Development of a Chromatographic Strip Assay for Detection of Porcine Antibodies to 3ABC Non-Structural Protein of Foot-and-Mouth Disease Virus Serotype O

Tsu-Han CHEN1), Chu-Hsiang PAN1), Ming-Hwa JONG1), Hsiu-Min LIN2), Yu-Liang HUANG1), Kuang-Pin HSIUNG2), Parn-Hwa CHAO1) and Fan LEE1)*

1) Division of Hog Cholera Research, Animal Health Research Institute 376 Chung-Cheng Road, Tamsui, Taipei County 25158 and 2) Taiwan Unison Biotechnology Inc. 22 Kedung 3rd Road, Chunan, Miaoli County 35053, Taiwan

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ABSTRACT. A chromatographic strip assay was developed for rapid detection of serum antibodies to non-structural protein of foot-and-mouth disease virus. The assay was based on Escherichia coli-expressed 3ABC non-structural protein and an immunochromatographic technique, which shortened the detection time to about one hour. The sensitivity of the assay was determined to be 96.8% for infected pigs; its specificity was 100% for naïve pigs and 98.8% for vaccinated pigs. In the experimentally infected pigs, anti-3ABC antibodies were detectable from eight days post-infection until the end of the study, 34 days post-infection. The performance of this assay was comparable to that of two commercial ELISA kits, Ceditest FMDV-NS and UBI FMDV NS EIA, and was better than that of CHEKIT FMD-3ABC po. Given its advantages of instant testing and quantitative measurement, this assay has potential as a useful tool for rapid on-farm diagnosis of foot-and-mouth disease.

KEY WORDS: antibody detection, chromatographic strip, foot-and-mouth disease, non-structural protein.

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals. The disease is characterized by fever, vesicular lesions and erosion in the mouth and on the tongue, muzzle, feet and teats [1]. Foot-and-mouth disease virus (FMDV), the etiological agent of FMD, is a non-enveloped, single-stranded, positive-sense RNA virus of the genus Aphthovirus in the family Picornaviridae. The viral genome is approximately 8.5 kb in length and encodes four structural proteins, VP1–4, and many non-structural proteins (NSPs). To date, seven FMDV serotypes have been recognized worldwide, known as serotypes O, A, C, Asia 1, SAT 1, SAT 2 and SAT 3 [14].

In 1997, a devastating FMD outbreak caused by the FMDV O/Taiwan/1997 strain occurred in Taiwan and resulted in severe economic losses [27]. The FMDV strain shows a porcinophilic phenotype with an altered non-structural protein 3A gene [1]. Another strain, O/Taiwan/1999, which had a full-length 3A coding region, was isolated from subclinically infected cattle in 1999 [15]. To eliminate FMD from Taiwan, compulsory vaccination and intensive serological surveillance have been implemented since 1997. With the implementation of these strategies, Taiwan regained its status in 2003 as an FMD-free country in which vaccination is practiced.

Anti-NSP antibodies, which are usually present only in FMDV-infected animals, have been considered to be indicators for discriminating infected from vaccinated animals [9]. Since NSPs are highly conserved among serotypes, detection of the antibodies has the additional advantage of serotype independence [10]. During the last decade, the use of immuno-enzymatic tests to detect anti-NSP antibodies and thus to assess virus circulation in susceptible populations has been studied extensively. Methods such as the latex beads agglutination test [26], enzyme-linked immunoelectrotransfer blot assay [2], enzyme-linked immunosorbent assay [2, 3, 5, 8, 9, 10, 12, 16, 17, 19, 23–25] and multiplexed Luminex assay [9] have been employed for this purpose. These assays all need to be performed in the laboratory with different instrument and technical requirements, which restricts the use of these methods in the field and hence prolongs the time between diagnosis and response to this rapidly transmitted disease.

In the present study, a chromatographic strip assay was developed for the detection of anti-3ABC antibodies. This assay could detect the antibodies in swine sera with high sensitivity and specificity and could be a good choice for FMD surveys in the field. With the employment of a portable point-of-care testing (POCT) scanner, qualitative and quantitative antibody detection could be performed with this assay.

