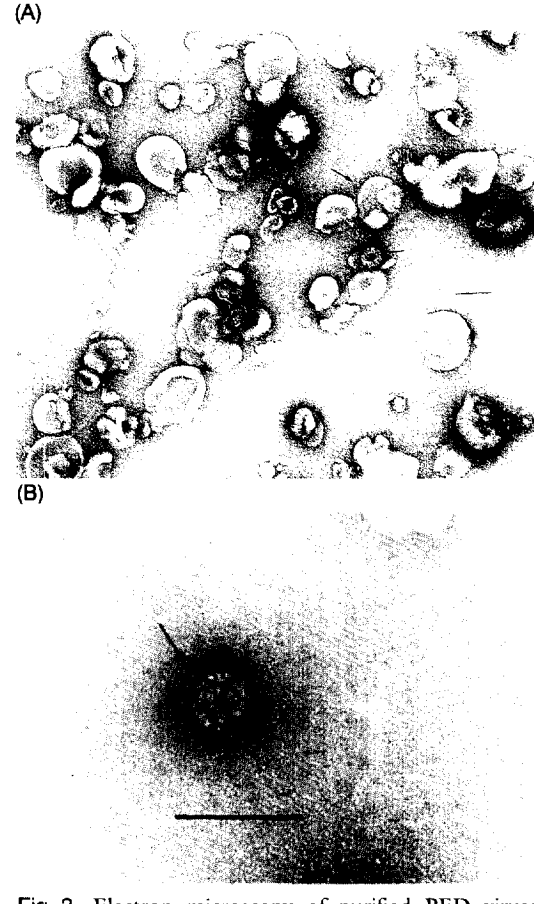

Fig 3. Electron microscopy of purified PED viruses after staining with uranyl acetate. Variance in size (A) and surface projections of virus (B) are arrow indicated. Bar represents 100nm.

cytoplasmic RNA extracted from both infected and control cells were subjected to denaturing gel electrophoresis and analysed after ethidium bromide staining. As shown in Fig 2, one large extra RNA band was only detected in the lane of RNA from virus infected cells. The estimated molecular weight (MW) was roughly more than 20Kb.

When the concentrated viral pellet was examined by electron microscopy, the presence of enveloped viral particles were detected with variance in size from 80 to 300nm (Fig 3, A). However, the electron microscopy of KPEDV-9 revealed the presence of surface projections with the size of 5-10nm, showing the typical morphology of coronavirus (Fig 3, B). Nevertheless, the heterogeneity in size and shape were usually observed throughout morphologial study

Table 1. Detection of PEDV infections by FA test from intestines of piglets with acute diarrhea

Province	Positive reactions/Cases examined*
Kyunggi	3/32
Chungnam	5/16
Chunbuk	1/13
Kyungbuk	3/3
Kyungnam	2/5
Kangwon	ND**/3
Chungbuk	ND/3
Inchun	ND/2
Jeju	ND/3
Total (%)	14/80(17.5)

*: Samples are separately collected from Apr 1992 to Jun 1993.
**: Not detected.

Table 2. Age distributions and clinical signs of positive cases

Positive cases*	Age	Major clinical signs
9	piglets within 2 weeks	diarrhea, vomitting
5	weaning or fattening pigs from 2 weeks to 6 months	diarrhea

*: Positive reaction by FA test.

as indicated in Fig 3.

Detection of PEDV infection from pigs with diarrhea : A total of 80 separate cases of intestines of swine having enteropathogenic diarrhea were examined for the presence of viral antigens by immunofluorescence test. Among those cases, 14 positive reactions were detected. Table 1 shows the regional distribution of positive cases, indicating that PEDV infections present almost throughout the country. However, about two third of positive cases were detected in piglets within two weeks old and the rest were found in the fattening or growing pigs as listed in Table 2.

Animal inoculation and ELISA : After setting up the basic conditions for ELISA, the sensitivity were tested using the sera from experimentally inoculated swine. Pigs were inoculated with virus at different passages and the sera were tested in the same plate by ELISA. As shown in Fig 4, A, the inoculated pig showed the rising ELISA titer at one week's postinoculation. In order to check the sensitivity, IFA test was conducted on virus infected and control cells. The IFA result also indicated 2 fold difference from first week after inoculation (Fig 4, B). For the specificity of ELISA, the sera from 4 weeks after virus inoculation were tested for the presence of antibody against TGEV and none showed the antibodies by neutralizing assay, thus validating the specificity of ELISA.

