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Journal of Virological Methods 225 (2015) 4–8

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

A novel diagnostic approach to detecting porcine epidemic diarrhea virus: The lateral immunochromatography assay

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Article history:
Received 28 March 2015
Received in revised form 31 August 2015
Accepted 31 August 2015
Available online 2 September 2015

Keywords:
Porcine epidemic diarrhea virus
Immunochromatography
Lateral flow test
Sensitivity
Specificity

A B S T R A C T

Porcine epidemic diarrhea virus (PEDV) causes acute diarrhea and dehydration in sucking piglets and has a high mortality rate. An immunochromatography (IC) assay, known as a lateral flow test, is a simple device intended to detect the presence of target pathogens. Here, we developed an IC assay that detected PEDV antigens with 96.0% (218/227) sensitivity and 98.5% (262/266) specificity when compared with real-time reverse transcriptase (RT)-PCR using FAM-labeled probes based on sequences from nucleocapsid genes. The detection limits of the real-time RT-PCR and IC assays were 1×10^2 and 1×10^3 copies, respectively. The IC assay developed herein did not detect non-specific reactions with other viral or bacterial pathogens, and the assay could be stored at 4 °C or room temperature for 15 months without affecting its efficacy. Thus, the IC assay may result in improved PED detection and control on farms, and is a viable alternative to current diagnostic tools for PEDV.

1. Introduction

Porcine epidemic diarrhea (PED), which is caused by porcine epidemic diarrhea virus (PEDV), is an acute, highly contagious, and devastating enteric disease characterized by vomiting, watery diarrhea, and dehydration, resulting in high mortality rates in suckling pigs (Pijpers et al., 1993). PEDV is an enveloped RNA virus belonging to the order *Nidovirales*, genus *Alphacoronavirus*, within the *Coronaviridae* family (Bridgen et al., 1998; Brian and Baric, 2005; Sergeev, 2009). The viral genome comprises a single-stranded positive-sense RNA of approximately 28 kb, which encodes six genes: replicase (Rep), spike (S), ORF3, envelope (E), membrane (M), and nucleoprotein (N) (Duarte and Laude, 1994; Utiger et al., 1995; Chen et al., 2008). PEDV was first reported in growing pigs in the United Kingdom in 1971 (Pensaert and de Bouck, 1978) and subsequently in a number of European countries (Nagy et al., 1996; Pritchard et al., 1999; Martelli et al., 2008); more recently, the virus has appeared in China, Japan, Thailand, Vietnam, and South Korea (Sueyoshi et al., 1995; Song et al., 2006; Puranaveja et al., 2009; Chen et al., 2010).

PED is clinically indistinguishable from other porcine gastroenteric diseases, including those caused by transmissible gastroenteritis (TGE), rotavirus, or bacteria. Confirmatory laboratory tests are therefore necessary to definitively identify the causative pathogen. Several methods of detecting PEDV have been published: reverse transcriptase polymerase chain reaction (RT-PCR), antigen enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry (Callebaut et al., 1982; van Nieuwstadt et al., 1988; Sueyoshi et al., 1995; Paton et al., 1997; Kim et al., 2000, 2007; Shibata et al., 2000; Rodák et al., 2005). Although these techniques have been used widely to diagnose PEDV infection, they are laborious, time-consuming, and require laboratory expertise and/or special equipment, making them unsuitable for field use. These methods are also unsuitable for the management of emergent PED outbreaks, thereby restricting their application to veterinary clinical diagnosis.

Immunochromatography (IC) assays were first described in the late 1960s and were originally developed to test for serum proteins (Kohn, 1968). Over the past decade, many IC assays have been developed to detect infectious diseases (Allwinn et al., 1999;

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http://dx.doi.org/10.1016/j.jviromet.2015.08.024
0166-0934/

Aidoo et al., 2001; Berdal et al., 2000; Grunow et al., 2000; Buser et al., 2001) and for use in veterinary medicine (Klingenberg and Esfandiari, 1996; Laitinen and Vuento, 1996; Oh et al., 2006; Meng et al., 2014). Lateral flow IC assays are generally not quantitative and provide only a yes/no answer; they also lack multiplexing (Zhou et al., 2012). Despite these disadvantages, the IC assay is straightforward to use because it simply requires that the user dilute the test agent in a sample buffer prior to detection. Thus, IC assays yield rapid and accurate results without the need for specialized equipment or trained personnel. The assays are also very stable and robust (they have a long shelf life and do not usually require refrigeration) and are relatively inexpensive to produce. These features make them ideal for both field and laboratory testing of samples.

