Evaluation of Monoclonal Antibody-Based Sandwich Direct ELISA (MSD-ELISA) for Antigen Detection of Foot-and-Mouth Disease Virus Using Clinical Samples

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Abstract

A monoclonal antibody-based sandwich direct ELISA (MSD-ELISA) method was previously developed for foot-and-mouth disease (FMDV) viral antigen detection. Here we evaluated the sensitivity and specificity of two FMD viral antigen detection MSD-ELISAs and compared them with conventional indirect sandwich (IS)-ELISA. The MSD-ELISAs were able to detect the antigen in saliva samples of experimentally-infected pigs for a longer term compared to the IS-ELISA. We also used 178 RT-PCR-positive field samples from cattle and pigs affected by the 2010 type-O FMD outbreak in Japan, and we found that the sensitivities of both MSD-ELISAs were about 7 times higher than that of the IS-ELISA against each sample (P<0.01). In terms of the FMD-positive farm detection rate, the sensitivities of the MSD-ELISAs were about 6 times higher than that of the IS-ELISA against each farm (P<0.01). Although it is necessary to conduct further validation study using the other virus strains, MSD-ELISAs could be appropriate as a method to replace IS-ELISA for FMD antigen detection.

Introduction

Foot-and-mouth disease (FMD) is caused by the FMD virus (FMDV), a member of the family Picornaviridae, genus Aphthovirus. FMDV is highly contagious and has the economic effect of limiting international trade in livestock and livestock products [1]. FMDV can cause blistering, vesicles and ulcers in the epithelia of the mouth, snout, feet, and teat. FMDV consists of seven immunologically distinct serotypes: O, A, C, Asia1, South African Territories (SAT) 1, SAT2 and SAT3. There are some genetically and geographically distinct evolutionary lineages (topotypes) which differ by at least 15% in their VP1 sequences within various serotypes. For example, FMDV type O can be divided into eight topotypes [2]. Antigenic diversity often influences immunoassays for FMDV diagnosis and/or vaccine selection [3,4]. The FMDV antigenic diagnostic methods mentioned in the World Organization for Animal Health's Office International des Epizooties (OIE) manual [5] are virus isolation, immunological methods—i.e., indirect sandwich–enzyme-linked immunosorbent assays (IS-ELISAs) and the complement fixation test—and nucleic acid detection methods such as reverse transcription-polymerase chain reaction (RT-PCR) and real-time RT-PCR. However, the IS-ELISA is able to do serotyping FMDV, but it does not have sufficient sensitivity [6-8].

In an extensive outbreak, it is difficult to collect vesicular fluid and/or vesicular epithelial samples from every suspect farm. In fact, in the 2010 FMD outbreak in Japan, most of the diagnostic samples were oral or nasal swabs, and initial diagnosis was conducted only by RT-PCR for the reason of sensitivity of IS-ELISA which is appropriate for vesicular fluid and/or vesicular epithelial samples. Thus, an antigen-detection ELISA which has high sensitivity enough to detect a viral antigen in samples of saliva and/or nasal discharge must be valuable for the case like 2010 outbreak in Japan.

In our previous study [7], monoclonal antibody (MAb)-based sandwich direct ELISAs (MSD-ELISAs) were developed for multiserotypes (MS) and single serotypes (SS) for FMDV types O, A and Asia1. The MSD-ELISAs were able to detect the different FMDV strains except for MSD-ELISA/SS/Asia1, which showed a weak cross-reaction to type O antigens. In clinical samples, MSD-ELISA/MS and SS/O were able to detect specific FMDV antigens from the saliva and plasma of pigs inoculated with O/TAW/97 (Cathay topotype) [7], and the detection limits of these assays were about 100 to 1000 PFU, as determined by real-time RT-PCR results.

In this study, we evaluated the sensitivity and specificity of the MSD-ELISA reported and compared it with the currently used IS-ELISA, using both experimental samples of other topotypes of serotype O and serotypes A and Asia1 and field samples from the 2010 outbreak of serotype O FMD in Japan.

