Development of monoclonal antibody-Based Immunofluorescence assay for PEDV Detection

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ABSTRACT

The variant porcine epidemic diarrhea virus (PEDV) is the etiological agent for the re-emergency of porcine epidemic diarrhea (PED) worldwide since 2010. To establish a rapid and specific detection method of variant PEDV, three monoclonal antibodies (McAbs) against truncated recombinant spike (S) protein of PEDV that are expressed in Escherichia coli were generated, characterized and evaluated. The titers of the three McAbs were all above 1:10^6 and immunoglobulin subtypes were IgG 2a with κ light chain, respectively. Three McAbs showed good reactivity with S1 protein expressed in baculovirus expression system and recognize the native PEDV in the infected cells, while no cross reaction was observed with other general enteric viruses, such as transmissible gastroenteritis virus (TGEV) and porcine rotavirus (RV). The McAb-based IFA was established to rapidly detect PEDV in intestinal tissues. The PEDV can be specifically recognized in intestinal tissues of PEDV-infected piglets. A total of 102 intestinal tissues samples were separately detected and compared by our in-house IFA and RT-PCR methods. The positive detection rates of IFA and RT-PCR were 97.1 and 92.2%, respectively. The high level of agreement between IFA and RT-PCR indicated its potential application for clinically rapid detection of PEDV.

Key words: Variant porcine epidemic diarrhea virus(PEDV), McAbs, in-house IFA and RT-PCR methods.

INTRODUCTION

Porcine epidemic diarrhea (PED) is a contagious swine enteric disease, characterized by watery diarrhea, vomiting, dehydration and high mortality in sucking piglets, resulting in devastating economic losses to swine industry (Pensaert and de Bouck, 1978; Song and Park, 2012). The disease was first reported in Europe in 1970s and thereafter spread to Asia. Severe outbreak of PED was reported in China since late 2010, which was caused by variant PEDV with higher virulence (Li et al., 2012; Wang et al., 2013). The new PED outbreaks were then successively reported in the United States, South Korea, Canada, Vietnam, Germany, Portugal, Ukraine, Austria, etc., causing significant economic losses (Wang et al., 2014; Lee and Lee, 2014; Pasick et al., 2014; Kim et al., 2015; Stadler et al., 2015; Mesquita et al., 2015; Dastjerdi et al., 2015; Steinrigl et al., 2015). The etiological agent, porcine epidemic diarrhea virus (PEDV), is an enveloped, single-stranded positive-sense RNA virus that belongs to Coronaviridae family (Kocherhans et al., 2001). PEDV genome encodes four structural proteins, including the spike (S), envelope (E), membrane (M), nucleoprotein (N), and at least one accessory protein (ORF3) (Chen et al., 2012; Li et al., 2013). The S protein is located at the outer surface of the virion, containing 1386 amino acid, and can be divided into the N-terminal S1 and the C-terminal S2. The S protein plays an important role in receptor attachment and virus-cell membrane fusion (Gallagher and Buchmeier, 2001).

Due to the economic importance of PED, a variety of diagnostic methods have been established for PEDV detection, such as electron microscopy (EM), immunofluorescence (IF) tests, immunohistochemical
(IHC) techniques, and enzyme-linked immunosorbent assay (ELISA) (Song and Park, 2012), reverse transcription polymerase chain reaction (RT-PCR) and loop-mediated isothermal amplification (LAMP) method. Rapid antigen detection is urgently needed for timely diagnosis of PEDV. However, the antigen detection methods that have been developed for detection of variant PEDV are using polyclonal antibodies as detection antibody and are time-cost.

In this study, the McAbs were prepared, characterized and further used for development of a rapid and visible IFA method for detecting PEDV in intestinal tissues. The method was compared with our in-house RT-PCR for detection PEDV in clinical samples. The results show that IFA can be a promising method.

MATERIALS AND METHODS

Ethics Statement

The methods and animal experiments used in this study were carried out in strict accordance with the approved guidelines by the Ethics Committee of Department of Veterinary Medicine of Huazhong Agricultural University, Wuhan City, Hubei Province, P. R. China.

