*Polish Journal of Veterinary Sciences Vol. 21, No. 1 (2018), 139–147*

DOI 10.24425/119032

*Original article*

# Development of a rapid immunochromatographic assay for detection of antibodies against porcine epidemic diarrhea virus

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## Abstract

An immunochromatographic test strip was developed for the detection of antibodies against porcine epidemic diarrhea virus (PEDV) in porcine serum. In this test, the recombinant nucleocapsid protein of PEDV was expressed in soluble form and purified by using Ni-NTA resin and gel filtration chromatography. The purified recombinant N protein conjugated with colloidal gold was dispensed on a conjugate pad as the detector. Staphylococcal protein A and rabbit anti-N protein IgG were blotted on a nitrocellulose membrane for the test and control lines, respectively. The immunochromatographic test strip specifically detected PEDV antibodies within 10 min and had higher sensitivity (96.0%) and specificity (90.8%) than those of commercial enzyme-linked immunosorbent assay (ELISA) kits. Our newly developed strip has great potential for the early diagnosis of PEDV infection.

**Key words:** porcine epidemic diarrhea virus, immunochromatographic test strip, nucleocapsid protein, diagnosis

## Introduction

Porcine epidemic diarrhea (PED) is an acute and highly contagious swine viral enteric disease manifested by vomiting, watery diarrhea, and dehydration. Frequent PED outbreaks have occurred in many pig-producing countries (Choudhury et al. 2016). PED is caused by porcine epidemic diarrhea virus (PEDV), which was first discovered in Europe in 1971 (Wood 1977). Since then, PEDV outbreaks have been reported in many countries in Europe and Asia. PED first emerged with a high morbidity and mortality in neonatal piglets in the United States in May 2013, dramatically affecting the pork industry (Huang et al.

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2013). PED subsequently appeared in pig farms in Canada and Mexico (Ojkic et al. 2015, Trujillo-Ortega et al. 2016). In China, PEDV outbreaks have been documented in the majority of pig farms since late 2010 (Sun et al. 2012, Li et al. 2014, Chen et al. 2016, Fan et al. 2017). In a mixed infection, recombination is a key source of genetic variation and a driving force for the rapid evolution of PEDV. Recombinant strains between PEDV and transmissible gastroenteritis virus (TGEV) have appeared in Italy (Boniotti et al. 2016). We recently detected a recombinant strain CH/HNQX-3/14 in intestinal samples from piglets in Henan, China. This epidemic recombinant strain likely resulted from a recombination between a vaccine strain and naturally circulating PEDV variants (Li et al. 2016), indicating the complex evolution of PEDV. The nucleocapsid (N) protein of coronaviruses can package the viral genome to form ribonucleoprotein (RNP) complexes for viral assembly. Multiple copies of N protein interact with viral genomic RNA and subgenomic RNA molecules for viral transcription and translation (McBride et al. 2014, Lee 2016). A recent study indicated that PEDV N protein specifically interacts with nucleolar phosphoprotein nucleophosmin (NPM1) and positively modulates PEDV growth (Shi et al. 2017). High levels of antibodies are produced against N protein at the early stages of PEDV infection. Therefore, N protein could be a suitable candidate as an antigen for the early diagnosis of PEDV infection (Song and Park 2012). In the present study, we developed the immunochromatographic test strip, which is a simple and easy-to-use rapid assay for the detection of antibodies against PEDV N protein for the early diagnosis of PED.

## Materials and Methods

### Serum, reagents and materials

The positive reference sera for *transmissible gastroenteritis virus* (TGEV), *classical swine fever virus* (CSFV), *porcine reproductive and respiratory syndrome virus (PRRSV)*, *foot and mouth disease virus* (FMDV) and *porcine pseudorabies virus* (PRV) were supplied by the Key Laboratory of Animal Immunology of the Ministry of Agriculture (Zhengzhou, China). These sera were retested with the ELISA kits following their corresponding manufacturer's instructions and no cross-reactivity with other swine pathogens were found. The PEDV-positive and -negative control sera were purchased from Veterinary Medicine Research Development Inc., USA and then stored in our laboratory. The PEDV-positive and -negative clinical sera were collected in Henan Province, China from 13 swine herds. Chloroauric acid was purchased from Sigma-Aldrich Inc (St. Louis, MO, USA). Bovine serum albumin (BSA) was purchased from Roche (Basel, Switzerland). Nitrocellulose membranes, glass fibers, sample pads, and absorbent pads were purchased from Millipore Corporation (Bedford, MA, USA). The dispensing platform and cutting module were purchased from Bio-Dot (Irvine, CA, USA). The scanning densitometer, CS-9300PC, was from Shimadzu (Kyoto, Japan).

