Research paper

Development of a Luminex assay for the detection of swine antibodies to non-structural proteins of foot-and-mouth disease virus

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ABSTRACT

Foot-and mouth disease (FMD), swine vesicular disease (SVD), and vesicular stomatitis (VS) are highly contagious vesicular diseases of swine but are not easy to differentiate clinically. For the purpose of instant detecting of FMD and differentiating it from the other vesicular diseases, a Luminex assay was developed. Sera from 64 infected, 307 vaccinated, and 280 naïve pigs were tested by the Luminex assay. Diagnostic sensitivity of the assay was 100%. Diagnostic specificity of the assay was 98.7% in vaccinated pigs and 97.5% to 100% in naïve pigs. Agreement between the results from the Luminex assay and those from a 3ABC polypeptide blocking ELISA was 96.3% with kappa statistics of 0.92. The Luminex assay can detect the immune response to NSP-3ABC in swine as early as eight days post-infection. Moreover, all of the 15 vaccinated but unprotected pigs were all detected by the Luminex assay. The results indicated that the Luminex assay has potential with specificity in detecting antibodies to FMDV 3ABC NSP and in distinguishing FMDV-infected pigs from with either SVDV or VSV.

1. Introduction

Foot-and-mouth disease (FMD), swine vesicular disease (SVD), and vesicular stomatitis (VS) are serious vesicular diseases that have devastated swine populations throughout the world. FMD, caused by FMD virus (FMDV; genus *Aphthovirus*, family *Picornaviridae*) (Clavijo et al., 2004a; Perkins et al., 2007a, 2007b; OIE, 2012; Oem et al., 2009), is one of the most contagious diseases in cloven-hoofed animals. Species susceptible to FMDV include swine, sheep, goats, and many wild ruminants. In 1997, a devastating FMD outbreak caused by O/TW/97 strain occurred in Taiwan and resulted in severe economic losses. The FMDV strain shows a porcinophilic phenotype with a deleted non-structural protein 3A gene (Yang et al., 1999; Beard and Mason, 2000). Another strain O/TW/99, having a full-length 3A coding region, was isolated from subclinically infected cattle in 1999 (Huang et al., 2002). SVD is caused by SVD virus (SVDV; genus *Enterovirus*, family *Picornaviridae*) (OIE, 2008). VS, affecting horses, cattle, and pigs, is caused by VS virus (VSV; genus *Vesiculovirus*, family *Rhabdoviridae*) (OIE, 2010). These diseases are clinically indistinguishable from FMD. To diagnose them correctly, advanced laboratory techniques are therefore required to detect and differentiate antibodies to these different viruses.

When susceptible host animals are infected with FMDV, antibodies against both viral structural proteins (SPs) and non-

structural proteins (NSPs) are elicited. In contrast, animals administered with inactivated FMD vaccines that contain no or only trace amounts of NSPs are unlikely to induce NSP antibodies. Therefore, for FMD diagnosis, the NSP antibodies can be used as a marker to differentiate infected from vaccinated animals. Detecting NSP antibodies has an additional advantage that they are serotype independent, because the NSPs are predominantly conserved between serotypes of FMDV (Clavijo et al., 2004b). Currently, many methods for detecting NSP antibodies have been used, including agar gel immunodiffusion (O'Donnell et al., 1997), latex bead agglutination test (Sugimura et al., 2000), enzyme-linked immunoelectrotransfer blot (Bergmann et al., 1993, 2000; Bronsvoort et al., 2006), enzyme-linked immunosorbent assay (ELISA) (De Diego et al., 1997; O'Donnell et al., 1997; Sørensen et al., 1998, 2005; Shen et al., 1999; Chen et al., 2011), and chromatographic strip assay (Chen et al., 2009).

The first use of flow cytometry for analysis of microsphere immunoassay was published in 1997 (Fulton et al., 1997). More recently, two types of high-level multi-analyte profiling (xMAP) and high-throughput diagnostic methods using microspot arrays and bead arrays have been successfully developed to complement single-analyte assays. Antibodies and antigens are directly or indirectly coupled to fluorescent microspheres composed of a variety of materials, such as latex, polystyrene, polyacrylamide, and glass. Luminex liquid array technology is one of the detection systems, and it employs microspheres with carboxyl, amine/hydrazide, and maleimide groups on their surfaces to which the molecules such as antibodies, proteins, oligonucleotides, polysaccharides, lipids, or small peptides can bind the microspheres and be subsequently measured by a fluorochrome-conjugated detection molecule. The platform enables simultaneous detection of numerous analytes in a single assay, and there are now many examples of the creative use of this technology (Kellar and Iannone, 2002; Clavijo et al., 2006; Perkins et al., 2006).

In the present study, a new application of the Luminex liquid array technology was developed, and to address the limitations of existing antibody detection methods of sensitivity and specificity to FMDV, a single-signature Luminex assay was developed to detect and differentiate antibodies against SVDV and VSV. These tests were also compared with other methods of the same purpose.

2. Materials and methods

2.1. Serum samples

For studying the diagnostic sensitivity of the tests, FMD-positive serum samples were produced by experimental infection in pigs. Sixty-four 8-week-old SPF pigs comprised two groups of 32 pigs. Ear tag numbers in group 1 were 601–632. Ear tag numbers in group 2 were 1513–1528, 1530–1533, 1536–1546, and 1548. They were intradermally injected with 10^5 $TCID_{50}$ (in a volume of 500 μL) of FMDV O/TW/97 strain into the heel bulb of their right foot. Blood samples were collected in group 1 and group 2 at 28 and 34 days post infection (dpi), respectively. A panel of sequentially sampled from group 2 of 32 pigs was experimentally tested during 0 to 34 dpi. These swine sera were used to evaluate sensitivity and specificity of the Luminex assay.