Materials and Methods

Cells and viruses

The virus strains FMDV O/JPN/2000 (ME-SA topotype) [9,10], O1 BFS 1860 (EURO-SA topotype), A15 TAI 1/60 (ASIA topotype), and Asial Shamir ISR were used for animal
samples were used for the detection of FMD viral antigens in clinical signs were definitely observed, and undiluted saliva samples were collected by cotton swab until 6 days when the occurrence of vesicular lesions on feet was confirmed after inoculation. Field samples collected by veterinarians for FMD diagnosis occurred in 2010 in Japan. These oral and nasal swabs and vesicular epithelial tissues were collected by veterinarians in accordance with the guidelines of Act on Domestic Animal Infectious Diseases Control.

Laboratory clinical samples
Animal experiments were conducted in a biosafety level 3-ag-approved biocontainment facility at our institute. For each virus strain, six or two two-month old pigs were inoculated intradermally with $10^7$ TCID$_{50}$ at the right and front heel bulbs. Saliva samples were collected by cotton swab until 6 days when the clinical signs were definitely observed, and undiluted saliva samples were used for the detection of FMD viral antigens in each assay.

Field samples
In addition to the samples from animal experiments, a total of 178 RT-PCR-positive samples (135 oral swab samples, 7 nasal samples, 24 oral and nasal swabs soaked in about 10-times volumes of PBS (about 2 ml) and 12 samples of 10% emulsion of homogenized epithelial tissues) collected from cattle and pigs from 78 farms that were affected by the 2010 type O FMD outbreak in Japan caused by O/JPN/2010 (SEA topotype) [12] were used for the comparative studies. Field samples were submitted from Miyazaki prefecture for diagnosis of FMD occurred in 2010 in Japan. These samples were collected by veterinarians in accordance with the guidelines of Act on Domestic Animal Infectious Diseases Control in which the veterinarian should collect samples such as epithelium or swabs from a lesion and soaked them in 2 ml of PBS.

Monoclonal antibody-based sandwich direct eELISA for foot-and-mouth disease virus antigen detection
In MSD-ELISAs, the MSD-ELISA for multiserotypes (MS) and the MSD-ELISA for single serotypes (SS) for each serotype (O, A, Asial), MAb 1H5 (which was produced against O/JPN/2000), which reacts with all seven serotypes of FMDV is used as an antigen-capture-antibody. For the detection of each antigen, the MAbs 1H5, 70C4 (which was produced against O/JPN/2000), 16C6 (which was produced against A15 TAI 1/60), and 12C7 (which was produced against Asial Shamir ISR) were used as horseradish-peroxidase (HRPO)-labeled-MAbs for MS, SS for O, A and Asial, respectively. To improve the specificity of SS for Asial, MAb 12C7 was used in this study instead of MAb 7C2, which showed slight cross-reaction with the type O strains in previous study. In addition, for the detection of all seven FMDV serotypes in MS, MAb was changed from 71F2 (which was produced against O/JPN/2000) to 1H5. The protocol of the MSD-ELISAs is described in detail in our previous report [7].

Foot-and-mouth Disease Virus Antigen Detection

Indirect Sandwich ELISA
The IS-ELISA by the World Reference Laboratory of FMD was conducted in accord with the OIE manual [5]. The reagents of IS-ELISA (rabbit anti-sera and guinea pig anti-sera) in a lot which we used for this study are as follows: type O (O Taiwan 98 (Cathay topotype)), type A (A 4164 (Asia topotype)), and type Asial (Asia1 CAM 9/80).

Statistics
For analyzing the statistical significance of the differences in virus detection rates between the MSD-ELISAs and IS-ELISA, the Pearson’s chi-square test was used.

RT-PCR
For the RT-PCR for detection of FMDV nucleic acid, the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA) and primers for the 3D region were used [9].