### Construction of expression vectors for the nucleocapsid gene of PEDV

The PEDV variant CH/HNQX-3/14 (GenBank accession number: KR095279.1) was isolated from a clinical PEDV-positive sample and was confirmed to be of a recombination PEDV strain (Li et al. 2016). The viral genomic RNA was isolated for cloning of the full-length nucleocapsid (N) gene by reverse transcriptase polymerase chain reaction (RT-PCR). The N gene was cloned by a forward primer (5'-GGGGATCCTCACTATCTGTGAGAACC-3') and a reverse primer (5'-GCGAGCTCGACCGGCTTATTCTGGC-3') containing underlined restriction enzyme sites (BamHI and SacI). Reverse transcription was performed at 50°C for 30 min. PCR of N gene was performed as follows: initial denaturing at 95°C for 5 min; followed by 30 cycles at 95°C for 30 s, 55°C for 45 s, and 72°C for 1 min; and a final extension at 72°C for 7 min. The amplified products were visualized using agarose gel electrophoresis, purified by a QIAquick Gel Extraction kit (Qiagen China Co., Shanghai, China), and ligated into the pET28a(+) vector (Novagen Inc., Darmstadt, Germany) designated as pET28a(+)-N). The positive colonies harboring the correct insert were confirmed by restriction enzyme digestion and sequencing.

### Preparation of recombinant PEDV nucleocapsid protein

The recombinant plasmid pET28a(+)-N was transformed into *E. coli* $BL_{21}$ (DE3) competent cells. A positive clone was grown at 37°C in Luria-Bertani broth (LB) supplemented with 100 μg/mL kanamycin to an optical density of 0.6 at 600 nm. The recombinant N protein fused with His-tag was produced by addition of 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma, St. Louis, USA). Twenty hours after IPTG induction at 25°C, the cells were harvested and lysed by sonication. The solubilized proteins containing the His-tag were firstly purified using

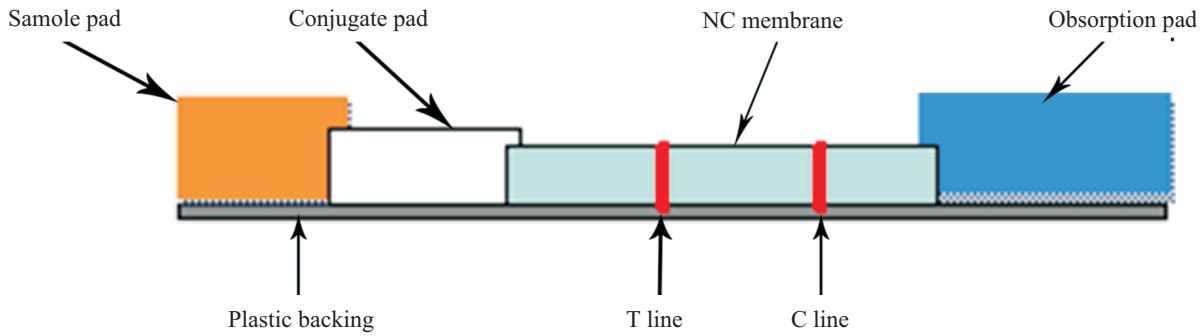



Fig. 1. Schematic diagram of the immunochromatographic strip.

nickel-nitrilotriacetic acid (Ni-NTA) resin following the manufacturer's protocol, and further purified by gel filtration chromatography (Superdex 75) in the AKTA prime plus instrument (GE Healthcare Life Sciences, Marlborough, USA). The purified protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie brilliant blue R250 and Western blot. For the Western blot, the recombination N protein was incubated with PEDV positive sera and probed with HRP-conjugated polyclonal goat (anti-porcine IgG) antibody, which was coupled with dimethylaminobenzidine (DAB) substrate solution.