MATERIALS AND METHODS

Sera: For study of the sensitivity of the tests, 62 FMD-positive swine sera samples from pigs experimentally infected with FMDV O/Taiwan/1997 strain at 14 days post-infection (dpi) were used. For study of the specificity, 254 sera from naïve pigs and 167 sera from vaccinated pigs were used. The 254 swine sera comprised 96 sera collected from specific pathogen-free pigs and 158 sera collected from commercial pigs before 1997. The 167 swine sera, kindly provided by Professor W. B. Chung, were collected from...
167 non-infected pigs that had been vaccinated twice with a commercial FMD vaccine of serotype O. In addition, six antisera against the UKG/27/72 strain of swine vesicular disease virus were purchased from the Institute for Animal Health, Pirbright, United Kingdom, for evaluation of possible serological cross-reactivity between FMDV and swine vesicular disease virus. For comparison of the detection ability of the chromatographic strip assay with those of ELISA, a serum panel containing 320 swine sera collected sequentially from 32 secondary specific-pathogen-free pigs experimentally infected with the FMDV O/Taiwan/1997 strain was used. The sera in the panel were sampled at 0, 2, 4, 6, 8, 10, 14, 21, 28 and 34 dpi. Convalescent swine sera from pigs experimentally infected with either the FMDV O/Taiwan/1997 strain or O/Taiwan/1999 strain as well as a negative control swine serum (GIBCOBRL, Invitrogen, Carlsbad, CA, U.S.A.) were used for western blotting and standardized of the chromatographic strip assay.

Reverse transcription polymerase chain reaction (RT-PCR): A primer pair was designed for RT-PCR amplification of the 3ABC region (GenBank accession No. AJ539137; nucleotides 5595 to 6119) in the genome of the FMDV O/TAW/2/99 BOV strain. The forward primer was 5’-CACCGGATCTTTGCGGATACTCGCAAGAGACGAGCATCAG-3’ and contained a BamHI restriction site, and the reverse primer was 5’-CCCAGATTCGCACGTCTTCCCGTGAGGATGACGCTC-3’ and contained an EcoRI restriction site. Extracted FMDV RNA was used as the template. The RT-PCR mixture was prepared with the SuperScript™ One-Step RT-PCR System in combination with Platinum® Pfx DNA Polymerase (Invitrogen, Carlsbad, CA, U.S.A.). The reaction, performed in a GeneAmp PCR system 2400 thermocycler (Applied Biosystems, Foster City, CA, U.S.A.), was started by incubation at 42°C for 40 min followed by pre-denaturation at 94°C for 50 sec. The pre-denaturation was followed by 35 cycles of denaturation (94°C for 30 sec), annealing (55°C for 30 sec) and extension (68°C for 1 min). A final extension at 72°C for 7 min was performed before holding the sample at 4°C. The RT-PCR product was stored at −20°C until use, and 10 μL of the product was analyzed by 2% agarose gel electrophoresis and visualized by ultraviolet transillumination.

Cloning of the 3ABC gene: To insert the RT-PCR product into an expression vector, the product in the agarose gel was extracted with a Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., Taipei County, Taiwan), digested with the restriction endonucleases BamHI and EcoRI, and then ligated to pET 32a (+) vector (Novagen, EMD Biosciences Inc. San Diego, CA, U.S.A.), which was also digested with restriction endonucleases mentioned above. Competent E. coli cells BL21 (DE3; Novagen, EMD Biosciences Inc. San Diego, CA, U.S.A.) were transformed with the ligation product as previously described [22] and then plated onto Luria-Bertani agar supplemented with 100 μg/mL ampicillin. Positive clones were selected, and the plasmids in each clone were extracted with a QIAprep® Spin Miniprep Kit (QIAGEN Inc., Valencia, CA, U.S.A.) and then screened for the presence of the desired insert. The sequence of the insert was determined by a commercial sequencing service using a 3730 DNA Analyser (Applied Biosystems, Foster City, CA, U.S.A.).