Real-time RT-PCR
A TaqMan probe and primers for 3D region of FMDV were designed according to the OIE manual [5]. The sequences were as follows: forward primer 5'- ACT GGG TTT TAC AAA CCT GTG A -3', reverse primer 5'- GCG AGT CCT GCC ACG GA -3', TaqMan probe 5'-FAM- TCC TTT GCA CGC GTG GG AC -TAMRA-3'. The program was 48°C for 30 min, 95°C for 10 min, and 40 cycles of 60°C for 15 seconds and 95°C for 1 min. Serial 10-fold dilutions of each FMDV virus containing $10^6$ plaque forming unit (PFU)/0.1 ml were used as the positive samples to construct the standard curve.

Evaluation of ELISAs for FMD Virus Antigen

| Laboratory clinical samples | Table 1 shows the FMDV antigen detection by the MSD-ELISAs and the IS-ELISA obtained using FMDV (O/JPN/2000, O1 BFS 1860, A15 TAI 1/60 and Asial Shamir ISR)-inoculated pig saliva samples. On average, about 0.3 ml of saliva samples were recovered from experimental cotton swabs. In these viruses, O/JPN/2000, A15 TAI 1/60 and Asial Shamir ISR are homologous to MAbS used for the MSA-ELISA/SS and heterologous to rabbit and guinea-pig immune sera use in IS-ELISAs. However, O BFS 1860 is heterologous antigen for both of the MSD-ELISAs and IS-ELISA. The MSD-ELISAs (especially the MSD-ELISA/SSs) were able to detect each FMDV serotype antigen with high sensitivity and specificity compared to the IS-ELISA. Among the inoculated viruses, the FMDV O/JPN/2000 strain was a low pathogenic virus that showed lower levels of clinical signs compared to the other inoculated FMDV strains (data not shown), and the virus excretion levels of the O/JPN/2000 strain were also lower than those of the other strains (Table 1). Therefore, the IS-ELISA did not show positive results against most of the samples of O/JPN/2000-virus-inoculated pigs. Regarding pigs inoculated with the other FMDV strain (O1 BFS 1860, A15 TAI 1/60 and Asial Shamir ISR), the MSD-ELISAs were able to detect FMDV antigens for a longer term compared to the IS-ELISA. The two MSD-ELISAs could detect FMDV antigen at about the same time when the obvious vesicular appeared except for the inoculation site and some samples of inoculated pigs with O1 BFS 1860 and A15 TAI 1/60 showed positive before the vesicular forming. It was generally able to detect about 2 to 3 days after vesicular forming and becoming |
### Table 1. Comparison of the results of FMDV antigen detection methods using saliva of FMDV-inoculated pigs.