## Conjugation of recombinant N protein with colloidal gold

Colloidal gold with a mean diameter of 20 nm was prepared using a method described previously (Pong et al. 2007). Briefly, 3 mL of 1% trisodium citrate solution (w/v) was added rapidly to 100 mL of 0.01% chloroauric acid solution (w/v) preheated to the boiling point (176°C). The solution was boiled for 5-10 min with constant stirring until the mixture's color changed from light yellow into black and eventually brilliant wine red. The obtained colloidal gold solution was cooled to room temperature and the average particle diameter was checked with a transmission electron microscope. Finally, the gold colloidal solution was stored with 0.02% (w/v) of sodium azide at 4°C for further use. The colloidal gold-labeled recombinant N protein was prepared according to a previously described protocol (Jin et al. 2012). Briefly, 1.6 ml of purified recombinant N protein (1.2 mg/mL) was incubated with 20 ml of colloidal gold solution (pH 9.0) with rapid stirring. After the mixture was stirred vigorously for 30 min and then cooled to 4°C for 30 min, 1 ml of 10% BSA solution was added to the stirred solution then stabilized for another 60 min at 4°C. The resulting suspension was centrifuged (15,000 rpm) at 4°C for 30 min. Finally, the colloidal gold-labeled antigen was re-suspended with 2 ml of 0.02 M sodium borate buffer (containing 1% BSA, 1% sucrose, 0.1% $NaN_3$), and stored at 4°C.

## Preparation of immunochromatographic test strip

The colloidal gold-labeled recombinant N protein was micro-sprayed onto a nitrocellulose membrane at 1 µL/cm using the dispensing platform to produce the conjugate pad. The sample pad was saturated with a buffer (pH 8.0) containing 0.02 M sodium borate, 0.1% Tween-20, and 0.1% $NaN_3$ and dried at 42°C for 2 h, and stored in a desiccator at room temperature. Staphylococcal Protein A (1.0 mg/mL) and rabbit anti-N protein IgG (3.0 mg/mL) were dispensed on the nitrocellulose (NC) membrane as the test (T line) and control lines (C line), respectively. The two lines were approximately 0.5 cm apart.

The strip consisted of the NC membrane, antigen-gold conjugate pad, sample pad, and absorbent pad. The NC membrane was pasted at the center of an adhesive plastic backing card. The absorbent pad and conjugate pad were then pasted by overlaying 1 mm on the upper and bottom of the NC membrane, respectively. The sample pad was then pasted by overlaying 2 mm on the bottom of the conjugated pad (Fig. 1). The master card was cut into 4.0 mm wide strips using an automatic cutter. Each strip was assembled on a plastic cassette and sealed with a desiccant (0.5 g) in an aluminum pouch. The test strips were stored at a broad temperature range (4-30°C) prior to use.

## Immunochromatographic strip test procedure

To use the immunochromatographic strip test, a sample of swine serum was diluted 50-fold with application buffer (30 mM Tris, 336 mM NaCl, 9 mM EDTA, 1% Triton X-100, pH 9.3), and 50 µL of the

