Development of a multiplex Luminex assay for detecting swine antibodies to structural and nonstructural proteins of foot-and-mouth disease virus in Taiwan

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KEYWORDS
antibody detection; foot-and-mouth disease virus; multiplex Luminex assay; nonstructural protein; structural protein

Background/Purpose(s): Foot-and-mouth disease (FMD) and swine vesicular disease (SVD) are serious vesicular diseases that have devastated swine populations throughout the world. The aim of this study was to develop a multianalyte profiling (xMAP) Luminex assay for the differential detection of antibodies to the FMD virus of structural proteins (SP) and nonstructural proteins (NSP).

Methods: After the xMAP was optimized, it detected antibodies to SP-VP1 and NSP-3ABC of the FMD virus in a single serum sample. These tests were also compared with 3ABC polypeptide blocking enzyme-linked immunosorbent assay (ELISA) and virus neutralization test (VNT) methods for the differential diagnosis and assessment of immune status, respectively.

Results: To detect SP antibodies in 661 sera from infected naive pigs and vaccinated pigs, the diagnostic sensitivity (DSn) and diagnostic specificity (DSp) of the xMAP were 90.0–98.7% and 93.0–96.5%, respectively. To detect NSP antibodies, the DSn was 90% and the DSp ranged from 93.3% to 99.1%. The xMAP can detect the immune response to SP and NSP as early as 4 days postinfection and 8 days postinfection, respectively. Furthermore, the SP and NSP antibodies in all 15 vaccinated but unprotected pigs were detected by xMAP. A comparison of SP and NSP antibodies detected in the sera of the infected samples indicated that the results from the xMAP had a high positive correlation with results from the VNT and a 3ABC polypeptide blocking ELISA assay. However, simultaneous quantitation detected that xMAP had no relationship with the VNT. Furthermore, the specificity was 93.3–94.9% with 3ABC polypeptide blocking ELISA for the FMDV-NSP antibody.

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Conclusion: The results indicated that xMAP has the potential to detect antibodies to FMDV-SP-VP1 and NSP-3ABC and to distinguish FMDV-infected pigs from pigs infected with the swine vesicular disease virus.

Introduction

Foot-and-mouth disease (FMD), swine vesicular disease (SVD), vesicular stomatitis (VS), and vesicular exanthema of swine are serious vesicular diseases that have devastated swine populations throughout the world. Foot-and-mouth disease is caused by the foot-and-mouth disease virus (FMDV), a virus in the genus Aphthovirus within the family Picornaviridae. The disease is a very contagious disease in cloven-hoofed animals, and it affects animal species such as cattle, pigs, sheep, goats, elephants, yaks, sambar, spotted deer, African buffaloes, antelopes, and wild boars.  

Five antigenic sites have been identified on the type O FMDV, and three of the sites (sites 1, 3, and 5) have been mapped at the VP1. The amino acid residues that are critical for these antigenic sites are residues 144, 148, 154, and 208 for site 1; residues 43, 44, and 45 for site 3; and residue 149 for site 5. Changes in these critical residues may lead to antigenic variation.

In 1997, a devastating FMD outbreak caused by the O/TW/97 strain occurred in Hsinchu County, Taiwan. The incursion resulted in severe economic losses. The FMDV strain shows a porcinephilic phenotype with a deleted nonstructural protein 3A gene. Sequence analysis of the VP1 coding region showed that the viruses isolated in Taiwan between 1998 and 2009 were most similar to the O/TW/97 strain and to viruses isolated from Hong Kong and Vietnam in 1991–1996; this was also supported by phylogenetic analysis. Furthermore, substantial mutations were present in the viruses isolated in 2009. Some of these changes may result from vaccine pressure in the field. The serum neutralization test also supports that the viruses isolated in 2009 have significant changes in antigenicity.

When susceptible host animals are infected with FMDV, antibodies are elicited against viral structural proteins (SPs) and nonstructural proteins (NSPs). By contrast, animals administered inactivated FMD vaccines that lack or contain only trace amounts of NSPs are unlikely to induce FMDV antibodies. Therefore, for FMD diagnosis, the NSP antibodies can be markers to differentiate infected from vaccinated animals (DIVA), providing the vaccine used is of high purity. Detecting NSP antibodies has the additional advantage of being serotype-independent because the NSPs are predominantly conserved between the serotypes of FMDV. Many methods of single signature assays for detecting NSP antibodies have been used, including agar gel immunodiffusion, the latex bead agglutination test, enzyme-linked immunoelectrotransfer blot, enzyme-linked immunosorbent assay (ELISA), and chromatographic strip assay. For FMDV-SP diagnosis, different immunoassay formats have been established such as the virus neutralization test, liquid-phase blocking ELISA, solid-phase blocking ELISA, and competition ELISA. Recombinant antigens and synthetic peptides derived from FMDV SPs and NSPs have been developed as an alternative to the inactivated virus antigen.