Here, we describe a novel diagnostic approach to detecting PEDV antigens in diarrhea samples from piglets naturally infected with PEDV. We also evaluated the assay as a potential alternative to current diagnostic methods.

2. Materials and methods

2.1. Reference panel and field samples

Reference panels comprised PED virus strains (K14JB01, SM98, and DR13) and non-PED samples containing transmissible gastroenteritis (TGE), porcine coronavirus (PRCV), or bacteria (Supplemental Fig. 1). K14JB01 is a novel variant PEDV strain isolated from an infected pig in South Korea in 2013 (Cho et al., 2014). All of the reference panels were obtained from the Korea Veterinary Culture Collection (KVCC) (http://kvcc.kahis.go.kr) and were used to test the specificity of the developed IC assay. A total of 493 fecal samples (324 diarrhea and 169 normal feces) were collected from piglets in Chungnam, Kangwon, Gyeongbuk, and Gyeongnam provinces in South Korea. Of the 493 field samples, 77.3% (381/493) came from piglets less than 3 weeks old and 22.7% (112/493) came from piglets more than 3 weeks old.

2.2. Real-time RT-PCR

Total RNA was extracted from fecal samples using the Qiagen viral RNA kit (Hilden, Germany), according to the manufacturer's instructions. The following primers were used for real-time RT-PCR: PED-F, CGCAAAGACTGAACCCA CTAATTT; PED-R, TTGCCTCTGTTGTTACTTGGAGAT; and PED-FAM, FAM-TGTTGCCATTGCCACGACTCCTGC-BHQ (Kim et al., 2007). The real-time RT-PCR mixture was prepared using the Taq^{TM} Universal Probes One-step kit (Bio-Rad Co., Cat no. 172-5141, USA) and comprised 5 μL of extracted RNA, 1 μL of each primer (F, R) and probe, 10 μL of 2× buffer, 0.5 μL of 25× enzyme, and 1.5 μL of nuclease-free water in a total reaction volume of 20 μL. The PCR conditions were as follows: reverse transcription for 30 min at 48 °C, followed by denaturation for 5 min at 95 °C, and 45 cycles at 95 °C for 10 s and 60 °C for 1 min. PCR was performed in a LightCycler® 96 real-time PCR machine (Roche Diagnostics, Basel, Switzerland).

2.3. IC antigen kit for detecting PEDV

The IC kit for diagnosis of PEDV comprised an IC device, dilution buffers, and droppers. Dilution buffers comprised 50 mM borax buffer, Tween 20, sodium azide, and 1.2 N NaCl. The IC device comprised a sample pad, a fecal separation pad, a gold-conjugate pad, a nitrocellulose membrane, and an absorbance pad. The control line of the IC strip was coated with 2.6 μg of a goat anti-mouse antibody, and the test line was coated with 2.1 μg of an anti-PEDV monoclonal antibody (5F15 mAb). The gold-conjugate pad was coated with another PEDV monoclonal antibody (8D28 mAb) conjugated to gold nanoparticles. The monoclonal antibodies (5F15 and 8D28) were identified to bind the nucleocapsid protein of PEDV. The monoclonal capture antibody was immobilized on the nitrocellulose membrane and dispensed using the dispenser (model no. Matrix 1600) provided by KINEMATIC AUTOMATION. The monoclonal antibodies were generated against the SM-98 strain of PEDV. The IC antigen kit (VDRG® PEDV Ag Rapid Kit, Catalog no. PS-PED-11) was purchased from Median Diagnostics Inc. (Chuncheon, South Korea). Briefly, porcine fecal samples (50–100 mg) were placed in sample collection tubes containing 1 mL of dilution buffer (50 mM Borax buffer (pH 9.0), 0.4% Tween-20, 1.2 M NaCl, and 0.1% sodium azide) and mixed vigorously. The tube was then left to stand for 3 min at room temperature to allow particles to settle. Finally, four drops of sample (100 μL) solution were loaded into the loading hole using a disposable dropper and the results were interpreted after 10 min. Three technicians read the results obtained by IC assay.

2.4. Storage stability test

The long-term stability of an IC assay is an important consideration if it is to be applicable in the field. The stability of antibodies (8D28 mAb and 5F15 mAb) immobilized on the IC strip was tested by storing the assay at 4 °C, 28 °C, or 45 °C for 0, 3, 6, 9, 12, and 15 months. Each experiment was repeated three times. After each time interval, the diagnostic accuracy was tested using a PEDV-positive reference sample (SM98 strain) at concentrations of 10^3 and 10^2 $TCID_{50}$/mL. PEDV-negative reference samples (confirmed by real-time RT-PCR) were also tested.