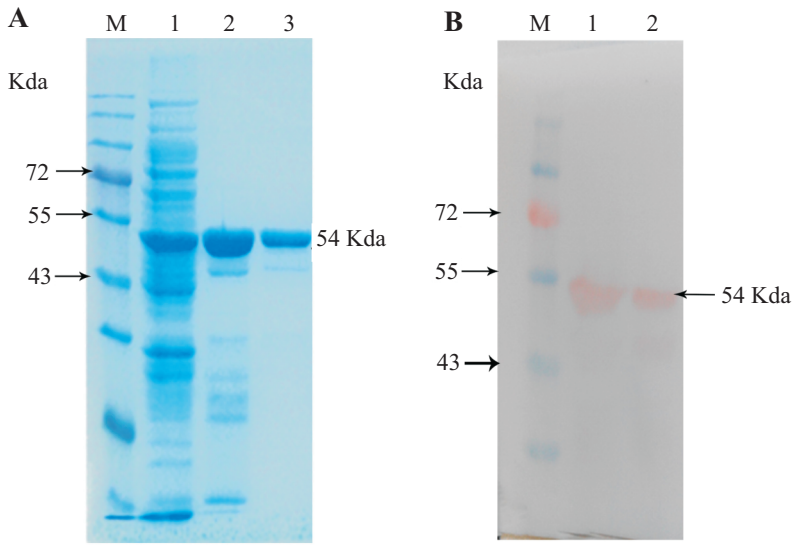



Fig. 2. Expression and purification of recombination N protein. (A) Purification profile of N protein. Lane M: pre-stained protein molecular weight marker (10-180 KDa); lane 1: unpurified recombination N protein; lane 2: purified by Ni-NTA affinity chromatography; lane 3: purified by superdex 75 gel filtration chromatography. (B) Analysis of purified recombinant N protein by Western blot. Lane M: Pre-stained protein molecular weight marker; lane 1: His mAb; lane 2: PEDV positive serum.

diluted sample was added to the sample well. The result was read by naked eyes between 5-10 min after the addition of the sample. A definite visible band must appear at the C location for an acceptable immunochromatographic strip test. When the color red is shown on both T and C lines in the result window, the result is positive, and when red appeared only on the C line, the result is negative. Failure to produce a visible line at the control line indicates an invalid test.

### Sensitivity and specificity of the immunochromatographic test strip

The sensitivity of the immunochromatographic test strip was examined by using a serially diluted PEDV positive control serum. The titer of the positive serum for PEDV was determined by using a commercial ELISA kit (Biovet, Inc., Canada) for the antibody against PEDV N protein in accordance with manufacturer's instructions. The strip was tested under two-fold serial dilutions of PEDV positive serum in PBS (1:200 to 1:12800). Three independent experiments were conducted. The specificity of the test strip was evaluated on five PEDV-negative, five PEDV-positive, five TGEV-positive, nine CSFV-positive, seven PRRSV-positive, three PRV-positive, and three FMDV-positive serum samples. Approximately 50 µL of each sample was added to the sample chamber and left to stand for 5-10 min. A result was considered positive when a red band appeared at both the test (T) and control (C) lines, and considered negative when a red band appeared only at the C line.

### Application of the immunochromatographic strip in the field

The immunochromatographic test strip was applied to the testing of 201 serum samples collected from different farms in Henan province. These serum samples were also evaluated for anti-N antibody using the commercial ELISA kit following the manufacturer's instructions. All of the 201 sera were stored at -20°C quickly thawed for tests. Cohen's kappa value was introduced as a measure of agreement between the strip and ELISA kit (Sim and Wright 2005).

## Results

### Expression and purification of PEDV N protein in *E. coli*

The cloned N gene of PEDV was expressed as a soluble protein with a N-terminal His-tag. The PEDV N gene exhibited a higher expression level at the position corresponding to the molecular mass of 54 kDa (Fig. 2A) after the cells were induced with 0.8 mM IPTG (25°C for 20 h) and purified through affinity chromatography in accordance with the manufacturer's instructions (QIAGEN). The recombinant N protein bound to Ni-NTA was eluted with 250 mM imidazole and further purified through gel filtration chromatography (Superdex 75). The peak of the N protein occurred at 50 min after the sample injection (50 mL of the buffer flow had passed). SDS-PAGE (Fig. 2A) and Western blot results

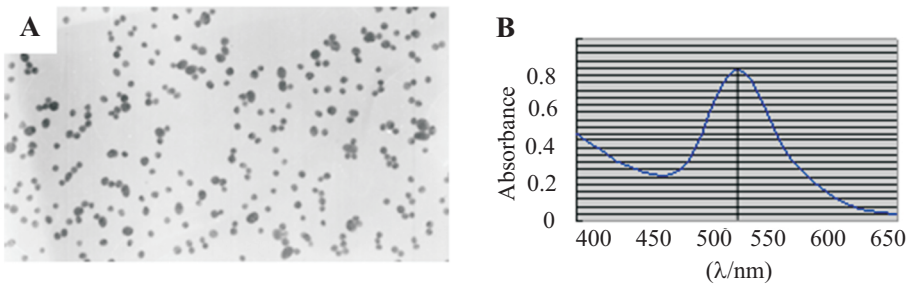
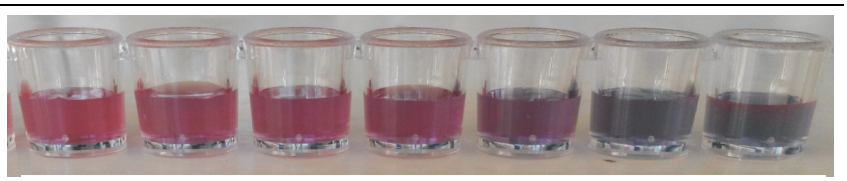



Fig. 3. Analysis of prepared colloidal gold. (A) Determination of colloidal gold by transmission electron microscope (Bar=100 nm). (B) Determination of colloidal gold by spectrophotometry.