For studying the diagnostic specificity, 280 sera from naïve pigs and 307 sera from vaccinated pigs were used. The 280 naïve pig sera comprised 120 sera collected from SPF pigs and 160 sera collected from commercial pigs before the first FMD outbreak in Taiwan in 1997. The 307 sera from vaccinated pigs were collected from non-infected commercial pigs that had been vaccinated twice with commercial FMD O/TW/97 vaccine.

For evaluating possible serological cross-reactivity between FMDV and SVDV/VSD in Luminex assay, six swine antisera (RS1, RS2, RS3, RS4, RS5, and RS6) against SVDV UKG/27/72 strain (EU SVD reference serum batch 2002) and six bovine antisera against FMDV serotypes A, C, Asia 1, SAT 1, SAT 2, and SAT3 were purchased from the Institute for Animal Health, Pirbright, United Kingdom. Two antisera against the New Jersey and Indiana strains of VSV and one VSV-negative serum were purchased from the National Veterinary Services Laboratories, United States of America.

For comparing the detection ability of the Luminex assay with other methods, a serum panel of group 2 containing 320 swine sera collected sequentially from 32 eight-week-old SPF pigs was experimentally infected with FMDV O/TW/97 strain as aforementioned. The sera in the panel were sampled at 0, 2, 4, 6, 8, 10, 14, 21, 28, and 34 dpi.

One (ear tag numbers in group 2 was 1536) and five (ear tag numbers in group 3 were 1155-1, 1155-2, 1170, 1171, 1172) 8-week-old SPF pigs, were intradermally injected with 10^5 $TCID_{50}$ (in a volume of 500 μL) of FMDV O/TW/97 and O/TW/99 strains into the heel bulb of their right foot. Blood samples were collected finally at 34 and 28 dpi, respectively. The No.1536 serum sample was used as the inter-assay repeatability and the analytical sensitivity of positive control (PC), and the five O/TW/99 sera were used as serotypes in identifying the developed Luminex assay. The analytical sensitivity was tested through from $10\times$ serial dilution of 10^{-1}–10^{-8} to No.1536 antiserum of positive control (PC). Neutralizing antibody titers of the positive control sera ranged from 1:256 to 1:512. One out of 120 SPF swine serum was tested negative by western blotting and ELISA was used as the negative control (NC) of inter-assay of the Luminex assay.

2.2. Virus neutralization test (VNT)

To confirm the infection of the experimentally infected pigs employed for establishing a positive serum panel, the sera collected were tested by the VNT as previously described (OIE, 2012; Chen et al., 2009).

2.3. Preparation of 3ABC-coupled microspheres

Production and purification of recombinant NSP 3ABC were described previously (Chen et al., 2009, 2011). Briefly, the recombinant NSP 3ABC was produced by expression of cloned PCR-amplified 3ABC fragment of FMDV O/TW/2/99 strain (GeneBank accession no. AJ539137; nucleotides 5595 to 6119) within *E. coli*. The expressed soluble 3ABC polypeptide was covalently coupled to a unique carboxylate bead class (Luminex Corp., BIO-RAD, Hercules, California, U.S.A.). The amine coupling assay was conducted according to the instruction manual (Bio-Plex™, BIO-RAD).

For a one-fold scale coupling reaction, 100 μL of mono-disperse carboxylated polystyrene microspheres (1.25×10^6 beads) (Bio-Plex) was transferred to one of the coupling reaction tubes. The beads were centrifuged at 14,000 ×g for 4 min, removed and discarded the supernatant. The microspheres were resuspended in 100 μL of bead wash buffer by vortexing and sonicating for 10 s. The microspheres were then centrifuged at 14,000 ×g for 4 min to remove the supernatant and were resuspended in 80 μL of bead activation buffer. The beads were vortexed and sonicated by bath sonication for 30 s. Freshly prepared 10 μL of a bead activation buffer solution of sulfo-N-hydroxysuccinimide (S-NHS; 50 mg/mL) (Pierce Biotechnology, Thermo Fisher Inc., Rockford, Illinois, U.S.A.) and N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC; 50 mg/mL) (Pierce Biotechnology) were added to each tube. The tubes were vortexed for 30 s. and gently agitated at room temperature in darkness for 20 min. After the agitation, they were centrifuged at 14,000 ×g for 4 min to remove the supernatant and washed with 100 μL of PBS (pH 7.4) once, repeating this step. Following the washing step, the microspheres were centrifuged at 14,000 ×g for 4 min to remove the supernatant, resuspended in a concentration of 5–12 μg/500 μL recombinant 3ABC protein solution by vortexing for 30 s, and gently agitated at 4 °C overnight in darkness, allowing the 3ABC protein molecules to couple to the microspheres. The coupled beads were centrifuged at 14,000 ×g for 4 min to remove the supernatant. And then the beads were washed with 500 μL of PBS (pH 7.4), centrifuged at 14,000 ×g for 4 min, and discarded the supernatant. The coupled beads were resuspended with 250 μL of blocking buffer by pipetting, and gently agitated at room temperature in darkness for 30 min. Then, the beads were centrifuged at 14,000 ×g for 4 min and discarded the supernatant. The 3ABC-coupled microspheres were washed with 500 μL PBS (pH7.4) and centrifuged at 16,000 ×g for 6 min, and then the supernatant was discarded. The 3ABC-coupled microspheres were resuspended with 150 μL of PBS (pH7.4) for formulation to make a coupled microsphere stock and stored at 4 °C in darkness. To prepare a working microsphere suspension, the coupled microsphere stock was diluted to a final concentration of 100 microspheres of each set in PBS, and 50 μL of working microsphere suspension was required for each reaction.