Expression and purification of FMDV 3ABC polypeptide in E. coli: Transformed E. coli with the desired pTH 162-B plasmid were cultivated in Luria-Bertani medium supplemented with 100 μg/mL ampicillin at 37°C with vigorous shaking. Isopropylthiogalactoside (IPTG) was added to a final concentration of 1 mM when the culture reached mid-logarithmic growth, and the culture was then incubated for an additional four hours to induce expression of the 3ABC polypeptide. Following the four-hr induction, cells were harvested by centrifugation and then lysed by B-PER® II Bacterial Protein Extraction Reagent (Pierce, Rockford, IL, U.S.A.). The soluble 3ABC polypeptide in the lysate was purified by a HisTrap HP affinity chromatography column (Amersham Biosciences, Little Chalfont, Buckinghamshire, England).

SDS-PAGE and Western blotting: The purified 3ABC polypeptide was analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a discontinuous Tris-glycine buffer system [22]. After being resolved by the SDS-PAGE, the proteins were electrically blotted onto a nitrocellulose filter, Sequi-Blot PVDF (Bio-Rad, Hercules, CA, U.S.A.), and examined for serological reactivity to the strong positive swine serum by Western blotting [22]. The primary antibody used in Western blotting was 1:100 diluted positive swine serum, and the secondary antibody was 1:5,000 diluted alkaline phosphatase-conjugated goat-anti-swine IgG (Santa Cruz Biotechnology Inc,Santa Cruz, CA, U.S.A.). The substrates of alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Bio-Rad, Hercules, CA, U.S.A.), were used for color development.

Strip manufacture: The strip was prepared by adhering a nitrocellulose membrane (12 μm, AE-100, Whatman, Kent, UK), absorption pad (cellulose, 470, Whatman, Kent, UK), conjugate pad and sampling pad (glass fiber paper, 8964, Ahlstrom, Helsinki, Finland) to a supporting plastic film. The conjugate pad contained dried goat-anti-swine IgG (Jackson ImmunoResearch Laboratories Inc, West Grove, PA, U.S.A.) conjugated to colloidal gold. The test line’s sample was prepared by dissolving 3ABC polypeptide in 3% methanol to a concentration of 4.5 mg/mL. The control line’s sample was prepared by dissolving rabbit-anti-goat IgG (KPL, Gaithersburg, MD, U.S.A.) in 3% methanol to a concentration of 2 mg/mL. Two detection bands representing the test and control lines, located at 5.0 cm and 6.05 cm, respectively, from the bottom of the nitrocellulose membrane stick were constructed by drawing 0.5 μL/cm of the samples with a XYZ 3050 Platform (BioDot, Irvine, CA, U.S.A.) and BioJet Quanti dispenser (BioDot, Irvine, CA, U.S.A.). The nitrocellulose membrane stick was cut into 8 cm long and 0.5 cm wide strips with a CM1000 cutter (BioDot, Irvine, CA, U.S.A.). Each strip was assembled into a plastic cartridge, dried and then preserved in an alumi-
num foil package with desiccant.

Serological testing by the chromatographic strip assay: Ten microliters of serum sample and 190 μL of dilution buffer (Taiwan Unison Biotechnology Inc., Miaoli County, Taiwan) were mixed, and 120 μL of the mixture was applied to the sample pad of the chromatographic strip. After a 50-min incubation at room temperature, the strip was read by a UNISCAN™ point-of-care testing (POCT) Scanner (Fig. 1). The scanner was able to detect the color density of the developed strip and produce a corresponding analog test signal. With a signal amplifier and an analog/digital converter in the scanner, the analog signal was then amplified and converted into a digital signal. A computing unit within the scanner received the digital signal, analyzed it, and output a concentration value for the analyte. The time required to read one strip was 15 to 20 sec. To standardize the results among different batches of strips, each value read by the scanner was divided by a modified factor supplied with the kit to be a standardized value. The cut-off value of positive reactivity was determined as the mean derived from 286 negative swine sera plus 3.5 standard deviations of the mean. The 286 sera used to determine the cut-off value were composed of 254 sera from naïve pigs and 32 sera from 0 dpi sera in the aforementioned serum panel.