Table 1. Optimization of experimental conditions for preparation of colloidal gold-N conjugates.

| Serial number | 1 | 2 | 3 | 4 | 5 | 6 | BC |
|---|---|---|---|---|---|---|---|
| N protein (μL) | 40 | 20 | 10 | 5 | 2.5 | 1.25 | 0 |
| Colloidal gold (μL) | 125 | 125 | 125 | 125 | 125 | 125 | 125 |
| NaCL (μL) | 125 | 125 | 125 | 125 | 125 | 125 | 125 |
| Color | red | red | purple | purple | Light blue | blue | Dark blue |



confirmed the successful purification of the N protein, which showed a predominant band at 54 kDa (Fig. 2B).

## Analysis of prepared colloidal gold

TEM imaging revealed that the particle size had an average diameter of 20 ± 1.13 nm (n = 100) (Fig. 3A). The absorbance of the generated colloidal gold particles was measured by using a UV-vis spectrometer and plotted against the wavelengths. The maximum absorbance occurred at 523 nm (Fig. 3B), which was within the typical absorbance range of colloidal gold particles used in immunochromatographic assays (Sun et al. 2005). These results indicated that the generated gold particles were suitable for producing colloidal gold-conjugated recombinant N protein and ultimately generating the immunochromatographic strip.

## Determination of the optimal amount of colloidal gold-conjugated N protein

The concentration of the recombinant N protein conjugated with colloidal gold was optimized with various amounts (0-40 μL) of recombinant N protein (1.2 mg/mL in PBS) after 125 μL colloidal gold and 10 g/L NaCl were added. The decrease in red color (from red-purple to dark blue) indicated the reduced efficacy of coating N protein on the surface of the colloidal gold particles. Results showed that 10 μL recombinant N protein was optimal for 125 μL colloidal gold (Table 1). Therefore, 96 μg recombinant N protein conjugated with 1.0 mL colloidal gold was determined to produce the optimal detecting efficacy for recombinant N protein on an immunochromatographic strip.

## Sensitivity and specificity of the immunochromatographic strip

To determine the limit of detection of the immunochromatographic test strip, the positive control sera against PEDV N protein were serially diluted and examined with the strip. Two red bands were clearly developed at the T and C lines, and the highest titers of the sera against PEDV reached up to 1:6400 (Fig. 4). These results indicated that the endpoint titer for the strip was 1:6400 for detecting PEDV antibodies.

The specificity of the test strip was determined by using the anti-PEDV positive sera and positive sera against the TGEV, CSFV, PRRSV, PRV, FMDV, and anti-PEDV negative sera. When the anti-PEDV positive sera were assayed by the test strip, two red

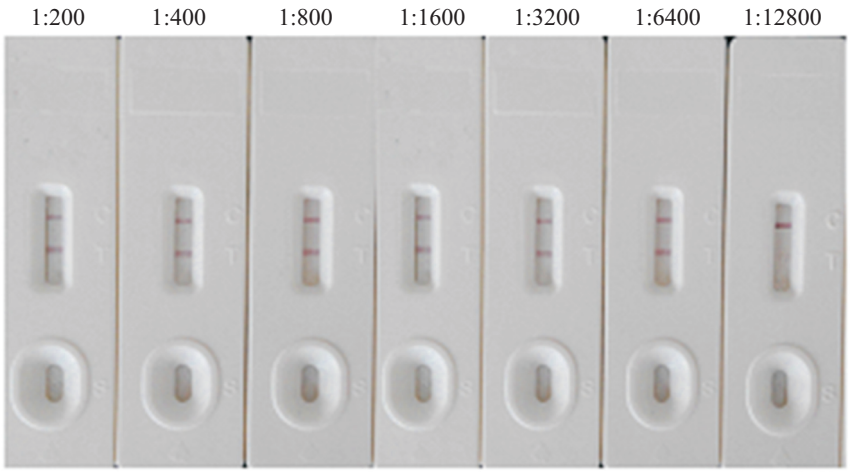



Fig. 4. Sensitivity of the immunochromatographic strip test.