2.4. Single-signature Luminex assays

A 96-well MultiScreen® HTS 1.2 μm filter plate (EMD Millipore, Billerica, Massachusetts, U.S.A.) was prewetted by 100 μL/well of PBS and aspirated by vacuum manifold. A volume of 50 μL of the working microsphere suspension was added to the appropriate wells of the wetted filter plate. Blocking buffer [1% (w/v) casein; Hammer-sten grade] in 100 mM sodium phosphate (150 mM NaCl, pH 7.4, containing Kathon® antimicrobial agent) (50 μL/well) was added to a well as the blank. In addition, 50 μL of sera from experimentally FMDV-infected pigs (PC), SPF swine serum (NC), SVDV antisera and test serum samples were 50-fold diluted with blocking buffer and added to the appropriate wells as controls and detected test serum samples. The plate was incubated for 60 min at room temperature on a plate shaker.

For detecting swine and bovine serum samples, biotin-SP-conjugated goat-anti-swine IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, U.S.A.; 1.9 mg/mL) and biotin-conjugated goat-anti-bovine IgG (KPL, Gaithersburg, MD, U.S.A.; 0.5 mg/mL) as secondary antibodies, were a 1:3000 and 1:500–1:1000 diluted with PBS at optimal concentration, respectively. A volume of 50 μL of the diluted secondary antibody was added to the wells of the filter plate with incubated controls and serum samples, after the previous 60-minute incubation. The filter plate was then covered with adhesive film and incubated for 60 min at room temperature on a plate shaker.

R-phycoerythrin conjugated streptavidin (Jackson Immuno-Research Laboratories, U.S.A.; 0.5 mg/mL), as a reporter reagent, were a 1:1500 diluted with PBS at optimal concentration against reporter antibodies for swine and bovine, respectively. A volume of 50 μL of the diluted reporter reagent was added to the appropriate wells of the filter plate, after the addition of secondary antibody and incubation. The filter plate was covered with adhesive film and incubated for 30 min at room temperature on a plate shaker.

After the 30-minute incubation, 50 μL of reaction in each well was analyzed by Luminex analyzer Bio-Plex™ 200 System (BIO-RAD) according to the operation manual. The median fluorescence intensity (MFI) for 100 microspheres for each specific protein was recorded for each well. To normalize the results obtained from different tests, results were expressed as a test/control (T/C) index.

2.5. Tests for comparison

Three commercially available ELISA kits, one in-house ELISA, and one chromatographic strip assay were employed to compare their detection performance with that of the developed Luminex assay. The three commercially available ELISA kits were PrioCHECK FMDV-NS kit (Prionics Lelystad B.V., Lelystad, The Netherlands), which is a 3ABC recombinant protein-based blocking ELISA; UBI FMD NS EIA (United Biomedical Inc., Hauppauge, New York, U.S.A.), which is a 3B synthetic peptide-based indirect ELISA; and IDEXX FMDV 3ABC Ab Test (IDEXX Laboratories Inc., Westbrook, Maine, U.S.A.), which is a 3ABC recombinant protein-based indirect ELISA. The kits were conducted according to the manufacturers' instructions.

In-house sandwich ELISA for the O serotype was established to detect serum antibodies against 3ABC NSP (Chen et al., 2011). Shortly, 96-well microtiter plates were coated with 3ABC NSP specific mAb and incubated. The 3ABC recombinant protein was then added and incubated. Diluted test pig serum was added and incubated. Diluted horseradish peroxidase-labeled goat anti-swine IgG was added and incubated. The substrate, TMB solution, was added and incubated. Finally, stop solution, sulfuric acid, was added to stop the color development. Each incubation step was followed by washing. Results were expressed as a test to control (T/C) index.

In addition, in-house the 3ABC-based chromatographic strip assay (Chen et al., 2009) is described previously. The serum sample and dilution buffer were mixed, and the mixture was applied to the sample pad of the chromatographic strip. After incubation at room temperature, the strip was read by a UNISCAN™ point-of-care testing (POCT) Scanner. 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 s. To standardize the results among different batches of strips, each value read by the scanner was divided by a modified factor supplied with the batch to be a standardized value.

Agreement between Luminex assay and ELISA kits was evaluated using kappa statistics.

2.6. Detection of vaccinated but infected pigs by Luminex assay

Fifteen sera were collected from pigs that received one dose of commercial available vaccine serotype O/TW/97 FMD vaccine (No.1 vaccine, V1: FGBI "ARRIAH"/Russia; No.2 vaccine, V2: Biogenesis-Bago S.A./ Argentina) at 12 weeks old and were intramuscularly injected with 10^5 TCID$_{50}$ FMDV at 31 weeks old. The 15 pigs, from two farms, were assigned into four vaccinated groups. As shown in Table 3, the digit following W indicates the farm number, and the digit following V indicates the commercial vaccine administered. Additionally, two pigs in the control group, given PBS instead of vaccine, were infected as the vaccinated groups.