Luminex assays are based on multianalyte profiling (xMAP) technology that simultaneously detects and quantifies multiple RNA or protein targets. The xMAP system combines a flow cytometer, fluorescent-dyed microspheres (i.e., beads), lasers, and digital signal processing to multiplex up to 100 unique assays within a single sample efficiently. In the present study, a new application of xMAP was developed. To address the limitations of sensitivity and specificity to FMDV in existing antibody detection methods, xMAP was developed to detect and differentiate antibodies against the swine vesicular disease virus (SVDV). These tests were also compared with 3ABC polypeptide blocking ELISA and virus neutralization test (VNT) methods for the differential diagnosis and assessment of the immune status, respectively.

Materials and methods

Serum samples

To study the diagnostic sensitivity of the tests, sera were used from 32 eight-week-old specific pathogen-free (SPF) pigs experimentally infected with the FMDV-O/TW/97 strain. To compare the detection ability of the Luminex assay with that of other methods, the serum panel contained 320 swine sera that were collected sequentially from 32 eight-week-old SPF pigs (the ear tag numbers were 1513–1528, 1530–1533, 1536–1546, and 1548). The pigs were intradermally injected with 10⁵ Tissue Culture Infectious Dose 50 (TCID50) (in a volume of 500 μL) of the FMDV-O/TW/97 strain into the heel bulb of the right foot. Blood samples were collected at 34 days postinfection (dpi). The sera in the panel were sampled at 0 days, 2 days, 4 days, 6 days, 8 days, 10 days, 14 days, 21 days, 28 days, and 34 days postinfection (dpi).

The performance of the xMAP in detecting SP and NSP antibodies against the FMDV-O/TW/97 strain was examined. Five 8-week-old SPF pigs (with ear tag numbers 1155-1, 1155-2, 1170, 1171, and 1172) were intradermally injected with 10⁵ TCID50 (in a volume of 500 μL) of the
Fifteen pigs were assigned to four vaccination groups: (1) W2V1 group, three pigs from farm #2 were vaccinated with the No. 1 commercial vaccine; (2) W1V2 group, three pigs from farm #1 were vaccinated with the No. 1 commercial vaccine; (3) W2V1 group, three pigs from farm #2 were vaccinated with the No. 1 commercial vaccine; and (4) W2V2 group, five pigs from farm #2 were vaccinated with the No. 2 commercial vaccine. Each pig received one dose of FMD vaccine at 12 weeks old and was challenged with $10^5 \text{TCID}_50$ O/TW/97 FMDV at 31 weeks old. All 15 vaccinated pigs showed typical vesicle lesions on the feet and snout at 4 days postinfection. Two pigs in the control group were administered phosphate buffered saline instead of the vaccine and were challenged as the vaccination groups.

Virus neutralization test

To confirm the infection of the experimentally infected pigs used to establish a positive serum panel, the collected sera were tested with the VNT, as previously described. A titer of 1/45 or more of the final serum dilution in the serum/virus mixture is interpreted as positive. A titer of less than 1/16 is interpreted as negative. Titters of 1/16–1/32 are considered doubtful.

Expression of recombinant FMD-SP and NSP in Escherichia coli

Recombinant FMDV-SP-VP1 and NSP-3ABC were produced by the expression of the cloned reverse transcription polymerase chain reaction (RT-PCR)-amplified VP1 fragment of the FMDV-O/TW/97 strain (GenBank accession no. NC_004004; nucleotides 3600–3731) and the 3ABC fragment of the FMDV-O/TW/2/99 strain (GenBank accession no. AJ539137; nucleotides 5595–6119) within Escherichia coli (E. coli). The generation of the NSP was published previously; the SP was generated in the same way. The forward primer for the FMDV-SP-VP1 contained a BamHI restriction site, and the reverse primer contained an EcoRI restriction site (Table 2). Extracted FMDV RNA was used as the template. The RT-PCR reaction mixture was prepared with the SuperScript One-Step RT-PCR System in combination with Platinum PfX DNA Polymerase (Invitrogen, Carlsbad, CA, USA). The reaction, which was performed in a GeneAmp PCR system 2400 thermocycler (Applied Biosystems, Foster City, CA, USA), was started by incubation at 42°C for 40 minutes, followed by predenaturation at 94°C for 30 seconds. The predenaturation was followed by 35 cycles of denaturation (at 94°C for 30 seconds), annealing (at 55°C for 30 seconds), and extension (at 68°C for 1 minute). A final extension at 72°C for 7 minutes was performed before the sample was held at 4°C. The RT-PCR