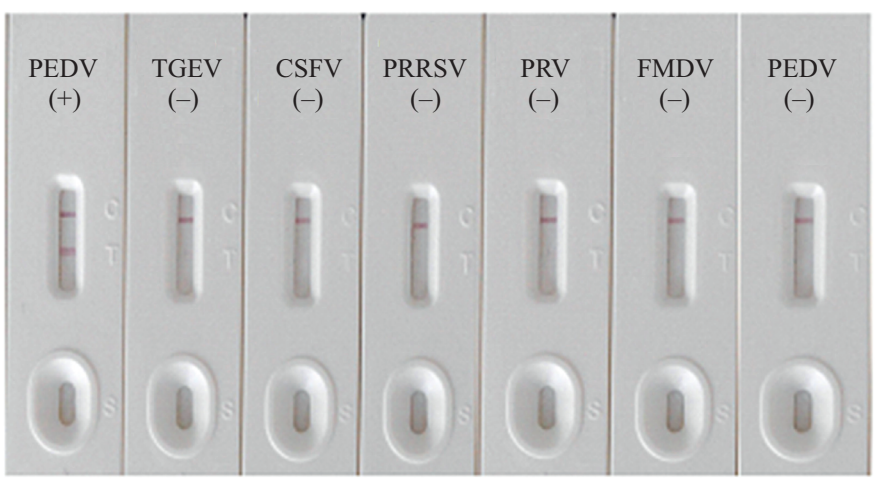



Fig. 5. Specificity of the immunochromatographic strip test. Note: (+) and (-) indicates the positive and negative results, respectively. The sera positive for *transmissible gastroenteritis virus, classical swine fever virus, porcine reproductive and respiratory syndrome virus, porcine pseudorabies virus*, *foot and mouth disease virus*, and PEDV-negative sera were simultaneously tested by using the immunochromatographic strip.

Table 2. Comparison of blocking N strip with ELISA kit (Biovet).

| | | ELISA kit | | | |
|---|---|---|---|---|---|
| | | Positive | Negative | Total | AP[b] (strip) |
| N strip[a] | Positive | 120 | 7 | 127 | 0.632 |
| | Negative | 5 | 69 | 74 | 0.381 |
| | Total | 125 | 76 | 201 | |
| | AP[b] (ELISA) | 0.622 | 0.378 | | |

*Note*: A total of 201 samples were tested by the strip and ELISA kit. The top left and bottom right cells indicate the number of samples that generated similar data for both assays. The top right and bottom left cells indicate the number of samples that generated different data for the assays.

* Relative sensitivity = 120/125, or 96.0%; relative specificity = 69/76, or 90.8%.

[a] Observed proportional agreement: (120 + 69)/201 = 0.94; chance proportional agreement: (0.622 × 0.632) + (0.378 × 0.381) = 0.537; observed minus chance agreement: (0.94 – 0.537) = 0.403; maximum possible agreement beyond chance level: (1 – 0.537) = 0.463; agreement quotient (k): (0.403/0.463) = 0.870.

[b] Apparent prevalence (AP) of the strip or ELISA: positive ≡ positive number/total number; negative ≡ negative number/total number.

bands clearly appeared at both the T and C lines. One red band appeared on the strip at the C line when the anti-PEDV negative sera were tested. All of the positive sera against TGEV, CSFV, PRRSV, PRV, and FMDV displayed no positive results at the T line, indicating that the PEDV N protein did not share

a cross-reaction with the anti-TGEV, CSFV, PRRSV, PRV, and FMDV sera (Fig. 5). The test strip demonstrated a high specificity in detecting anti-PEDV-specific IgG without cross-reactivity to that of other porcine viruses.

### Comparison with ELISA

The relative sensitivity and specificity of the strip compared with that of ELISA were 96.0% (120/125) and 90.8% (69/76), respectively (Table 2). For the 201 clinical serum samples, the results obtained by the strip agreed well with those obtained through ELISA. Cohen's kappa value was calculated by using the formula described in Table 2. The *p* and *pe* values were calculated as 0.94 and 0.537, respectively. The agreement quotients (*k* value) for the strip was 0.87, which implied a perfect agreement.

