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Isolation of porcine epidemic diarrhea virus(PEDV) in Korea

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돼지 유행성 설사 바이러스(porcine epidemic diarrhea virus)의 국내 분리주 작성에 관한 연구

권창희·권병준·정태성·기영진·허동호·황의경
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초록 : 설사로 폐사한 자돈의 장 절편을 이용하여 돼지 유행성 설사바이러스(PEDV), 돼지 전염성 위장염 바이러스(TGEV), 돼지 로타 바이러스(PRV)에 대한 병인학적 검사를 형광항체반응을 통하여 조사하였던 바 돼지 유행성 설사 바이러스의 감염에 의한 자돈의 폐사를 확인하였다.

간접형광항체검사시 돼지 유행성 설사 바이러스에 대한 양성반응을 보인 가검재료를 이용 Vero세포에 연속 계대한 후 plaque assay를 통하여 크로닝된 돼지 유행성 설사 바이러스 KPEDV-9주를 작성하였다.

돼지 유행성 설사 바이러스에 대한 면역혈청과 바이러스가 분리된 농장에서 채취된 돼지 혈청을 이용 돼지 유행성 설사 바이러스에 대한 구조단백성분을 분석하였던 바 88K(M.W.), 74K, 70K, 58~54K, 54~46K, 44~40K 및 33~32K에 상당하는 단백성분을 검출할 수 있었다.

Key words : porcine epidemic diarrhea virus, derivation of KPED-9 strain, immunoblotting analysis.

Introduction

Porcine epidemic diarrhea virus(PEDV) is known to cause similar clinical symptoms of transmissible gastroenteritis virus(TGEV) infection.[1] It is also reported that experimental inoculations of PEDV can cause enteropathogenic character both in piglets and fattening swine.[2] Although biophysical studies indicate that PEDV showed general characteristics of Coronaviridae family,[3] the exact epidemiological mechanism of infection and its pathogenicity are not clear partly because of poor replication in cell culture system.

Recently, Vero(African green monkey kidney) cell has been reported to support the growth of PEDV in the presence of trypsin and several immunological methods are available to detect the presence of PEDV.[4~7] The present study is carried out to detect and isolate PEDV from piglet died from acute diarrhea.

Meterial and Methods

Virus and cell : Vero cell from American type culture collection(ATCC) was regularly maintained in EMEM with 5% fetal bovine sera and used for propagation and isolation of PEDV in this study. Standard PEDV strain

was kindly provided by Dr. Hofmann[6] and used for the preparation of rabbit anti-PEDV hyperimmune sera. For purification of virus, the virus-inoculated cells and supernatant were treated with polyethylene glycol 8000 (M.W.) and purified according to the procedures of Chu et al.[8]

Production of diagnostic reagent on PEDV : Rabbit sera against PEDV were collected after four immunization with partially purified PEDV as described.[9] The serum was then conjugated with NHS-LC-Biotin (Pierce, U.S.A.) according to the procedures of manufacturer. Direct FITC conjugates on Porcine rotavirus (PRV) and TGE were provided from Central Veterinary Service Station(CVSL, Ames, Iowa, U.S.A.).

Isolation of virus : The cryostat-microtome sectioned intestines of piglet died from acute diarrhea were examined by fluorescence antibody(FA) test for the detection of PEDV, porcine rotavirus and TGEV. The intestine, which showed the positive immunofluorescence against PEDV, was further chosen for the isolation of virus. The inoculated cells were freezed-thawed three times and the supernatant was passaged in roller culture tube with supplement of trypsin as described before.[4,6] At the same time, the sample was separately inoculated into Vero cell on coverslip and examined for the detection of PEDV viral antigen by IFA. The inoculum, which showed the cytopathic effect (CPE) and positive reactions on PEDV by IFA, was further selected for plaque assay. For plaque assay, the virus inoculated cells were overlayed with 1% Seaplaque agarose (FMC, U.S.A.) containing 1 μg of trypsin and stained with 0.01% neutral red as described before.[10]

SDS PAGE and Immunoblotting : Partially purified PEDV was subjected to SDS-PAGE in 10% slab gel according to the method of Laemmli.[11] For immunoblotting the SDS-PAGE separated proteins were elctrophoretically transferred to nitrocellulose paper as described by Towbin et al.[12] After transfer, the nitrocellulose paper was reacted with biotinylated rabbit anti-PEDV immunesera or porcine sera and the subsequent immunodetection procedures were described previously.[13]

Result

Detection of PEDV infection from piglet died from diarrhea : In order to know the case of PEDV infection, total 37 cases of piglets died from acute diarrhea were examined for the presence of viral antigens by immunofluorescence test. Two other viral agents, TGEV and PRV, were also tested at the same time. Although rotavirus infections were most frequently observed followed by the infections with TGEV, the positive reactions against PEDV were also detected in four cases, thus, indicating that PEDV infections have already had in this country(Table 1, and Fig 1).