<table>
<thead>
<tr>
<th>Groups</th>
<th>Pig, no.</th>
<th>VNT (SN50)</th>
<th>3ABC blocking ELISA kit</th>
<th>xMAP (SP-VP1)</th>
<th>xMAP (NSP-3ABC)</th>
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<tr>
<td>W1V1</td>
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<td>–</td>
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<td>+</td>
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<tr>
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<tr>
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<td>–</td>
<td>+</td>
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<tr>
<td>W2V2</td>
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<td>+</td>
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<tr>
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<td>+</td>
<td>+</td>
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<tr>
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<td>+</td>
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</tbody>
</table>

**Table 1** Sera collected from four FMD-vaccinated groups and one control group were tested using the virus neutralization test, the newly developed multianalyte profiling assay, and the 3ABC blocking ELISA kit.

### Footnotes:

1. Fifteen pigs were assigned to four vaccination groups: (1) W1V1 group, four pigs from farm #1 were vaccinated with the No. 1 commercial vaccine; (2) W1V2 group, three pigs from farm #2 were vaccinated with the No. 2 commercial vaccine; (3) W2V1 group, three pigs from farm #2 were vaccinated with the No. 1 commercial vaccine; and (4) W2V2 group, five pigs from farm #2 were vaccinated with the No. 2 commercial vaccine. Each pig received one dose of FMD vaccine at 12 weeks old and was challenged with $10^5 \text{TCID}_50$ O/TW/97 FMDV at 31 weeks old. All 15 vaccinated pigs showed typical vesicle lesions on the feet and snout at 4 days postinfection. Two pigs in the control group were administered phosphate buffered saline instead of the vaccine and were challenged as the vaccination groups.

2. ELISA = enzyme-linked immunosorbent assay; FMD = foot-and-mouth disease; NSP = nonstructural protein; SN50 = serum neutralizing test titer; SP = structural protein; VNT = virus neutralization test; xMAP = multianalyte profiling.
product was stored at $-20^\circ$C until use, and 10 $\mu$L of the product was analyzed with 2% agarose gel electrophoresis. It was then visualized with ultraviolet transillumination.

To insert the RT-PCR product into the expression vector, the product in the agarose gel was extracted using a Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan), digested with the restriction endonuclease $\text{Bam}^\text{HI}$ and $\text{EcoRI}$, and then ligated to the pET 32a (+) vector (Novagen, EMD Biosciences Inc., San Diego, CA, USA), which was also digested with the restriction endonucleases $\text{Bam}^\text{HI}$ and $\text{EcoRI}$. Competent $\text{E. coli}$ cell BL21 (DE3; Novagen, EMD Biosciences Inc.) was transformed using the ligation product, as previously described. It was then plated onto Luria-Bertani agar supplemented with 100 mg/mL ampicillin. Positive clones were selected. The plasmids in each clone were extracted using a QIAprep Spin Miniprep Kit (Qiagen Inc., Valencia, CA, USA), 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 analyzer (Applied Biosystems, Foster City, CA, USA).

Transformed $\text{E. coli}$ with the desired FMDV-SP plasmid were cultivated in Luria-Bertani medium supplemented with 100 mg/mL ampicillin at $37^\circ$C with vigorous shaking. Isopropyl $\beta$-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1mM when the culture reached mid-logarithmic growth. The culture was then incubated for an additional 4 hours to induce the expression of the SP. After the 4-hour induction, the cells were harvested by centrifugation and then lysed with the B-PER II Bacterial Protein Extraction Reagent (Pierce, Rockford, IL, USA). The soluble SP polypeptide in the lysate was purified with a HisTrap HP affinity chromatography column (Amersham Biosciences, Little Chalfont, Buckinghamshire, England). The purified SP was analyzed with 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a discontinuous Tris-glycine buffer system. After being resolved by the SDS-PAGE, the proteins were electrically blotted onto a nitrocellulose filter (Sequi-Blot PVDF; Bio-Rad, Hercules, CA, USA) and examined for serological reactivity to the strong positive swine serum with Western blotting. The primary antibody used in Western blotting was 1:100 diluted positive swine serum, and the secondary antibody was 1:5000 diluted alkaline phosphatase-conjugated goat-anti-swine immunoglobulin G (IgG) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The substrates of alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Bio-Rad), were used for color development.