## Discussion

PEDV is a highly infectious disease that currently poses a serious threat to the swine industry. A rapid and simple diagnostic kit is necessary for the prevention of the spread of PEDV. Routine diagnosis of PEDV currently relies on gross observation of clinical symptoms, laboratory tests by RT-PCR, immunochromatochemistry, ELISA, virus neutralization (VN) tests, and virus isolation (OIE 2014). Unfortunately, these methods are time consuming and require specialized equipment operated by trained technicians, suggesting the urgent need for a novel quick and easy-to-use diagnosis method. Given that PEDV is a fast-replicating and quick-spreading virus, the on-site and rapid test of PEDV infection and the surveillance of PED in pig herds are significant measures for the economics of swine production.

Colloidal gold particle-based immunoassay has been widely used for diagnosing various infectious diseases and detecting bioactive molecules, hormones, haptens, etc (Heeschen et al. 1998, Allwinn et al. 1999, Zhang et al. 2015). Several immunochromatographic strips for detection of antivirus antibodies, such as PRRSV, PCV-2, PRV, and FMDV, have been developed and applied to the diagnostics and evaluation of antibody titers (Cui et al. 2008, Jin et al. 2012, Guo et al. 2015, Yang et al. 2015).

Obtaining N protein with high concentration and purity is necessary for the successful labeling with colloidal gold. In this study, the conditions for the expression of N protein, including IPTG concentration, temperature, and incubation time after induction by IPTG, were optimized. Finally, we obtained a fusion N protein with His-tag in soluble form after 20 h of induction with 0.8 mmol/L IPTG at 25°C. The soluble His-fused N protein was purified by using Ni-NTA resin and gel filtration chromatography (Superdex 75), and N protein with 95.7% purity was obtained (data not shown). Western blot hybridization confirmed that the purified recombinant N protein could react specifically with PEDV positive serum and anti-His monoclonal antibody, indicating that the recombinant N protein possessed good immunoreactivity and can be used as an antigen for detecting PEDV antibodies. Before being conjugated with colloidal gold, the purified N protein was pretreated by dialysis with normal saline to minimize interference by $Ni^{2+}$, organic molecules, and salt ions, and consequently provide a good basis for the development of an immunochromatographic test.

In our previous studies, PEDV was found to be the predominant causative pathogen contributing to outbreaks of clinical diarrhea in central China (Li et al. 2014). In the present study, swine serum samples from different farms with precisely known infection statuses were used for the evaluation of the immunochromatographic strip. A total of 120 positive serum samples were collected at the onset of diarrhea from piglets that had been previously confirmed by RT-PCR to be PEDV-infected through their feces samples. All PEDV-positive samples were detected by the strip. To validate the immunochromatographic test strip on sera collected at different times after infection, we experimentally inoculated pigs with PEDV CH/HNQX-3/14 strain orally ($TCID_{50}$ $=10^{6.5}$/mL), and the antibody response to N protein was determined by the immunochromatographic test strip. The earliest IgG antibody response was observed at day 7 post-challenge, but no antibodies against N protein were detected in pigs after day 10 post-infection (data not shown). These data are consistent with a previous report (Gimenez-Lirola et al. 2017). Further investigations are needed to elucidate the induction mechanism of the PEDV N antibody.

## Conclusions

We developed a novel immunochromatographic test by using a recombinant PEDV N protein conjugated with colloidal gold as detector. The test strip displayed high specificity and good sensitivity for the specific antibodies of the PEDV N protein. Furthermore, the results obtained by the strip well agreed with those attained with the commercial ELISA kit, but the time required for the strip is considerably faster than that obtained by ELISA or RT-PCR assays. The immunochromatographic test reported in this

study is a convenient and effective alternative method for the early clinical diagnosis of PEDV infection and thus can be used for the monitoring and minimization of the spread of PEDV.

## Acknowledgements

This study was supported by grants from the Key Research Project of Henan Higher Education Institutions (No. 18A230004), Doctoral Scientific Research Foundation of Henan Institute of Science and Technology (No. 206010617002) and China Agriculture Research System (No. CARS-36), China.

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