3. Results

3.1. Titration of neutralizing antibodies in experimentally infected pigs

All experimentally infected pigs demonstrated neutralizing antibody titers ranging from 1:16 to 1:1024 after 4 dpi, confirming the establishment of infection (Table 1).

3.2. Assessment of microsphere coupling efficiency

The two-fold serial dilution of positive and negative control sera against FMDV-NSP by the single-signature Luminex assay is depicted in Fig. 1. A dilution of 1/40–1/80 was the best in discriminating positive from negative sera as well as minimizing false positive and false negative results. The detection limit for the PC serum (MFI > 1000) was 1/640. By double duplex test,

Table 1
Frequency distribution of the antibody titers of 32 experimentally infected pigs by virus neutralization test.

Ab titer (SN$_{50}$)	Days of post infection (dpi)									
	0	2	4	6	8	10	14	21	28	34
≦1:3	30	31	13	1						
1:4	1		12	3	2	1				
1:8	1	1	6	6	4	3				
1:16			1	5	12	16	3	1		
1:32				13	7	7	9	4		1
1:64				4	6	5	12	4	2	2
1:128							7	9	7	6
1:256					1		1	9	12	13
1:512								4	8	7
1:1024								1	3	3

The result was indicated as the virus neutralization test antibody titers over 1:16 to 1:1024 after 14 dpi and experimentally pigs were exposed in clinical syndrome.
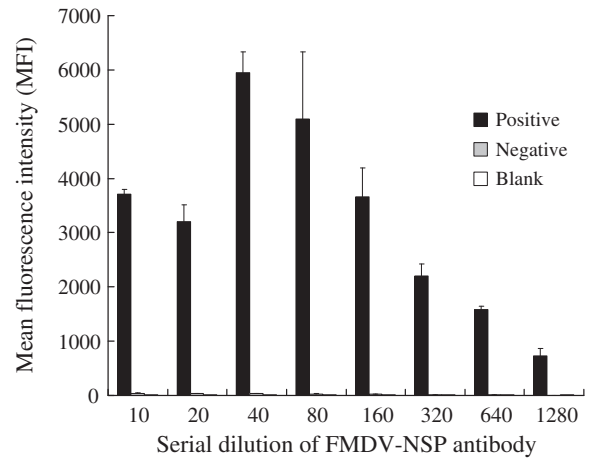

Fig. 1. Two-folded serial dilution of positive (black), negative (gray), and blank (empty) control sera were tested against foot-and-mouth disease virus non-structural protein (FMDV-NSP) by the single-signature Luminex assay.

the MFI values of positive, negative, and blank were 1577.4 ± 71.42, 8.4 ± 3.54, and 8.5 ± 2.12, respectively (Fig. 1). The analytical sensitivities of the developed Luminex assay were 10^{-3} fold (data not shown).

3.3. Determination of cutoff value for the Luminex assay

To differentiate infected from noninfected pigs clearly, the 64 PC serum samples that from group 1 and group 2 were used in this experiment taken at 28 and 14 dpi, and 120 NC sera were as illustrated in Fig. 2, the cutoff value was determined to be T/C ratio as 0.07 (equivalent to 916 MFI value), which equals the mean of median fluorescence intensities (MFIs) of the NC
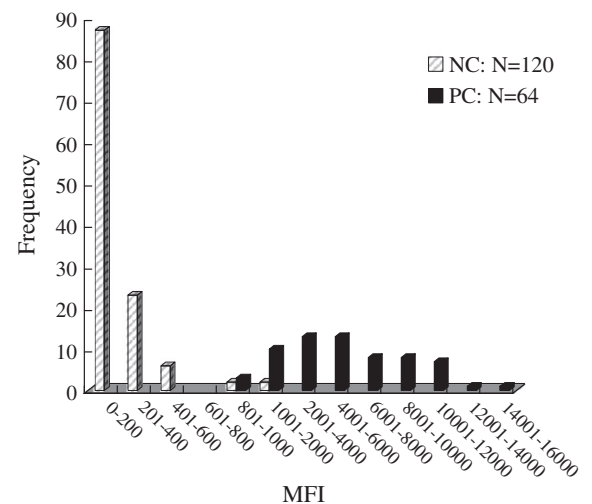

Fig. 2. Sixty-four positive control (PC) sera (group 1 and group 2) and 120 negative control (NC) sera were tested against foot-and-mouth disease virus non-structural protein (FMDV-NSP) by the single-signature Luminex assay to determine its cutoff value.

sera plus three standard deviations (SD) of the NC sera. Based on the cutoff, a sample was interpreted as negative when T/C ratio ≦ 0.07, and positive when T/C ratio > 0.07.