**Preparation of recombinant protein-coupled microspheres**

The expressed soluble SP-VP1 and NSP-3ABC polypeptide were covalently coupled to a unique carboxylate bead class (Luminex Corp; Bio-Rad). Beads account for 50% (v/v) of this reaction with the same bead ID always coupled to the

<table>
<thead>
<tr>
<th>Products</th>
<th>SP-VP1</th>
<th>NSP-3ABC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>5'-CGCGGATCC TGTCGTGTCTTAGCGACCGTCTACAAC-3'</td>
<td>5'-CGCGGATCC TGTCGCGAGACTCGCAAGAGACAGCAG-3'</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5'-GGGGATCG CGCGCAAGCACCGAAGTTGAAGGAGGTAGG-3'</td>
<td>5'-GGGGATCG GCACGTCTTCCCGTCGAGGATGAGCTC-3'</td>
</tr>
<tr>
<td>Size (b.p.; a.a.)</td>
<td>132; 44</td>
<td>525; 175</td>
</tr>
</tbody>
</table>

- **a** The GenBank accession number is NC_004004. The positions of the nucleotides are 3600–3731 within the FMDV-O/TW/97 strain.
- **b** The GenBank accession number is AJ539137. The positions of the nucleotides are 5595–6119 within the FMDV-O/TW/99 BOV strain.
- **c** Length of the polymerase chain reaction products and the expressed polypeptides are shown in base pairs (b.p.) and amino acid (a.a.), respectively.
same proteins: 3ABC to ID-a and VP1 to ID-b. The amine coupling assay (Bio-Plex; Bio-Rad) was conducted in accordance with the instruction manual.41

xMAP assays

A 96-well Multiscreen HTS 1.2 µm filter plate (EMD Millipore, Billerica, MA, USA) was prewetted with 100 µL/well of phosphate buffered saline (PBS), and the PBS was then aspirated using a vacuum manifold. A volume of 50 µL of the working microsphere suspension was added to the appropriate wells of the prewetted filter plate. Blocking buffer [1% (w/v) casein; Hammer-sten grade in 100mM sodium phosphate (150mM sodium chloride, pH 7.4, containing Kathon antimicrobial agent, Pierce, Rockford, IL, USA)] was added to a well as the blank (50 µL/well). In addition, 50 µL of sera from experimentally FMDV-infected pigs (i.e., the PC), SPF swine serum (i.e., the NC), SVDV antisera, and test serum samples were diluted 50-fold with blocking buffer and added to the appropriate wells as the controls and the test serum samples. The plate was incubated for 60 minutes at room temperature on a plate shaker. For detecting swine serum samples, 1.9 mg/mL biotin–SP-conjugated goat-anti-swine IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) and 0.5 mg/mL biotin-conjugated goat-anti-bovine IgG (KPL, Gaithersburg, MD, USA) used as the secondary antibodies were 1:2000 and 1:500 diluted with PBS at the optimal concentration, respectively. A volume of 50 µL of the diluted secondary antibody was added to the appropriate wells of the filter plate with the incubated controls and serum samples, after the previous 60 minutes of incubation. The filter plate was then covered with adhesive film and incubated for 60 minutes at room temperature on a plate shaker. R-phycoerythrin-conjugated streptavidin (0.5 mg/mL; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), used as the reporter reagent, was 1:2000 diluted with PBS at optimal concentration against reporter antibodies for swine. After the secondary antibody was added and incubated, a volume of 50 µL of the diluted reporter reagent was added to the appropriate wells of the filter plate. The filter plate was covered with an adhesive film and incubated for 30 minutes at room temperature on a plate shaker. After the 30-minute incubation, 50 µL of reaction in each well was analyzed with the Luminex analyzer Bio-Plex 200 System (Bio-Rad) in accordance with the operational manual instructions. The instrument was calibrated using CAL1/CAL2 calibration microspheres (Bio-Rad), according to the manufacturer’s directions. The CON1/CON2 controls were also run for confirmation. The amount of antibody bound to the microspheres was determined by using biotin-conjugated anti-swine IgG, which reacted with phycoerythrin-labeled streptavidin. When the microspheres were excited at a wavelength of 635 nm diode by the classification laser, the phycoerythrin emitted light at a wavelength of 532 nm YAG (yttrium aluminium garnet) by the reporter laser. The median fluorescence intensity (MFI) at 532 nm is directly proportional to the amount of antibody bound to the microsphere. Because the analyte specificity and position of each bead classification in the array are known, a single fluorescent reporter molecule could be used to measure the antibody levels for the protein-coupled microspheres. The MFI for 100 microspheres for each specific protein was recorded for each well. To normalize the results obtained from different tests, the results were expressed as the test/control (T/C) index.