Serological testing by ELISAs: Three commercially available ELISA kits were employed in our study to compare their antibody detection ability with that of our chromatographic strip assay. Ceditest FMDV-NS (Cedi-Diagnostics B.V., Lelystad, The Netherlands) is a blocking ELISA based on baculovirus-expressed 3ABC antigen [24, 25]. UBI FMD NS EIA (United Biochemical Inc., Hauppauge, NY, U.S.A.) is an indirect ELISA based on synthetic 3B peptide [23]. CHEKIT FMD-3ABC po (IDEXX Laboratories Inc., Westbrook, ME, U.S.A.) is an indirect ELISA based on E. coli-expressed 3ABC polypeptide [12]. Use of the kits and interpretation of the results were according to the manufacturers’ instructions.

Virus neutralization test: To confirm infection of the experimentally infected pigs employed for establishing the positive serum panel, the sera collected were tested by virus neutralization test as previously described [19].

Sensitivity and specificity of the tests: For each assay in the present study, the sensitivity was the percentage of positive sera that tested positive in the assay; the specificity was the percentage of negative sera that tested negative in the assay.

RESULTS

Expression of 3ABC polypeptide in E. coli: A 525-bp DNA fragment corresponding to the 3ABC polypeptide was amplified by RT-PCR. A plasmid, pTH 162-B, was constructed by inserting the RT-PCR-amplified fragment into the prokaryotic expression vector pET 32a (+). E. coli transformed with the pTH 162-B plasmid were induced with IPTG to express soluble 3ABC polypeptide with an expected molecular weight of 40 kDa, which was verified by the Western blots (Fig. 2).

Detection of anti-3ABC antibodies: The mean read values for the 286 negative swine sera was 6 ± 2.7, and the cut-off value was therefore determined to be 15.5. Based on this cut-off value, all negative sera produced negative results. The frequency distribution of the 62 positive sera is illustrated in Fig. 3. Of the 62 positive sera, two were interpreted as false negative, and the read value for both these sera was 13.3.

A positive serum panel of sequentially sampled swine sera was first tested by virus neutralization test to confirm infection of the sampled pigs and was subsequently tested by the present strip assay and by the ELISA kits for comparison of their detection abilities (Fig. 4). All experimentally infected pigs demonstrated virus neutralization test titers of over 1:16 by 14 dpi, confirming that infection had been established. As determined by our assay, anti-3ABC antibodies were first detected in 32 experimentally infected pigs at 8 dpi. All pigs tested seropositive by 10 dpi, and the positive percentage stayed over 90% throughout the period of the animal experiment. The mean standardized values of our assay reached a peak at 14 dpi and decreased rapidly. We used the data obtained between 14 and 34 dpi to estimate the trend of antibody decay. The mean standardized value was found to decrease to 15.5, the cut-off value, at 42.4 dpi.

Similar results were obtained with the Ceditest and UBI kits but not from the CHEKIT kit. The antibodies were first detected by the CHEKIT kit at 14 dpi, and the positive percentage stayed at 80% or lower during the experiment.

Sensitivity and specificity: The sensitivity and specificity of the chromatographic strip assay and a comparison with those of the three commercially available ELISA kits are shown in Table 1. The sensitivity of the chromatographic strip assay was comparable to those of the Ceditest and UBI
kits and was better than that of the CHEKIT kit. The specificities of our assay and the ELISAs were all satisfactory, except for that of the UBI kit for testing vaccinated pigs. The chromatographic strip assay produced negative results for the six antisera against the swine vesicular disease virus (data not shown).