Cells, virus, and reagents

African green monkey kidney cell line (Vero-E6) and SP2/0 myeloma cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1640 medium supplemented with 20% FBS (Gibco), respectively at 37°C under 5% CO₂. PEDV strain CH/YNKM-8/2013 (Accession no. KF761675) was propagated in Vero-E6 cells with serum-free DMEM containing 8 μg/mL trypsin (Invitrogen). Freund’s Complete Adjuvant (FCA), and Freund’s Incomplete Adjuvant (FICA) were purchased from Sigma. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was purchased from ABclonal Inc. Alexa Fluor 488 Donkey Anti Mouse IgG was purchased from AntGene Inc.

RT-PCR detection

The triplex enteric pathogens (PEDV, TGEV and RV) of clinical samples were detected by previously reported method (Zhang and He, 2010).

Generation of McAbs against S protein

The E.coli BL21 (DE3) containing recombinant plasmid pET32a-S1D (636-789aa of PEDV S protein) was conserved in our laboratory. Protein expression and purification were performed as previously described with little modification (Liu et al., 2009).

The McAbs were produced according to the established techniques (Sun et al., 2013). Briefly, 200 μg of rS1D protein mixed with complete Freund’s adjuvant was used to immunize each BALB/C mice and two booster vaccinations (100 μg of rS1D protein) were performed at interval of two weeks. The spleen of the immunized mice with highest titer of serum antibody was gently homologized to yield single cells which were further used to fuse with the myeloma SP2/0 cells. The presence of antibody in the supernatant of the hybridoma cells was checked and further limited dilution was carried out to purify the hybridoma cells.

To screen the hybridomas antibody generation, rS1D protein-based indirect ELISA and PEDV-infected Vero cell-based IFA were performed using the supernatant of the fusion cells. McAbs were isotyped using the Pierce® Rapid ELISA Mouse mAb Isotyping Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer’s instructions. The mice were handled and maintained under strict ethical conditions for animal welfare.

Purification of the McAbs

The ascites fluid was harvested from the mice that were intraperitoneally injected with antibody-producing hybridoma cells. Then the McAb was purified using caprylic saturated ammonium sulfate and affinity chromatography on a protein G column (Smart-lifesciences) according to the manufacturer’s instructions.

Western blot analysis

The PEDV-S1 protein produced through baculovirus expression system was separated by 12% SDS-PAGE. Thereafter, the proteins were electrotransferred to 0.45 μm PVDF membranes (Millipore). Membranes were blocked with 5% (w/v) skim milk-TBST at room temperature for 2 h and then incubated overnight at 4°C with the purified McAb. The membranes were washed with TBST and then incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (ABclonal, Inc.) at 37°C for 1 h. The signals were obtained using the clarity-enhanced chemiluminescence (ECL) reagent (Bio-Rad, Hercules, CA).

Preparation of intestine freezing ultrathin section

The fresh intestinal sample collected from PEDV-infected pigs with clinical signs of severe diarrhea was covered with
Table 1: Comparison of PEDV detection between RT-PCR and IFA. The pigs were regarded as positive for PEDV if the RT-PCR and/or IFA were positive.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive in RT-PCR and IFA</td>
<td>92</td>
</tr>
<tr>
<td>Positive in RT-PCR only</td>
<td>2</td>
</tr>
<tr>
<td>Positive in IFA only</td>
<td>7</td>
</tr>
<tr>
<td>Negative in RT-PCR and IFA</td>
<td>1</td>
</tr>
<tr>
<td>Prevalence</td>
<td>97.1%</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>92.9%</td>
</tr>
<tr>
<td>Specificity</td>
<td>33.3%</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>97.9%</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>12.5%</td>
</tr>
<tr>
<td>Kappa value</td>
<td>0.15</td>
</tr>
<tr>
<td>Level of agreement</td>
<td>Slight</td>
</tr>
<tr>
<td>Odds ratio</td>
<td>6.6</td>
</tr>
</tbody>
</table>

O.C.T. embedding compound (Sakura Finetek USA, Inc.) and then was handled into freezing ultrathin section with 6 mm using freezing microtome (Leica CM 1950, www.leica-microsystems.com) according to its instructions.