Comparison of the xMAP to other assays

A commercially available blocking ELISA kit and VNT were used to compare their ability to detect antibodies to FMDV-NSP 3ABC and FMDV-SP VP1 with that of the developed multiplex Luminex assay. The commercially available ELISA kit was the PrioCHECK FMDV-NS kit (Prionics Lelysted B.V., Lelysted, the Netherlands), which is a 3ABC recombinant protein-based blocking ELISA used to detect antibodies against NSP-3ABC. The kit was used in accordance with the manufacturer’s instructions. The SN50 titer of FMDV antibody was measured by the VNT. Agreements between the assays were evaluated using statistics and linear regression analysis.

Results

Titration of neutralizing antibodies in experimentally infected pigs

After 4 dpi, all experimentally infected pigs demonstrated neutralizing antibody titers that ranged from 1:16 to 1:1024, which confirmed that infection had been established.

Expression of SP and NSP polypeptides in E. coli

The 132 bp and 525 bp DNA fragments corresponding to the VP1 and 3ABC polypeptides, respectively, were amplified by RT-PCR. Plasmids, FMDV-SP-VP1, and FMDV-NSP-3ABC were constructed by inserting each RT-PCR-amplified fragment into the prokaryotic expression vector pET 32a (+) (Fig. 1, Table 2). After the induction with IPTG, E. coli transformed with the FMDV-SP-VP1 and FMDV-NSP-3ABC plasmids, expressed soluble VP1 and 3ABC polypeptides with the

![Figure 1. Agarose gel electrophoresis of the reverse transcription-polymerase chain reaction (RT–PCR) yields the amplified structural protein VP1 and nonstructural protein 3ABC gene of the foot-and-mouth disease virus. Lane M is the molecular weight standard, which ranges from 100 bp to 1000 bp (Sigma, Saint Louis, MO, USA). Lanes 1–4 express VP1 (132 base pairs) and lanes 5–8 express 3ABC (525 base pairs). bp = base pair.](image-url)
expected molecular weights of 25 kDa and 40 kDa. This was verified using SDS-PAGE and Western blotting (Fig. 2).

Assessment of microsphere coupling efficiency

The two-fold serial dilution of the positive and negative control sera was tested against FMDV-NSP with the monoplex Luminex. A dilution of 1/40 to 1/80 was best in discriminating positive sera from negative sera and minimizing false-positive and false-negative results. For FMDV-NSP, the MFI values for "positive" (i.e., 1533-34 and 1536-34), "negative" (i.e., SPF and SS), and "blank" were 5046.3 (1533-34), 5527.8 (1536-34), 274.3 (SPF), 261.3 (SS), and 48.0 (blank), respectively. For FMDV-SP, the MFI values for "positive", "negative", and "blank" were 5610.9 (1533-34), 2814.4 (1536-34), 165.4 (SPF), 372.4 (SS), and 12 (blank) (Fig. 3). The analytical sensitivities of the developed Luminex assay were $10^{-3}$-fold for FMDV-SP and FMDV-NSP.

Determination of the cutoff value for the xMAP assay

To differentiate clearly infected pigs from uninfected pigs, as illustrated in Figs. 4 and 5, the cutoff values of the T/C ratio were 0.12 (MFI values equivalent to 388.1 as 400) for FMDV-SP and 0.18 (MFI values equivalent to 974.2 as 1000) for FMDV-NSP, which is equal to the mean of the MFIs plus three standard deviations of the NC sera. Based on the cutoff values, a sample was interpreted as FMD-SP antibody-positive when the T/C ratio was greater than 0.12. A sample was interpreted as FMD-NSP antibody-positive when the T/C ratio was greater than 0.18.

Comparison of the xMAP to the commercially available ELISA and VNT in detecting sequentially sampled sera

The panel of sequentially sampled swine sera was tested by the xMAP, by blocking ELISA, and by VNT. According to the Luminex assay, positive results were first administered at 4 dpi for the FMDV-SP antibody and at 8 dpi for the FMDV-NSP antibody; the mean standardized values of the assay reached a peak at 14 dpi and then decreased rapidly (Fig. 4). To compare the SP and NSP antibodies detected in the sera of the 14 dpi-infected samples, the xMAP had a high positive correlation with those from the VNT and the blocking ELISA. Results of quantitation detected the xMAP had a 0.33 of $R^2$ value to the results of the VNT (Fig. 6).