Isolation of PEDV : The intestine, which showed positive immunofluorecence with PEDV conjugate, was further used as the source for virus isolation.

The supernatant from grinded intestine was inoculated into Vero cells. After three blind passages, the supernatant was subjected to multiple dilution followed by plaque assay. The plaque-isolated virus was examined for reactivity with various FITC conjugates against Hog Cholera virus(HCV), Aujeszky virus(ADV), TGEV, Porcine rotavirus, Japanese encephalomyelitis virus and PEDV. Since positive immunofluorescence was observed only against PEDV conjugate as shown in Fig 2, the viral strain was designated as KPEDV-9.

Identification of PEDV polypeptides by immunoblotting : For the identification of PED viral polypeptides, partially purified PEDV was subjected to SDS-PAGE and analysed by immunoblotting. The result showed that at least seven polypeptide bands were detected either standard PEDV or KPEDV-9. The estimated molecular weight(M.W.)s were 88K, 74K and 70K in both strains and slight differences in low molecular weight polypeptides were observed in 58~54K, 54~46K, 44~40K and 33~32K. Further reactions with the porcine

Table 1. Detection of viral antigens by FA test from intestines of piglet died from acute diarrhea

Number of intestines diagnosed	Positive case*				ND**
	PEDV	PRV	TGEV	PRV & TGEV	
37	4	16	4	8	5

* : FA test using cryostat-microtome section of intestine.

** : Negative result against PEDV, PRV and TGEV.

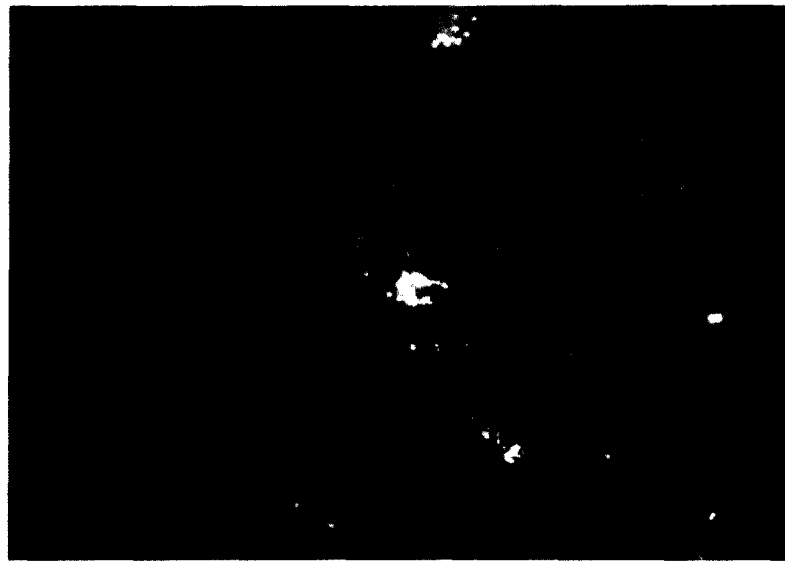

Fig 1. Immunofluorescence of cryostat-microtome section of intestine from piglet died from acute diarrhea, reacted with biotinylated anti-PEDV conjugate x 150.