2.5. PEDV animal challenge test

Three-day-old PED-negative piglets were purchased from a PED-free farm and fecal swabs were taken prior to PEDV challenge. Each piglet was then challenged (orally) with the K14JB01 strain (7×10^6 copies). Fecal samples were obtained by anal swabbing at 1, 2, 3, 4 and 5 days post-challenge (dpc), and real-time RT-PCR and IC assays were performed simultaneously.

2.6. Calculation of diagnostic sensitivity and specificity

All 493 field fecal samples were tested simultaneously by real-time RT-PCR and in the IC assay. Samples were classified as "true positive" (TP), "true negative" (TN), "false positive" (FP), or "false negative" (FN) according to the real-time RT-PCR results. The diagnostic sensitivity and specificity of the IC assay were calculated as follows: sensitivity = TP/(TP + FN) × 100, and specificity = TN/(TN + FP) × 100. The results were expressed as percentages.

2.7. Calculation of Cohen's kappa value

Cohen's kappa was introduced as a measure of agreement. Cohen's kappa adjusts the observed proportional agreement to take account of the levels of agreement between real-time PCR and IC assay that would be expected to occur by chance.

The following calculation was used: Kappa = $(p - pe)/(1 - pe)$ (where p is the proportion of units showing agreement; pe is the proportion of units expected to agree by chance).

3. Results

3.1. PED antigen detection limit and non-specific reactions

The threshold of the real-time RT-PCR assay was set at a C_T value of 35 cycles according to the sigmoid profile obtained at SM98 concentrations from 10^6 to 10^1 copies (data not shown). The mean C_T

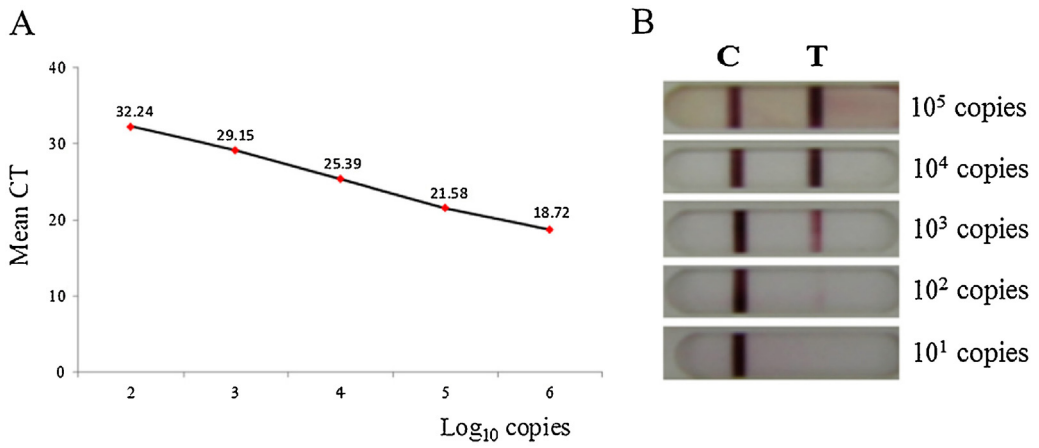

Fig. 1. Standard curve for the real-time RT-PCR assay, and the detection limit of the IC assay for the SM98 strain of PEDV. (A) The virus was serially diluted (10-fold) from 10^2 to 10^6 copies prior to assay by RT-PCR. The equation for the curve is as follows: $y = -3.461x + 35.799$; $r^2 = 0.9977$. (B) Detection limit of the IC assay. PEDV was serially diluted (10-fold) before application to the test strips. The control line (C) and test lines (T) were examined visually.

values at 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 copies were 18.72, 21.58, 25.39, 29.15, 32.24, and 0, respectively. The linear equation was as follows: $y = -3.461x + 35.799$; $r^2 = 0.9977$ (Fig. 1). Strong bands were observed in the IC assay at 10^6, 10^5, 10^4, and 10^3 copies. Although weak bands were observed at 10^2 copies (Fig. 1), we set the detection limit at 10^3 copies to improve accuracy in later experiments.

We also examined cross-reactivity with antigens derived from viral and bacterial pathogens. Thirty reference panels, which included three PEDV (K14JB01, SM98, and DR13) and 27 viral and bacterial pathogens (Supplemental Fig. 1), were evaluated. Only the three PEDV antigens generated bands on the IC test strips (Supplemental Fig. 1). These results indicate that the antibodies immobilized on the IC strip are specific for PEDV antigens.