| Inoculated Pig | Days post-inoculation | Inoculated Pig | Days post-inoculation |
|----------------|-----------------------|----------------|-----------------------|
| **Virus**      | **Methods**           | **no.**        | **0** | **1** | **2** | **3** | **4** | **5** | **6** | **0** | **1** | **2** | **3** | **4** | **5** | **6** |
| O/JPN 2000     | MS                    | 1              | -    | -    | ++ | +    | -    |        |        |        |        |        |        |        |        |        |        |
| SS             | -                    | -              | -    | -    | ++ | +    | -    |        |        |        |        |        |        |        |        |        |        |
| IS             | -                    | -              | -    | -    | -  | -    | -    |        |        |        |        |        |        |        |        |        |        |
| rPCR           | -                    | -              | +    | +    | ++ | +    | +    |        |        |        |        |        |        |        |        |        |        |
| 2              | MS                    | -              | -    | -    | -  | -    | +    |        |        |        |        |        |        |        |        |        |        |
| SS             | -                    | -              | -    | -    | +  | +    | -    |        |        |        |        |        |        |        |        |        |        |
| IS             | -                    | -              | -    | -    | -  | -    | -    |        |        |        |        |        |        |        |        |        |        |
| rPCR           | -                    | -              | +    | +    | ++ | +    | +    |        |        |        |        |        |        |        |        |        |        |
| 3              | MS                    | -              | -    | -    | -  | -    | -    |        |        |        |        |        |        |        |        |        |        |
| SS             | -                    | -              | -    | -    | +  | +    | -    |        |        |        |        |        |        |        |        |        |        |
| IS             | -                    | -              | -    | -    | -  | -    | -    |        |        |        |        |        |        |        |        |        |        |
| rPCR           | -                    | -              | +    | +    | ++ | +    | +    |        |        |        |        |        |        |        |        |        |        |
| 4              | MS                    | -              | -    | -    | -  | +    | -    |        |        |        |        |        |        |        |        |        |        |
| SS             | -                    | -              | +    | +    | +++| +    | -    |        |        |        |        |        |        |        |        |        |        |
| IS             | -                    | -              | -    | -    | -  | -    | -    |        |        |        |        |        |        |        |        |        |        |
| rPCR           | -                    | -              | +    | +    | ++ | +    | +    |        |        |        |        |        |        |        |        |        |        |
| 5              | MS                    | -              | -    | -    | -  | +    | -    |        |        |        |        |        |        |        |        |        |        |
| SS             | -                    | -              | +    | +    | -  | -    | -    |        |        |        |        |        |        |        |        |        |        |
| IS             | -                    | -              | -    | -    | -  | -    | -    |        |        |        |        |        |        |        |        |        |        |
| rPCR           | -                    | -              | +    | +    | ++ | +    | +    |        |        |        |        |        |        |        |        |        |        |
| 6              | MS                    | -              | -    | -    | -  | +    | -    |        |        |        |        |        |        |        |        |        |        |
| SS             | -                    | -              | +    | +    | -  | -    | -    |        |        |        |        |        |        |        |        |        |        |
| IS             | -                    | -              | -    | -    | -  | -    | -    |        |        |        |        |        |        |        |        |        |        |
| rPCR           | -                    | -              | +    | +    | ++ | +    | +    |        |        |        |        |        |        |        |        |        |        |
| O1 BFS1860     | MS                    | 1              | -    | +    | ++ | +    | -    |        |        |        |        |        |        |        |        |        |        |
| SS             | -                    | +++            | +++  | +++  | +  | -    | -    |        |        |        |        |        |        |        |        |        |        |
| IS             | -                    | -              | -    | -    | -  | -    | -    |        |        |        |        |        |        |        |        |        |        |
| rPCR           | -                    | ++            | ++   | ++   | +  | -    | -    |        |        |        |        |        |        |        |        |        |        |
| 2              | MS                    | -              | -    | -    | +  | -    | -    |        |        |        |        |        |        |        |        |        |        |
| SS             | -                    | +              | +    | -    | -  | -    | -    |        |        |        |        |        |        |        |        |        |        |
| IS             | -                    | -              | -    | -    | -  | -    | -    |        |        |        |        |        |        |        |        |        |        |
| rPCR           | -                    | ++            | ++   | ++   | +  | -    | -    |        |        |        |        |        |        |        |        |        |        |
| 3              | MS                    | -              | -    | +    | -  | -    | -    |        |        |        |        |        |        |        |        |        |        |
| SS             | -                    | +              | -    | -    | -  | -    | -    |        |        |        |        |        |        |        |        |        |        |
**Table 1.**

| Inoculated Pig Days post-inoculation | Inoculated Pig Days post-inoculation |
|------------------------------------|------------------------------------|
| Methods*                           | Methods*                           |
| IN -                                | IN -                                |
| rPCR -                              | rPCR -                              |
| 4MS -                               | 4MS -                               |
| SS -                                | SS -                                |
| IS -                                | IS -                                |
| rPCR -                              | rPCR -                              |
| 5MS -                               | 5MS -                               |
| SS -                                | SS -                                |
| IS -                                | IS -                                |
| rPCR -                              | rPCR -                              |
| 6MS -                               | 6MS -                               |
| SS -                                | SS -                                |
| IS -                                | IS -                                |
| rPCR -                              | rPCR -                              |

| IN -                                | IN -                                |
| rPCR -                              | rPCR -                              |
| 4MS -                               | 4MS -                               |
| SS -                                | SS -                                |
| IS -                                | IS -                                |
| rPCR -                              | rPCR -                              |
| 5MS -                               | 5MS -                               |
| SS -                                | SS -                                |
| IS -                                | IS -                                |
| rPCR -                              | rPCR -                              |
| 6MS -                               | 6MS -                               |
| SS -                                | SS -                                |
| IS -                                | IS -                                |
| rPCR -                              | rPCR -                              |