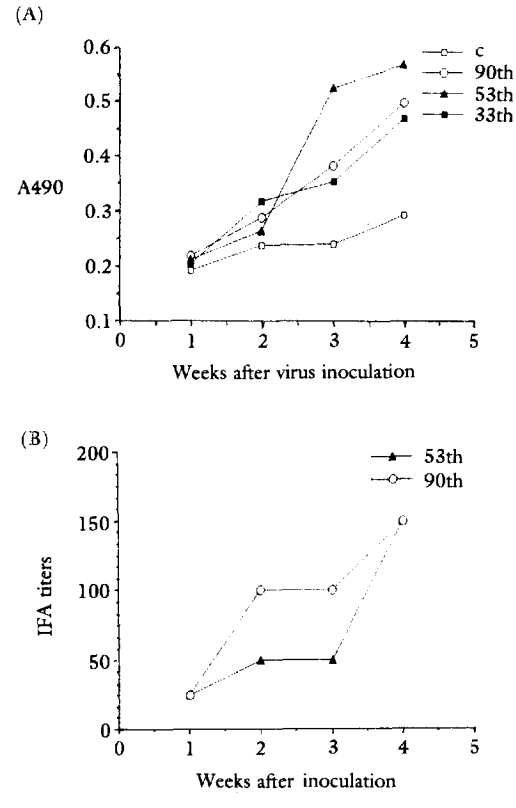

Fig 4. Immune responses of PEDV inoculated pigs by ELISA(A) and IFA test(B). Day of inoculation is expressed as 1, 1 week postinoculation (pi) as 2, 2 weeks pi as 3 and 3 weeks pi as 4, respectively.

Based on ELISA results from animal experiment, two fold difference at A_{490} (before and 3 weeks post inoculation) was considered as positive reaction against PEDV antibody. Porcine sera collected throughout the country showed the overall 45% positive rate. However, variances in positive rate were also observed with ranging from 17.6 to 79% (Table 3).

Discussion

In previous study, we reported the derivation of

Table 3. Antibody detection against PEDV by ELISA

Province	Kyunggi	Kangwon	Chungnam	Chunbuk	chunnam	Kyungnam	Jeju	total
Positive (P/N>2)*	28	26	15	25	17	21	79	211
Negative (P/N<2)	72	14	41	39	33	38	21	258
Positive(%)	28	65	17.6	39.1	34	35.6	79	45

*: $OD(A_{490})$ ratio to PEDV infected serum within one week postinoculation.

KPEDV-9 strain. Although the isolate replicated at low viral yields during low passages, the viral yields have steadily increased up to 10^{5-6} $TCID_{50}$/ml at the passage of 90th. Using cell adapted strain, we could identify the RNA of PEDV with molecular size of >20 Kb from the infected cell.[9]

The morphological examination also showed the overall morphological characteristics, which fit for coronavirus. However, variation in size and pleomorphic characteristic of virus was worthy to note. Like other enveloped virus, the way of purification might affect the result of electron microscopy.[10,11]

In this study, the etiological survey on swine with acute diarrhea indicated that about 17.5% of examined samples were turned out to be PEDV infection. Moreover, the result also confirmed the situations that the virus induce not only fatal cases in piglet, but also the severe diarrhea in growing or fattening pigs more than 2 weeks old. Furthermore study on antibody detection by ELISA showed about 45% positive responses on PEDV, suggesting that PEDV infections in some areas are endemic. Although it may not be exactly same with TGEV infection in the mode of transmission, but it is presumably similar to TGEV, especially in the aspect that both virus can be transmitted through fecal-oral route and could infest the swine without any severe symptoms.[12] In fact, recent study also indicated that PEDV infection could be continuous and cyclying partly due to the secretion of virus from infected herd wihtin the farm without typical signs.[13] Therefore, the high positive results from sera of certain area may not be surprising. However, the fact that PEDV can cause the enteric pathogenicity in all ages of swine indicates that the economic losses may be more than expected in this country.

Summary

Korean isolate, porcine epidemic diarrhea virus (KPEDV-9) was adapted through serial passages in vero cell. The viral yield reached up to 10^{5-6} $TCID_{50}$/ml at the passage level of 90th. The cell adapted virus was characterized through genetic and morphological examinations.

The RNA extracted from virus infected cell revealed the presence of RNA band with molecular size of >20Kb. The electron microscopic examination on purified virus showed the pleomorphic appearance of enveloped particles with 5-10nm surface projections, which fit with the shape of coronavirus.

The etiological survey on swine diarrhea by immunofluorescence test(FA) indicated 17.5% positive rate on the PEDV infection. In addition, the incidence were detected both in piglets within two weeks old as well as fattening pigs.

Serological survey by ELISA revealed the overall 45% positive result, thus, indicating the PEDV infection are widespread throughout this country.

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