3.2. Storage stability

As shown in Supplemental Table 1, all PEDV-positive samples generated strong bands on the test line, regardless of virus concentration (10^3 or 10^2 $TCID_{50}$/mL), storage temperature (4 °C, 28 °C, or 45 °C), and storage time (0, 3, 6, 9, 12, or 15 months). By contrast, PEDV-negative samples generated weak bands when the IC assay was stored at 45 °C for more than 6 months.

3.3. Comparison of real-time RT-PCR and IC assay results after PEDV challenge

All piglets challenged with 7×10^6 copies of K14JB01 developed diarrhea. The PEDV copy number increased significantly (up to 10^7 copies) in fecal samples taken at 1 dpc, and decreased gradually thereafter. The PEDV copy numbers in the fecal samples were as follows: $10^{7.06\pm1.24}$ at 1 dpc, $10^{6.69\pm1.33}$ at 2 dpc, $10^{5.68\pm1.13}$ at 3 dpc, $10^{5.06\pm1.37}$ at 4 dpc, and $10^{4.98\pm1.25}$ at 5 dpc. The piglets also suffered severe dehydration, resulting in mortality. The death rates were as follows: 0% (0/9) at 1 dpc, 22.2% (2/9) at 2 dpc, 33.3% (3/9) at 3 dpc, 44.4% (4/9) at 4 dpc, and 66.7% (6/9) at 5 dpc (Table 1). The IC assay showed that all piglets were PEDV-negative prior to challenge. All fecal samples obtained on Days 1, 2, 3, 4, and 5 post-challenge generated strong bands in the IC assay.

3.4. Sensitivity and specificity of the IC assay

Of the 493 field fecal samples tested (324 diarrhea and 169 normal feces), 46.0% (227/493) and 54.0% (266/493) were diagnosed as PED-positive and PED-negative, respectively, by real-time RT-PCR. Of the 324 diarrhea and 169 normal feces samples, 70.0% (227/324) and 0.0% (0/169), respectively, were diagnosed as PED-positive by real-time RT-PCR. All samples were re-tested in the IC assay to examine its sensitivity and specificity for PEDV. Of the 227 PEDV-positive samples (diarrhea) that tested positive by real-time RT-PCR, nine did not show a positive reaction in the IC assay (Fig. 2; triangles), whereas four (two diarrhea and two normal feces) of the 266 PEDV-negative samples (97 diarrhea and 169 normal feces) tested positive in the IC assay (data not shown). Thus, the sensitivity and specificity of IC assay were 96.0% (218/227) and 98.5% (262/266), respectively. Cohen's kappa value was calculated using the formula described in Section 2. The *p* and *pe* values were calculated as 0.9736 and 0.504, respectively. The value of Cohen's kappa coefficient was 0.9468. A value of Kappa above 0.81 means almost perfect agreement. The SE value was calculated to 0.015 using IBM Statistical Package for the Social Sciences (SPSS) statics 22. The 95% confidence intervals (Estimate ± 1.96SE) of the Cohen's kappa value were 0.9174 (lower limit) and 0.9762 (upper limit), respectively.

4. Discussion

The format of the lateral flow IC assay can vary depending on the target analyte. The two most common formats are the competitive assay and the double antibody sandwich assay. The competitive format is most suitable when the target analyte has low molecular weight or is a single specific antigen (Posthuma-Trumpie et al., 2008; Kim et al., 2011; Sathe et al., 2014), whereas the double antibody sandwich format is more suited to the detection of larger analytes such as bacterial pathogens and viruses, which have multiple antigenic sites (Xiang et al., 2012). Thus, the IC assay developed herein was based on the double antibody sandwich format.

The ideal label for lateral IC assays should have the following characteristics: simple conjugation chemistry without loss of biological integrity and activity; no (or low) non-specific binding under the selected salt, buffer, and detergent conditions; high stability at different pH values and temperatures; and be commercially available at low cost. Such labels comprise colored or fluorescent nanoparticles. Common labels are liposomes, colloidal carbon, or colloidal gold. Liposomes are vesicles formed by a lipid bilayer and have been used commercially in membrane-based lateral flow assays (Shukla et al., 2011, 2014). However, liposomes are relatively unstable and susceptible to lysis by surfactants. Colloidal carbon, in the form of India Ink, has been used in immunoassays since the 1970s (Geck, 1971; Waller, 1977; Chandler et al., 2000). Advantages include high stability and high color contrast on membranes;

Table 1
Comparison of real-time RT-PCR and IC assay results after PEDV challenge.