Diagnostic sensitivity and specificity to vaccinated pigs of the xMAP

Detecting the FMDV-SP antibody in the 78 experimentally vaccinated pigs with xMAP, the diagnostic sensitivity was 98.7% (77/78) and $R^2$ was 0.37. Detecting the NSP antibody in the 78 sera, the diagnostic specificity was 94.9% (74/78), and all T/C ratios were less than or equal to 0.18. The
results 94.9% agreed with those obtained from the 3ABC polypeptide blocking ELISA, which all tested negative (Fig. 7).

In detecting the SP antibody in the sera of the 30 field vaccinated pigs, the results of the xMAP 90.0% (27/30) agreed with the results from VNT, and $R^2$ was 0.40. The diagnostic sensitivity was 90.0% (27/30), of which three pigs were detected negative by xMAP. The results from SNT were detected positive in 30 vaccination pigs. In detecting the NSP antibody, the specificity results obtained with the developed assay 93.3% (28/30) agreed with the blocking ELISA, which tested all negative (Fig. 8).

### Diagnostic sensitivity and specificity of xMAP in experimental pigs

Table 3 indicates the diagnostic sensitivity and specificity of xMAP, based on tests of the sera obtained at 14 dpi from 30 experimentally infected pigs, 113 SPF pigs, 86 unvaccinated commercial pigs, 78 experimentally vaccinated commercial pigs, and 30 field-vaccinated pigs.

### Detection of SP and NSP antibodies in sera against the FMDV-O/TW/97 and the O/TW/99 strains

The performance of xMAP for detecting SP and NSP antibodies was examined against the FMDV-O/TW/97 strain or FMDV-O/TW/99 strain. All five FMDV-O/TW/99 sera demonstrated positive results, according to the xMAP. The ear tag numbers were 1155-1, 1155-2, 1170, 1171, and 1172 in animals that had high positive antibody reactions against SP and NSP of FMDV. The MFI values ranged 661.8, 5016.8, 2133.8, 4676.8, and 604.5, respectively, for FMDV-SP and ranged 9665, 5537.5, 5533, 4456.8, and 2250, respectively, for FMDV-NSP.
Analytical specificity of xMAP against SVDV antibody

The six antisera against SVDV UKG/27/72 strains obtained all negative results (i.e., MFI values were 400 or lower) in FMDV-SP and FMDV-NSP, based on the xMAP assay (data not shown).

Detection of VP1 SP and 3ABC NSP antibodies in bovine antisera

Diluted bovine antiserum against FMDV serotypes A, C, Asia1, SAT1, SAT2, and SAT3 gave positive results by the xMAP assay. Most antisera gave MFI values higher than 1000 to the FMDV-NSP antibody. The positive antibody reactions were high against NSP of the FMDV with MFI values ranging from 4898.6 to 11,666.8. In addition to the antibody to FMDV-SP, serotype O was detected; the antibodies of other serotypes were not measured (data not shown).

Detection of FMDV infection in vaccinated animals

All 15 vaccinated pigs that showed typical vesicle lesions on the feet and snout at 4 days postchallenge tested positive for FMDV-SP and FMDV-NSP, according to the xMAP. All sera samples had VNT antibody titers that ranged from 1:64 to 1:512 or greater, which confirmed infection. Nine of the 15 sera tested positive, according to the commercially available 3ABC blocking ELISA kit (Table 1).
Table 2), the optimal condition was established (Fig. 3), the soluble recombinant proteins for SP and NSP (Figs. 1 and 2; of FMDV. Through the detector studies performed using in the present study to detect antibodies against SP and NSP derived from vaccination or infection, xMAP was developed accuracy and sensitivity for detecting anti-FMDV antibodies in the xMAP platform simultaneously detects numerous analyts in a single assay. To develop a method with high accuracy and sensitivity for detecting anti-FMDV antibodies derived from vaccination or infection, xMAP was developed in the present study to detect antibodies against SP and NSP of FMDV. Through the detector studies performed using soluble recombinant proteins for SP and NSP (Figs. 1 and 2; Table 2), the optimal condition was established (Fig. 3), the early antibodies detection of kinetics were validated (Fig. 4), the ideal cutoff values of the assays were determined (Fig. 5), and the diagnosis specificity and diagnosis sensitivity were demonstrated (Figs. 4–8, Table 1 and 3).