3.4. Inter-assay repeatability

Inter-assay repeatability of the Luminex assay was evaluated for the high and low controls that are run in duplicate on 17 different plates to monitor plate-to-plate variation. The group 2 of the FMDV O/TW/97 serum panel of which the ear tag number 1536 was sampled at 34 dpi was used as a positive control (PC) for control of standardized. One out of 120 SPF swine serum was tested and used as a negative control (NC) of the Luminex assay. The mean of MFI values of PC was 12193.09 ± 966.83 (coefficient of variance, or CV = 7.93) and the mean of MFI values of NC was 196.58 ± 50.45 (CV = 25.66).
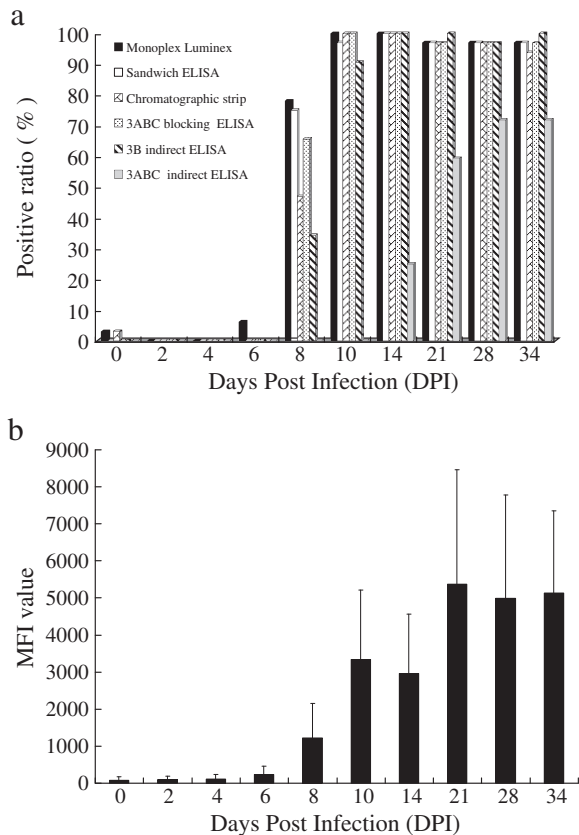
3.5. Comparison of the Luminex assay with the commercially available ELISA and in-house methods in detecting serially sampled sera

A set of serum panel of group 2 was tested from sequentially sampled 320 sera during the course 0 to 34 dpi for 32 experimentally infected pigs by the Luminex assay and the other methods. Positive results were given first at 8 dpi, and all pigs were tested positive by 10 dpi. From 10 dpi, positive percentage stayed over 90% throughout the period of the animal experiment by the Luminex assay. The 3ABC blocking ELISA kit, 3B indirect ELISA kit, in-house sandwich ELISA, and the chromatographic assay gave similar results (Fig. 3a). The agreement between the results of our Luminex assay and 3ABC blocking ELISA kit was 0.96 with kappa statistics of 0.92 (95% confidence interval: lower limit: 0.88, upper limit: 0.97). The agreement between the results of our assay and those of 3B indirect ELISA kit was 0.93 with kappa statistics of 0.87 (95% confidence interval: lower limit: 0.81, upper limit: 0.92). The agreement between the results of our assay and those of the 3ABC indirect ELISA kit was 0.65 with kappa statistics of 0.35 (95% confidence interval: lower limit: 0.28, upper limit: 0.42).

3.6. Diagnostic specificity of the Luminex assay in vaccinated pigs

Testing 307 vaccinated pigs by the Luminex assay, 303 out of 307 sera (98.7%) tested negative (T/C ratio ≦ 0.07), and all the MFI values were lower than 1000 (Fig. 4). All serum samples were tested negative by the 3ABC blocking ELISA kit.

3.7. Diagnostic sensitivity and specificity of the Luminex assay

With 64 sera evaluated from group 2 sampled at 14th dpi of 32 experimentally infected pigs and from group 1 sampled at 28th dpi of 32 infection pigs, 120 SPF pigs, 160 non-vaccinated commercial pigs, and 307 vaccinated commercial pigs, the

Fig. 3. (a) Kinetics of positive ratios of infected pigs detected by different non-structural protein-based immunoassays. The 32 pigs were experimentally infected with foot-and-mouth disease virus serotype O and serially sampled. The results obtained from the developer Luminex assay were compared to those from commercial kits, comprising two in-house assays (sandwich ELISA and chromatography strip) and three commercially available kits (3ABC blocking ELISA, 3B indirect ELISA, and 3ABC indirect ELISA). (b) The solid column indicates the standardized values measured by the developed assay with the MFI value. The error bar indicates the standard error of the mean at each day postinfection.
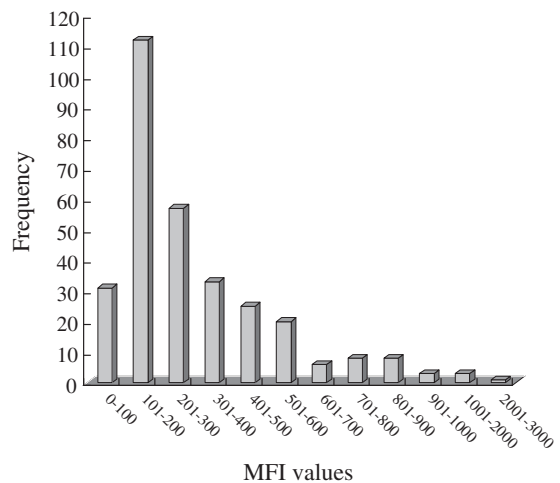

Fig. 4. Evaluation of the diagnostic specificity of the Luminex assay. The 307 serum samples from pigs vaccinated twice with commercial FMD serotype O/TW/97 vaccine were tested by the assay, and the frequency distribution of the obtained median fluorescence intensities (MFIs) values was illustrated.

Table 2

Sensitivity and specificity of the Luminex assay, in-house methods, and the commercially available ELISA kits for the detection of the FMDV infections in swine.