	Days post-challenge (dpc)					
	0	1	2	3	4	5
Real-time RT-PCR (copy number)	0	7.06 ± 1.24	6.69 ± 1.33	5.68 ± 1.13	5.06 ± 1.37	4.98 ± 1.25
IC assay (no. of positive samples)	0	9	7	6	5	3
No. of deaths/no. of survivors	0/9	0/9	2/7	1/6	1/5	2/3

Three-day-old piglets were challenged with 7×10^6 copies of the K14JB01 strain of PEDV via the oral route.

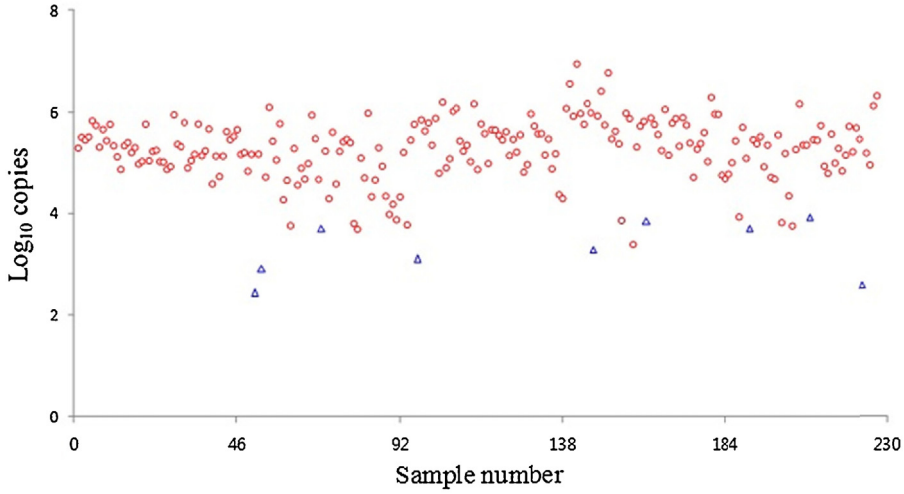

Fig. 2. Viral copy number in field samples by real-time PCR. Field samples that were PEDV-positive by real-time RT-PCR are represented by dots and triangles. Field samples that were PEDV-positive and PEDV-negative in the IC assay are represented by dots and triangles, respectively. (For interpretation of the references to color in the figure legend, the reader is referred to the web version of this article.)

however, conjugating protein to colloidal carbon takes longer time than to colloidal gold and, therefore, it is unsuitable for commercial immunoassays. Colloidal gold is the most widely used label in commercial lateral IC assays (Chandler et al., 2000). It is fairly easy and inexpensive to prepare, provides intense color, and can be visualized without the need for developing agents.

A positive test result is produced when a color change is observed in the test line of the IC strip. However, the appearance of a weak band in the test line may cause problems with interpretation; because the test is visualized with the naked eye, the final result can depend upon the interpretation of the user. Therefore, it is important to minimize non-specific bands in the test line. To avoid non-specific interactions, antibodies must be immobilized on the conjugate pad and test line at the appropriate density. If the density is too high, it can lead to non-specific binding and a lack of specificity.

Real-time RT-PCR is a sensitive method for detecting and quantifying the amount of target in a single tube. The low number of experimental steps associated with this assay minimizes the risk of contamination, thereby allowing better understanding of the epidemiology of an outbreak (Kim et al., 2007). Here, we showed that the real-time RT-PCR assay was 10-fold more sensitive than the IC assay (Fig. 1). However, the IC assay generates a result within 10 min, significantly faster than the time taken by real-time RT-PCR (>4 h). Rapid and accurate diagnosis of PED is paramount when deciding whether to initiate treatment. Such rapid responses will reduce economic losses in the animal industry. Rapid diagnosis and the instigation of timely control strategies before animals display clinical symptoms may prevent the spread of PEDV to other pig farms. Rapid diagnosis of PEDV has assumed greater significance because it can be of direct benefit to the swine industry in the absence of suitable therapeutic measures.

In conclusion, the high agreement between the IC and real-time RT-PCR assays suggests that the IC assay is a very useful method for the rapid and accurate diagnosis of pigs infected with PEDV. Thus, the IC assay may be a useful alternative to the current diagnostic tools used to detect PEDV.

Conflict of interest

The authors have no conflicts of interest to declare.

Acknowledgments

We are grateful for the technical assistance provided by Median Diagnostics Inc. (Chuncheon, South Korea). This study was supported by a grant (Project Code no. 313014-03-1-HD030) from the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries, Republic of Korea.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2015.08.024.

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