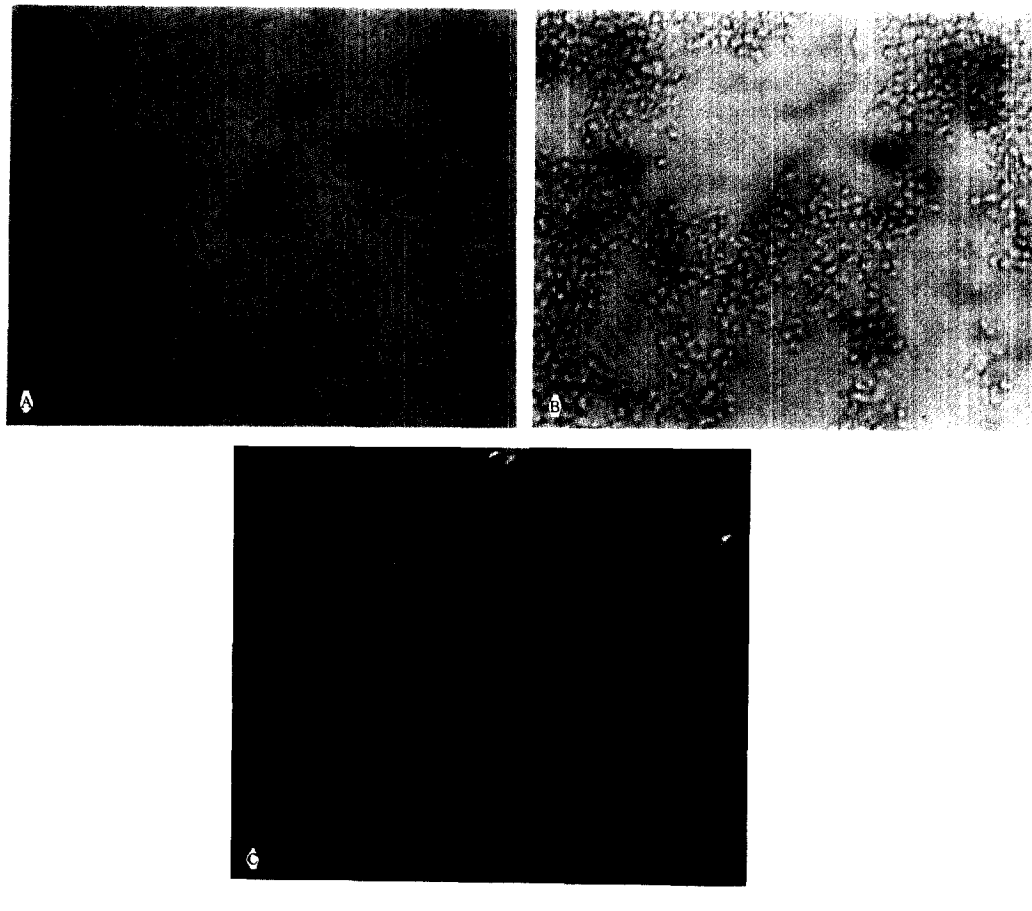

Fig 2. Cytopathic effect(CPE) and fluorescence of KPEDV-9 in Vero cell(a) : Uninfected Vero. × 100, (b) : infected Vero. × 100, and(c) : immunofluorescence of infected cell. × 320.