The diagnostic sensitivity and specificity of xMAP were 90.0–98.7% and 93.0–99.1%, respectively (Table 3). The analytical sensitivities of the xMAP, commercial 3ABC blocking ELISA, and VNT were diluted 10^{-3}-fold, 10^{-1}-fold, and 10^{-3}-fold, respectively. It has been reported that the analytical sensitivity of a Luminex assay can be as low as 1 pg/mL or less with high-titer antibodies. The limit of sensitivity of the antibody pairs falls in the low picogram range. This limit derives from high-affinity antibodies (K_d 10^{-8}–10^{-10}) that, in combination with liquid-phase kinetics and the high binding capacity of three-dimensional microspheres, produce a robust multiplexed immunoassay. The full range of these assays extends more than 3–4 logs, compared to 1–2 logs for ELISA tests. The sensitivity that researchers obtained from the cytokine FlowMetrixE assay in uniplexed and multiplexed formats is 3 pg/mL and 10 pg/mL, respectively. The sensitivity obtained from the serum in the ELISA and xMAP formats was down to 50 pg/mL and 0.1 pg/mL, respectively. These studies support that xMAP is more accurate, sensitive, and reproducible, compared to the conventional microtiter ELISA procedure.

The developed xMAP demonstrated its ability to detect antibodies against NSP and SP of FMDV early. The xMAP detected an NSP-specific antibody from infected pigs as early as 8 dpi (Fig. 4), which is similar to the chromatographic strip, sandwich ELISA, and uniplexed Luminex. In addition, the earliest antibody detection to the FMDV-SP was at 4–6 dpi (Fig. 4).

To assess a response to vaccination and for epidemiological surveillance of vaccinated and unvaccinated animals, 78 pigs that had been vaccinated twice were tested with the xMAP, and four vaccinated animals showed reactions of greater than 1000 MFI values to FMDV-SP, in which the MFI values were higher to the threshold than 1455.5–3126.5. By contrast, the specificity response decreased to 94.9%. This can be because of the presence of low levels of NSP in impure vaccine or the nonspecific reactions of the assay (Fig. 7). Thirty vaccinated pigs that had received one shot of vaccination similarly all tested negative, based on the blocking ELISA, and 93.3% (28/30) of these pigs tested negative for FMDV-NSP, based on xMAP. According to the VNT, 100% (108/108) had SN50 titers more than 1:16, which is equal to 3.70% (4/108) of the vaccination pigs with titers less than the 400 MFI value, based on xMAP. There were no correlations between the VNT and xMAP for anti-VP1 antibodies in the four vaccinated pigs. These studies proved that the production of the FMDV-SP antibody had some variability in different detection methods, which were inconsistently close to the threshold borderline of negative sera instead of positive samples.

The performance of indirect ELISA had also been evidenced (data not shown). The positive antibody against FMDV-SP consistently showed a MFI-value higher than 400 in 108 vaccinated animals. Agreement between the results obtained from the xMAP and those obtained from the VNT was 96.3%; however, the inconsistency of 3.70% could be because of differences in the conformation of proteins generated by different expression systems (Figs. 7 and 8).
Multianalyte profiling was similar to the chromatographic strip in detecting vaccinated but infected pigs. In all 15 sera from vaccinated but unprotected pigs, the SP and NSP antibodies were detected by xMAP. All sera samples had VNT antibody titers ranging from 1:64 to 1:512 or greater, which confirmed infection. By contrast, NSP antibodies were detected in nine of the 15 sera by the 3ABC blocking ELISA kit (Table 1), which suggests the ELISA result of this study showed slight sensitivity. The results indicated that xMAP was more sensitive and specific, as confirmed by the clinical diagnosis, and can be applied to detect SP and NSP antibodies in vaccinated animals. In addition, the VNT titers of 512 or higher and the positive reaction of xMAP were obtained by testing the pig sera collected at 2 weeks postchallenge. From the study it may only be concluded that one FMD vaccination could not confer sufficient protection to the vaccinated pigs, but the immune status before the challenge was not determined.

The new application provided a safer, faster, more economical, and easily performed platform in detection. Applying recombinant proteins could replace the use of traditional inactivated virus for antibody detection to FMDV by xMAP. Without the manipulation of live FMDV, a high-containment laboratory facility is not needed, and the method may therefore be applied in more laboratories. Regarding the testing time, ELISA is very time-consuming: it normally takes 4–6 hours to complete, and each test can measure only one subject matter. The VNT is serotype-specific, requires cell cultures and biocontainment facilities, and takes 2–3 days to obtain results. Low titer false-positive reactions are also observed in a small proportion of the sera; the proportion can be higher in animals under stress or with occasional disease and fever. In addition, the multiplexed assay is rapid and beneficial to automation, and a rough cost evaluation of reagents and supplies is approximately US $0.50 per assay, which is cheaper than ELISA. The xMAP takes only 1–2 hours using a single quantitative detection of multiple pathogens simultaneously with the subject matter of the antibody.