Tests	Sensitivity	Specificity		
	Infected[a]	SPF	Pre-outbreak	Vaccinated
Luminex	100% (64/64)	97.5% (117/120)	100% (160/160)	98.7% (303/307)
Sandwich ELISA[b]	98.4% (61/62)	100% (96/96)	100% (159/159)	100% (165/165)
Chromatographic strip[b]	96.8% (60/62)	100% (96/96)	100% (158/158)	98.8% (165/167)
3ABC blocking ELISA kit	98.4% (61/62)	100% (96/96)	100% (158/158)	100% (167/167)
3B peptide indirect ELISA kit	98.4% (61/62)	100% (96/96)	100% (158/158)	85.3% (93/109)
E. coli-expressed 3ABC polyprotein indirect ELISA kit	35.5% (22/62)	100% (96/96)	100% (158/158)	100% (167/167)

[a] The sensitivity was calculated to the 14th (dpi) from 32 infected serum panels of group 2 and the 28th (dpi) from 32 infection pig sera of group 1 by Luminex assay.

[b] Two other in-house methods developed in this laboratory.

diagnostic sensitivity and specificity of the Luminex assay are shown in Table 2.

3.8. Detection of 3ABC NSP antibody in bovine antisera

Diluted bovine antisera against FMDV serotypes A, C, Asia 1, SAT1, SAT2, and SAT3 gave positive results by the Luminex assay. Most antisera gave MFI values higher than 10,000 except that against the 1000× dilution of secondary antibody to antiserum SAT1, and the dilutions of 500× and 1000× of secondary antibody against the SAT2 give the MFI below 10,000 (Fig. 5).

3.9. Detection of 3ABC NSP antibody in sera against FMDV O/TW/97 and FMDV O/TW/99 strains

The performance of the Luminex assay in detecting specific NSP antibody against either the one of the 32 infected pigs for
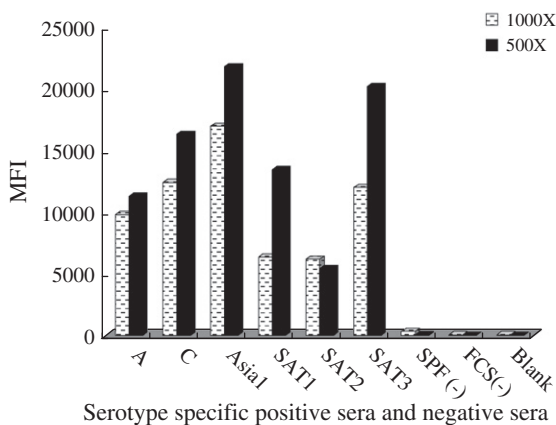

Fig. 5. Detection of non-structural protein antibodies in bovine infected with foot-and mouth disease virus serotypes other than serotype O by the Luminex assay. The biotin-conjugated anti-bovine IgG used as secondary antibody in the assay was diluted 500 fold and 1000 fold, respectively. SPF, specific-pathogen-free pig serum; FCS, commercially available fetal calf serum.

group 2 serum panel to FMDV O/TW/97 in 34 dpi or the five infected pigs for group 3 sera to FMDV O/TW/99 in 28 dpi were compared with those of the 3ABC blocking ELISA kit. It is noted that two of the five FMDV-O/TW/99 sera demonstrate positive results by the Luminex assay but not by the blocking ELISA kit (Table 3).

3.10. Analytical specificity of the Luminex assay

The six antisera against SVDV UKG/27/72 strains obtained negative results by the Luminex assay (data not shown). The one (RS5) of the six antiserum was evaluated to positive control of SVDV. Two antisera against the New Jersey and Indiana strains of VSV also tested negative by the Luminex assay (Table 3).

3.11. Detection of FMDV infection in vaccinated animals

All of the 15 vaccinated pigs that showed typical vesicle lesions on the feet and snout at four days post challenge tested positive by the Luminex assay. All serum samples had VNT antibody titers ranging from 1:64 to ≧1:512, confirming infection. The Luminex assay had a highly positive correlation with those generated by chromatographic strips and ELISAs. However, 11 out of the 15 sera were tested positive by the in-house sandwich ELISA. In contrast, only 9 of the 15 sera were positive by the commercially available 3ABC blocking ELISA kit (Table 4).

4. Discussion

A microsphere immunoassay was developed and validated to detect antibodies to 3ABC NSP of FMDV in the present study. The FMDV-3ABC antibody is a reliable indicator of FMDV infection for sero-epidemiological studies. Luminex offers advantages over this test such as sensitivity, specificity,

Table 3

Detection of NSP antibody in antisera against FMDV O/TW/97 strain, FMDV O/TW/99 strain, SVDV strain, two VSV strains by the developed Luminex assay, and the 3ABC blocking ELISA kit.

Antisera and sera	Luminex—MFI values[a]	3ABC blocking ELISA kit—PI values (%)[b]
FMDV—O/TW/97 (group 2)		
1536-34	10,866.4	92.1
FMDV—O/TW/99 (group 3)		
1155-1	10,647.4	54.8
1155-2	2242.9	42.9
1170	1766.9	41
1171	5706.4	58.2
1172	3827.9	69.5
SPF[c] pig	32.4	8.4
SVDV (RS5)	157.9	11.6
VSV—Indiana	7.5	−1.3
VSV—New Jersey	5.5	4.3
Blank	5	−1.6

[a] MFI: median fluorescent intensity. Based on the cutoff, a sample was interpreted as negative when T/C ratio ≦ 0.07 and positive when T/C ratio > 0.07, the means of the MFI values are equal to or higher than 1000 MFI as positive.