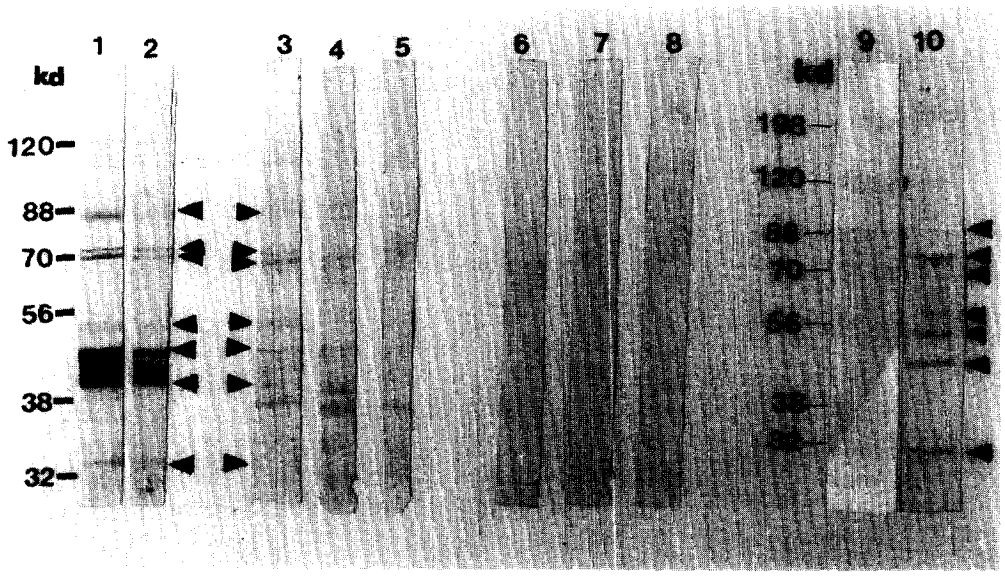

Fig 3. Immunoblotting analysis of purified PEDV using rabbit anti PEDV conjugate and porcine sera. Lane 1, 2 : Standard PEDV reacted with 500 x(1) and 1000 x(2) diluted biotinylated PEDV conjugate, respectively. Lane 3~8 : Standard PEDV reacted with 50 X diluted porcine sera. Lane 9 : Standard molecular marker. Lane 10 : KPEDV-9 reacted with biotinylated PEDV conjugate. The consensus of polypeptide bands, which detected with both biotinylated rabbit anti-PEDV conjugate and porcine sera, were indicated by triangle.

sera collected from farm, where the virus was isolated, showed polypeptide bands of same molecular weight. However, 120K polypeptide band was not reacted with the porcine sera, but extra polypeptide bands with difference in molecular weight were also detected(Fig 3, lane 1~10).

Discussion

Although TGEV like-outbreaks of diarrhea were observed in the farms having history of vaccination aganist TGEV, the exact reason was being suspected to be due to the presence of different viral agent like PEDV.

In this study, it was confirmed that the PEDV infection induce fatal case in piglet with acute diarrhea. The clinical signs of acute diarrhea are similar to TGEV infection and the diagnosis on PEDV infection was rather limited partly because of difficulty in isolation of this viral agent. In this respect, the immunofluorescence method can be a rapid diagnostic tool for differentiation. We also report the derivation of KPEDV-9 strain. Previous report showed that this viral agent can be grown in limited cells with the supplement of trypsin.[6] In fact, it was also shown that the isolate replicated in Vero cells at low viral yields. Recent study indicated that Vero cell adapted strain replicated at the titer of $10^{5.5}$ TCID 50/mℓ at the passage around 20.[4] However, the isolated strain showed rather low viral yield around 10^{2} ~ 10^{3} TCID 50/mℓ at the passage of 23(data not presented here). The reason is not known right now, but seems to be based on the difference in way of analysis. Although the presence of trypsin was essential for replication of virus, the high concentration of trypsin often resulted in CPE like effect in normal Vero cells, making titration ambiguous. Since the propagation of PEDV was rather laborious, little information is available on the structural component. Previous studies on CV777 strain indicated the presence of several viral polypeptides as the structural component.[14,15] Although the molecular weight of detected proteins were not exactly identical with CV777, it was possible to identify several polypeptides profiles of PEDV with reasonable consensus. The 88K, 74K and 70K polypeptides may presumably be corresponding to the category of peplomer of CV777 strain. Among other low molecular polypeptides, 58~41K protein was suspected to be RNA-binding protein.[14] However, it is not clear right now which one of three polypeptide bands are

the same protein, because only minor difference in low molecular weight proteins was detected, therefore the possible degradation of one polypeptide as well as difference in the way of viral preparations including heterogenicity through cell propagation should be considered. In addition, 120K polypeptide band was detected in the reaction with rabbit immunesera, but not with porcine sera and 115K band was barely visible in the reaction with KPEDV strain. For these reasons, it is possible to expect that there may exist other extra polypeptide bands as the separate viral component. Nevertheless, we could find that three protein bands of 88K, 74K and 70K in molecular weight turned out to be identical in both cell adapted strains. Further studies with monospecific antibodies will be necessary to explain the differences and functions of those polypeptides detected in this virus.

Summary

The etiological survey on porcine epidemic diarrhea virus(PEDV) by immunofluorescence antibody test(IFA) showed the positive result from the intestines of piglet died from acute diarrhea.

The viral agent of PED was also isolated from intestine, which showed positive reaction by immunofluorescence test. After passage in Vero cell, the viral agent was further cloned by plaque purification and designated as KPEDV-9. The immunoblotting analysis using hyperimmune sera and porcine sera revealed the presence of several polypeptide bands with molecular weight(M.W.) of 88K, 74K, 70K, 58~54, 54~46K, 44~40K and 33~32K, respectively.

Acknowledgement : The presence of PEDV in intestine was also confirmed through immunostaining using monoclonal antibody against glycopolypeptide of PEDV by Dr. Ackermann et al, Institute for virology, University of Zurich, Switzerland. We would like to express the deep thank for confirming the PEDV infection in Korea. The authors also appreciate Jong-myoung Lee' technical assitance for this project.

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