Immunohistochemical (IHC) analysis

The immunohistochemical analysis of piglet intestine was performed as previous described with little modification (Li et al., 2016). The dilution of the anti-PEDV monoclonal antibody was 1:500. The presence of virus-antibody combination was visualized using a Mouse SABC-AP kit (Boster, Wuhan, Hubei, China) according to the manufacturer’s instructions (Luo et al., 2005).

Hematoxylin-eosin (HE) staining

Freezing sections were fixed with methanol for 10 min. After rinsing for 3 min with running water, the sections were then stained with hematoxylin for 5 min, rinsed for 5 min, differentiated by hydrochloric acid for 30 s, back to blue by ammonium hydrxide for 10 s, rinsed with 80% ethanol for 3 min, stained with eosin for 50 s, rinsed with 95% ethanol for 2 min, dehydrated with ethanol (I) (80%) (II) (95%) (III) (100%) for 5 min, made transparent with dimethylbenzene (I) (II) for 5min, respectively, and finally sealed with neutral balsam.

Immunofluorescence assay

Freezing sections taking from -80°C were handled as described in HE staining above. After fixing and blocking, the tissue section was incubated with McAb (1:1000 dilution in PBS) for 1h at 37°C, and section were then washed three times with PBS before further incubation with FITC-labeled Goat Anti-Mouse IgG(H+L) (1:500 dilution in PBS) in the dark. After washing five times with PBS, the images were captured under a fluorescence microscopy.

Field samples and statistical analysis

One hundred and three intestinal samples collected from swine farms with acute diarrhea were detected by RT-PCR and the established IFA method in this study, respectively (Table 1). The results of our established IFA method with the RT-PCR method were compared and used to calculate positive predictive value, negative predictive value, relative risk, kappa value, odds ratio and 95% confidence intervals using a 2×2 contingency table (Altman and Bland, 1994). The level of agreement between the two testing method was compared using Kappa statistics (k) (Landis and Koch, 1977).

RESULTS

Generation of recombinant PEDV-S1D protein

As shown in Figure 1a, the recombinant PEDV-S1D protein was successfully expressed with a molecular mass of about 34 kDa. For further characterization and identification of PEDV-S1D protein, PED convalescent pig serum was used for Western blot analysis. The results indicated that the purified PEDV-S1D protein could be recognized by the PEDV antibody positive serum (Figure 1b).

Generation and characterization of McAbs

Indirect ELISA method was used to identify antibody-producing positive hybridoma clone. The anti-PEDV McAb hybridoma cells 1E1, 4B2, and 3E12 were identified and further purified (Figure 2A). All immunoglobulin classes of
Figure 1: The identification and characterization of purified PEDV-S1D protein. A: SDS-PAGE analyses of the purified S1D protein. Molecular weight ladder (M), PEDV-S1D protein (S1D). B: Identification of the reactivity of purified S1D protein by western blot assay. Molecular weight ladder (M), 1 and 2: negative control sera, 3: PED convalescent pig serum.

these three McAbs belong to the IgG 2a family and kappa chain. Good reactivity of McAbs, produced from all three hybridoma clones with both PEDV recombinant S1 protein (Fig.2B) and PEDV itself (Figure 2C), was observed in the IFA. The McAb 1E1 have the best reactivity with PEDV in the intestinal tissue collected from animals in the PEDV infection experiment by immunohistochemistry (Figure 2D).

**Specificity of the McAb 1E1**

All three McAbs have good reactivity with PEDV (Figure 3). No cross reaction was observed between McAb 1E1 and common enteric viruses, such as rotavirus (RV) and transmissible gastroenteritis virus (TGEV).

**Development and application of McAbs based IFA for PEDV detection in intestinal tissues**

Considering the best reactivity of McAb 1E1 with PEDV infected cells and intestine tissue by IHC, it was chosen for further development of IFA to detection of PEDV in the intestinal tissue. The HE staining presented a visual picture upon the status of injury in cells and tissues. The damage of PEDV-infected intestinal villus epithelial cells was mainly observed in cellular vacuolization (Figure 4). As shown in Figure 4, PEDV was determined in the cytoplasm of infected intestinal villus epithelial cells by IFA. There was no positive signal in PEDV free control samples. Co-localization analysis between the HE staining and immunofluorescence assay provided a comprehensive diagnosis for PEDV in intestinal tissue.