The xMAP method of the beads can be combined with efficient detection of FMDV SP and NSP antibodies. A previous publication showed that uniplexed Luminex and multiplexed Luminex observed values of the respective MFI multiplexed analysis found that some samples had slight diminished on the signal of reporter, but some samples were displayed in multiplexed analysis with uniplexed reaction as high signal performance in this study. These results are similar to those by Clavijo et al. In addition, the purpose of the assay was to detect anti-SP and NSP antibodies to identify previously immunized and infected animals in vaccination populations. As discovered through our experiments, the xMAP demonstrated satisfactory specificity, it was higher than 90%. The method distinguished antibodies produced by naturally infected animals from antibodies produced by vaccinated animals. Multianalyte profiling can specifically detect antibodies to SP and NSP of FMDV in swine sera. To evaluate the ability of xMAP to detect anti-NSP antibody elicited by FMDVs of other serotypes, bovine sera against serotypes other than serotype O were also tested. The results suggested that our xMAP assay was effective in detecting the NSP antibodies against other FMD serotypes; the results are similar to uniplexed Luminex. However, the anti-SP antibody is reversed, it can detect antibody against FMDV serotype O only (data not shown). Furthermore, with antisera against SVDV, the assay proved that it could specifically recognize antibodies against FMDV, but not cross-react with antibodies against SVDV, which indicates that the assay can be used in areas where the two diseases may be present.

In conclusion, the developed xMAP provided a sensitive, reliable, and effective platform for detecting SP and NSP of the FMDV. The method can be used for FMD diagnosis and surveillance, whether vaccination has been implemented. Therefore, xMAP has the potential to be used as the assay in vaccine development and assessing vaccine purity in future studies.

### Table 3  Sensitivity and specificity of xMAP for detecting antibodies against structural protein (FMDV-SP) and nonstructural protein (FMDV-NSP) of foot-and-mouth disease virus

<table>
<thead>
<tr>
<th></th>
<th>Naive Pre outbreak</th>
<th>SPF animals</th>
<th>Vaccination Experimentally infected</th>
<th>Field</th>
<th>Experimentally infected b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NSP SP</td>
<td>NSP SP</td>
<td>NSP SP</td>
<td>NSP SP</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>1 6</td>
<td>1 4</td>
<td>4 77</td>
<td>2 27</td>
<td>27 29</td>
</tr>
<tr>
<td>Negative</td>
<td>85 80</td>
<td>112 109</td>
<td>74 1</td>
<td>30 30</td>
<td>90.0 90.0</td>
</tr>
<tr>
<td>Total no.</td>
<td>86 86</td>
<td>113 113</td>
<td>78 98.7</td>
<td>90.0 90.0</td>
<td></td>
</tr>
<tr>
<td>Sensitivity (%) a</td>
<td>98.8 93.0</td>
<td>99.1 96.5</td>
<td>94.9</td>
<td>93.3</td>
<td></td>
</tr>
<tr>
<td>Specificity (%) a</td>
<td>100 92.3</td>
<td>100 94.2</td>
<td>100 92.5—97.0</td>
<td>92.5—100</td>
<td></td>
</tr>
</tbody>
</table>

95% CI = confidence interval; FMDV = foot-and-mouth disease virus; NSP = nonstructural protein; SP = structural protein; SPF = specific-pathogen-free; xMAP = multianalyte profiling.

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a Based on the cutoff value, a sample is interpreted as "negative" when the test to control (T/C) ratio is 0.12 or less and as "positive" when the T/C ratio is greater than 0.18 for FMDV-NSP. A sample is interpreted as "negative" when the T/C ratio is 0.18 or less and as "positive" when the T/C ratio is greater than 0.12 for FMDV-SP. The sensitivity is calculated with xMAP at the 14th day postinfection from 32 infected pig sera.

b The sensitivity is calculated with xMAP at the 14th day postinfection from 32 infected pig sera.
Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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References

26. Shen F, Chen PD, Walfield AM. Differentiation of convalescent animals from those vaccinated against foot and mouth disease by a peptide ELISA. Vaccine 1999;17:3039–49.