[b] PI: percentage inhibition obtained from PrioCHECK FMDV-NS kit. A sample is interpreted as positive when giving a PI value equal to or higher than 50%.

[c] SPF: specific-pathogen-free.

Table 4

Sera collected from four FMD vaccinated groups and one control group were tested by virus neutralization test (VNT), a newly developed Luminex assay, 3ABC blocking ELISA kit, sandwich ELISA, and the chromatographic strip method.

Groups[a]	Pigs of ear tag number	VNT (SN_{50})	Luminex	3ABC blocking ELISA kit	Sandwich ELISA	Chromatographic strip
W1V1	2654	≧1:512	+	−	−	+
	2659	≧1:512	+	−	−	+
	2661	≧1:512	+	+	+	+
	2662	≧1:512	+	−	−	+
W1V2	2668	1:256	+	+	+	+
	2670	≧1:512	+	−	−	+
	2671	≧1:512	+	−	+	+
W2V1	2679	≧1:512	+	+	+	+
	2680	≧1:512	+	−	+	+
	2682	≧1:512	+	+	+	+
W2V2	2688	≧1:512	+	+	+	+
	2689	≧1:512	+	+	+	+
	2690	≧1:512	+	+	+	+
	2691	≧1:512	+	+	+	+
	2692	≧1:512	+	+	+	+
Controls	2749	1:64	+	+	−	+
	2750	1:64	+	−	−	+

[a] Fifteen pigs were assigned into four vaccinated groups: (1) W1V1 group: four pigs from farm #1 were vaccinated with the No.1 commercial vaccine. (2)W1V2 group: three pigs from farm #1 were vaccinated with the No.2 commercial vaccine. (3) W2V1 group: three pigs from farm #2 were vaccinated with the No.1 commercial vaccine. (4) W2V2 group: five pigs 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 $TCID_{50}$ O/TW/97 FMDV at 31 weeks old. All of the 15 vaccinated pigs showed typical vesicle lesions on the feet and snout at 4 dpi. Two pigs in the control group were given PBS buffer instead of vaccine and were challenged as vaccinated groups.

simplicity, reliability, multiplexing capabilities, simultaneous quantitative determination, reducing the subjectivity, less time for data acquisition, time saving and reduced cost, statistical superiority, more flexibility, faster hybridization kinetic, greater accessibility to antibody, and no-wash assay (Clavijo et al., 2006; Dunbar, 2006; Oliver et al., 1998; Vignali, 2000). The assay is observed in some research, such as that for cytokines IL-2, -4, -6, -8, -10, -12; TNF-α; IFN-γ; antibodies to human immunodeficiency virus, avian influenza virus, cytomegalovirus, Epstein–Barr virus, and Rubella and Toxoplasma; soluble hepatitis B virus surface antigen in serum; and nucleic acid detection for single nucleotide polymorphism genotyping, genetic disease screening, gene expression profiling, infectious disease including HIV, SARS-CoV, hepatitis C virus, herpes simplex virus, HLA DNA typing, microbial detection, and other biomolecules (Watson et al., 2009; Willman et al., 2001; Kellar and Iannone, 2002; Dunbar, 2006; Lenhoff et al., 2008). In this study, a Luminex assay based on 3ABC NSP of FMDV serotype O was developed to test serum antibody against FMDV-3ABC in swine. Through the study, an ideal cutoff value of the assay was determined (Fig. 2), early detection was validated (Fig. 3), and high specificity and sensitivity were demonstrated (Figs. 4, 5, and Table 2).

The flow cytometer is capable of reading several hundred beads per second, and each analysis can be completed in as little as 15 s. Potentially up to 100 different analyses can be performed simultaneously, thereby providing a high-throughput platform. It is also conducive to automation, employing high throughput, liquid-handling robotic platforms that minimize human resources required for running routine screening (Dunbar, 2006; Perkins et al., 2006, 2007b). It has been reported that the analytical sensitivity of a Luminex assay can achieve as low as ≦1 pg/ml with high-titer antibodies (Kellar et al., 2006). High-affinity cytokine antibody in combination with liquid-phase kinetics and the high binding capacity of three-dimensional microspheres produce a robust

multiplexed immunoassay with a detection limit of these assays extending over three to four logs, as compared with one to two logs for ELISAs (Kellar and Iannone, 2002). In our single-signature Luminex assay, it is expected that similar results were obtained. The analytical sensitivities of the chromatographic strip assay, sandwich ELISA, and the newly developed Luminex assay were 10,000, and 10 and 100 times higher than that of the commercial 3ABC blocking ELISA kit (diluted 10^{-1} fold), respectively (Chen et al., 2011). This revealed that the chromatographic strip assay could rapidly accomplish the detection of NSP antibodies and was more suitably applied to clinical diagnosis. However, the Luminex and ELISAs could detect a larger number of samples at a time in a laboratory when used by skilled personnel.