**Primary detection of PEDV in clinical samples**

A total of 102 fresh intestinal tissues samples were independently detected by our in-house IFA and RT-PCR methods. The positive detection rates of IFA and RT-PCR were 97.1 and 92.2%, respectively. The statistics of clinical detection results obtained from RT-PCR and IFA are shown in Table 1. The Kappa value was about 0.15, indicating that the level of agreement of RT-PCR and IFA is slight and the detection rate of established IFA is higher than RT-PCR.

**DISCUSSION**

The variant PEDV-associated diarrhea poses a serious and continuous threat to the global swine industry. Thus, a more sensitive and quick detection method is essential for PED surveillance. Many diagnostic methods including both antigen and antibody detection have been developed for classical PEDV infection. For novel PEDVs, it is characterized by variations including insertions, deletions and mutations in its genomes, which is partly the reason for the immunization failure from the classical CV777-based vaccines. Thus, a new diagnostic method aiming to
detect the new PEDV is necessary for this disease control. A variety of physical observation, antigen, antibody, and genetic diagnostic methods have been established for PEDV detection, such as electron microscopy (EM) observation, enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay (IFA), immunohistochemical (IHC) techniques, fluorescent microsphere immunoassay (FMIA), fluorescent focus neutralization assay (FFN), reverse transcription polymerase chain reaction (RT-PCR), real time RT-PCR and loop-mediated isothermal amplification (LAMP) methods (Song and Park, 2012).

The spike protein is consisted of S1 and S2 domains, among which S1 is responsible for viral binding with the receptors in the cell surface and S2 for membrane fusion leading to the entry of PEDV into the host cells. Four antigen epitopes, S1D (636-789aa), COE (499-638aa), SS2 (748-755aa) and SS6 (764-771aa) have been established on PEDV spike protein. SD1 is the most conservative epitope. Thus, the monoclonal antibody against SD1 domain may recognize all the spike sequence with the highest possibility. Therefore, the negative detection of any PEDV including classical and variant strain will rarely occur. The recombinant SD1 protein was used as immunogen. The yielded monoclonal antibodies can recognize recombinant S1 protein produced by
baculovirus expressing system with Western blot and S protein in PEDV virion in the intestinal samples with IHC, indicating the diagnostic potential of the McAb. From three McAbs, 1E1 was selected for IFA development owing to its strongest signals in both IFA and IHC formats.

Since intestine is the most susceptible target organ of PEDV, intestines of piglets was chosen as the samples for IFA development. In this study, the McAb-based IFA using frozen section was established to rapidly detect PEDV in the intestines, while the pathological examination exhibited by HE staining was used as supporting evidence to confirm the infection and resultant lesion caused by PEDV. As the cell morphology may be damaged during traditional section preparation leading to the false detection or weak signals, the preparation of frozen section was used instead of the traditional method.

The positive detection rates of IFA and RT-PCR were 97.1 and 92.2%, respectively. The agreement level of RT-PCR and IFA detection method was very high in this study. Combing frozen section technique, our established method

**Figure 3:** The reactivity of three McAbs with PEDV and the McAb 1E1 with RV and TGEV.

**Figure 4:** HE staining and IFA analysis of PEDV presence in the piglet's intestinal sample.
is time saving and could help to specifically recognize PEDV in the intestines. According to the Kappa value, the level of agreement of RT-PCR and IFA was low and the detection rate of established IFA was higher than RT-PCR. PED is a worldwide economically important porcine enteric disease. Rapid and convenient diagnosis of PEDV is essential for effective PED prevention and control. In the present study, we reported the IFA, together with frozen section technique for PEDV infection detection in the intestinal tissue. This study could provide another time-saving and specific diagnostic tool for PEDV diagnosis and surveillance.

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REFERENCE


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