DISCUSSION

The detection ability of the chromatographic strip assay for the early FMDV infection in pigs was comparable to those of the commercially available ELISA kits. The assay was capable of detecting anti-3ABC antibodies in swine sera as early as 8 dpi, and over 90% of experimentally infected pigs tested positive between 10 dpi and 34 dpi (Fig. 3). These results were comparable to those obtained with the Ceditest and UBI kits and were better than those obtained with the CHEKIT kit, which has been shown to have relatively lower analytical sensitivity [18]. Two positive reactors were obtained on 0 dpi and 2 dpi in samples from the same experimentally infected pig. These two positive reactors tested negative in the virus neutralization test and the ELISAs, suggesting that these were false positive results.

The sensitivity and specificity of the assay were satisfactory. The assay showed a sensitivity of 96.8% for infected pigs and specificities of 100% and 98.8% for naïve pigs and vaccinated pigs, respectively (Table 1). The assay’s specificity was also highlighted by the fact that it did not generate a reaction for the six antisera against swine vesicular disease virus. The results we obtained with the three ELISA kits were consistent with reports by Bronsvoort et al. [4] and Chen et al. [6], which demonstrated that the Ceditest and UBI kits have greater diagnostic sensitivity than the CHEKIT kit (Table 1).

The chromatographic strip assay had the advantages of instant testing and quantitative measurement. As determined in this study, the assay was capable of detecting anti-3ABC antibodies in a serum sample within one hour. Indeed, ELISA-based laboratory tests would have a better capacity to handle large-scale screening than the chromatographic-
graphic strip assay; however, the chromatographic strip assay holds the advantage of rapid testing. Moreover, the chromatographic strip assay proved to be excellent for use not only in the laboratory but also in the field. Use of this assay on-farm could dramatically shorten the time between testing and responses, which is especially critical for rapidly transmitted diseases such as FMD. Additionally, use of a portable scanner instead of visual interpretation enables this assay to measure the relative amounts of antibodies in samples. This would be beneficial for comparison of the results between different sample sources or between samples collected from the same source at different times, which would therefore enable researchers to develop a deeper understanding of potential epidemics.

The antibodies against NSPs waned rapidly in the pigs. Previous studies reveal that anti-NSP antibodies in some FMDV-infected pigs are sustained for short or intermittent period of time [13, 18]. Chen et al. [7] reported a profile of the development of anti-3ABC antibodies in infected pigs and concluded that the strongest antibody response is in the first two months post-infection. In our study, as determined by the chromatographic strip assay, anti-3ABC antibodies were no longer detectable in two of the 32 experimentally infected pigs after a short period of time (data not shown). The mean standardized value also demonstrated a rapid decrease in the amount of antibodies, suggesting that the disappearance of anti-3ABC antibodies was due to not only variation between individuals but also the characteristics of the species. The shorter duration of antibody presence in pigs than that in cattle [12, 21, 23] might be a critical consideration when one intends to use anti-NSP antibody as an indicator for removal of previously infected pigs from herds.

The 3ABC polypeptide derived from the FMDV O/Taiwan/1999 strain could react with the antibodies elicited by infection with the FMDV O/Taiwan/1997 strain. The results of the Western blotting showed that the expressed 3ABC polypeptide could react well with the positive sera against both the O/Taiwan/1997 and O/Taiwan/1999 strains. The use of a large number of positive sera from experimentally infected pigs in this study further supports the conclusion that our chromatographic strip assay can be applied to detect antibodies against the O/Taiwan/1997 strain, which is porcinephilic and has seriously threatened the pig populations in Taiwan. Although the 3A region of the O/Taiwan/1999 strain is different from that of the O/Taiwan/1997 strain [1] and is included in the N-terminal portion of our expressed 3ABC polypeptide, our results suggest that this difference exerted no influence on the recognition by the anti-3ABC antibodies. Further studies on application of this assay to other FMDV serotypes and other susceptible species are being conducted.

In conclusion, the chromatographic strip assay we had developed may be useful as a sensitive, specific and user-friendly test for differentiating natively infected pigs from vaccinated ones. Without the need for laboratory instruments, this assay can be performed accurately and rapidly by less skilled personnel, reduce delays in laboratory diagnosis, and generate quantitative data. More importantly, it can decrease the time needed to trigger early responses to this rapidly transmitted disease.

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REFERENCES