In this study, the cutoff value was determined to be 0.07 in T/C ratio (equivalent to 1000 of the MFI value) based on testing sera from naïve and experimentally infected pigs (Fig. 2). To compare the relative sensitivities of NSP antibody assays, our laboratory generated a swine serum panel composed of 320 samples. The experimentally infected pigs at 8 dpi still had positive reactions of 80% and remained positive up to 34 dpi. The results indicate the peaks of positive ratios appeared on 14 dpi and 21 dpi, respectively (Fig. 3a and b). Three positive reactors were obtained on 0 and 6 dpi in samples from the same experimentally infected pigs. These positive reactors tested negative by the virus neutralization test and the ELISAs, suggesting that these were false positive results. However, three of the six methods, the Luminex assay, sandwich ELISA, and the 3ABC blocking ELISA kit showed earliest detection of NSP antibody in sequential serum samples on 8 dpi and gave positive ratios of 78%, 75%, and 66%, respectively. This is in agreement with most authors that report the appearance of specific antibodies by 7 dpi (Clavijo et al., 2006) or 8 dpi (Bergmann et al., 2003; Chen et al., 2009, 2011). In contrast, on the same time point the positive ratios given by the chromatographic strip assay and

3B indirect ELISA were less than 50%. The inconsistency could be due to some differences in conformation of proteins generated by different expression systems, thus suggesting that the ELISA platforms have a good capacity of detecting a small amount of antibodies in samples and are a suitable selection of serological mass screening. In fact, the 3ABC indirect ELISA can detect FMDV-infected pigs only on 14 dpi and later whereas the other ELISA assays showed high rates of positivity and earlier detection (Fig. 3a). Thus, the performances of these ELISA assays are assay-dependent. The Luminex assay also detected remarkable NSP antibody responses by 14 dpi in experimentally infected pigs, consistent with the results from the sandwich ELISA, 3ABC blocking ELISA kits, and 3B peptide indirect ELISA. It indicated the performance parameters of the Luminex assay and the good correlation with the ELISA recommend that we validate the Luminex assay for analyzing FMDV-NSP recombinant protein profiles in immunodiagnostic reagent studies in vitro (Fig. 3a). There are similar other immunoassay technologies as Luminex and blocking ELISA and indirect ELISA where the reagents are critically important (Shen et al., 1999; Sørensen et al., 1998, 2005; Chen et al., 2011; Clavijo et al., 2006; Codorean et al., 2010). Currently, collaborative efforts are underway to fully optimize and validate this Luminex assay for mass screening and confirmation.

Comparison of the performance for the detection of 320 experimentally infected pig sera between the Luminex and the 3ABC blocking ELISA, 3B indirect ELISA, and the 3ABC indirect ELISA kits demonstrated kappa statistics of 0.92, 0.87, and 0.35, respectively, demonstrating a high level of agreement against the former two.

To evaluate the ability of the Luminex assay to detect anti-NSP antibodies elicited by FMDVs of other serotypes, bovine sera against serotypes other than serotype O were also tested. The results suggested that our Luminex assay was effective in detecting the NSP antibodies against other FMD serotypes (Fig. 5), although the sera were from cattle and their numbers are very limited. The results showed that the Luminex assay using the 3ABC and anti-3ABC antibody derived from the FMDV O/TW/99 strain could detect antibodies induced by not only O/TW/99 but also O/TW/97 strains. The antibody detection for two out of the five showed the PI value under 50% for 42.9 and 41 as a negative reaction for the O/TW/99 strain antiserum by the 3ABC blocking ELISA. Indeed, both of these methods have distinct differences (Table 3).

As discovered through our experiments, the Luminex assay demonstrated that the sensitivity was higher and the specificity was lower with cutoff values of 0.06 and 0.07, and in contrast, sensitivity was lower and the specificity higher to the cutoff value 0.09, but all were higher than 90% (data not shown). Finally, evaluation of testing results was adopted based on the cutoff value in the T/C ratio of 0.07. Furthermore, with antisera against SVDV and VSV, the assay proved that it could specifically recognize antibodies against FMDV and did not cross-react with antibodies against SVDV and VSV (Table 3), indicating that it can be used in areas where the three viruses might exist.

The method was able to distinguish between antibodies elicited by naturally infected animals and those by vaccinated animals. Vaccination is one of the main methods that can be implemented to control FMD, and it is currently the primary control strategy used in Taiwan. With coverage through vaccination, highly sensitive methods are essential to discover virus activities, clinical and subclinical, in the field. In the present studies, we displayed 307 vaccinated pigs by the Luminex assay, and four vaccinated animals showed reactions over 1000 MFI (Fig. 4), three out of four of which were closer to the threshold less than 1500 MFI value. This can be due to the presence of low levels of NSP antibody or nonspecific reactions to contaminants in the antigens or maybe actually normal. That NSP antibody in all the 15 sera from vaccinated but unprotected pigs was able to be detected by the Luminex and the chromatographic strip. However, 11 out of the 15 sera were positive by the sandwich ELISA, and only 9 of the 15 sera were positive by the 3ABC blocking ELISA kit (Table 4). The results indicated that the former two methods were more sensitive, as confirmed by the clinical diagnosis, and worthy to be applied to the detection of NSP antibodies in vaccinated animals in the future. Thus, the Luminex developed here demonstrates the diagnostic application of recombinant FMDV NSP-3ABC in monitoring seroconversion following FMD vaccination.

To sum up, as such a Luminex-based immunoassay promises to be a sensitive and efficient method, we will, hopefully, be able to rapidly analyze field serum samples and effectively to diagnose the vesicular diseases in swine. Therefore, we can diagnose and then cull the infected animal in a very early stage.

Acknowledgments

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