**The OD results (average sample OD-average buffer OD) of the MS, SS and IS ELISAs were as [1]: ++, 1.0; ++, 0.5–1.0; +, 0.1–0.5; and −, <0.1.**

**The results-related plaque-forming unit of rPCR were as [1]: +++, 10^4; ++, 10^3–10^4; +, 10^2–10^3; and −, <10^2.**

**The pigs inoculated with virus were euthanized.**

**Squares mean the day the obvious vesicular appeared except for the inoculated site.**

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Discussion

Here we found that the sensitivity of the two MSD-ELISAs against oral and nasal swabs were higher than that of the conventional IS-ELISA. Since RT-PCR is one of the most sensitive diagnostic methods, it was generally used as the primary diagnosis tool for FMD diagnosis in the FMD free countries. However, RT-PCR cannot distinguish serotypes and is at risk for developing contamination. In addition to these reasons, the laboratory diagnosis should be performed by several methods especially for the disease causing severe economic loss for the country, such as FMD. Therefore, an MSD-ELISA could be an appropriate method to replace IS-ELISA, and it can perform an early serotyping of FMDV using saliva and oral or nasal swabs. Field samples

In addition to these samples from animal experiments, we used 178 RT-PCR-positive field samples (135 oral swab samples, 7 nasal samples, 24 oral and nasal swab samples, 12 samples of 10% emulsion of homogenized epithelial tissues) from cattle and pigs affected by the 2010 type O FMD outbreak in Japan to compare the sensitivity of the MSD-ELISAs and IS-ELISA. In the results, the positive sample detection rate of IS-ELISA was 8.52%, while on the other hand, those of MSD-ELISA/MS and MSD-ELISA/SS/O were 57.30% and 64.04%, respectively (Table 2). It means that the sensitivities of both MSD-ELISAs (MS and SS) were about 7 times higher than that of the IS-ELISA against each sample. However, the detection rates of IS-ELISA against oral and/or nasal swabs were low, it seems to depend on the amount of antigen of each sample.

Based on the sample detection results, we calculated the FMD-positive farm detection rate. In the epidemic diagnosis for the FMD free country, if the ELISA showed positive on at least one sample from FMD-suspected farm, it should be regarded as FMD-positive and conduct on immediately stamping-out for control and eradication of the disease. In terms of farm units, the IS-ELISA detected 14.1% of positive farms, and the MSD-ELISA/MS and MSD-ELISA/SS/O detected 84.62% and 87.18% of positive farms, respectively (Table 2). It means that the sensitivities of the MSD-ELISAs were about 6 times higher than that of the IS-ELISA against each farm.

Table 2. Sensitivities of the MSD-ELISAs and the IS-ELISA against the FMDV-positive field samples by RT-PCR.

| Sample                | MS-ELISA       | IS-ELISA       |
|-----------------------|----------------|----------------|
| Sample                | MS-ELISA       | IS-ELISA       |
| oral swab             | 56.30% (76/135) | 62.50% (85/135) |
| nasal swab            | 42.86% (3/7)   | 57.14% (4/7)   |
| oral/nasal swab       | 62.50% (15/24) | 70.83% (17/24) |
| epithelial tissue     | 66.67% (8/12)  | 66.67% (8/12)  |
| Total                 | 57.30% (102/178)| 64.04% (114/178)| 8.52% (15/176) |

The advantage of using MAbs is to be able to select highly efficient MAbs for high specificity and sensitivity, and uniform affinity to the antigen leads to minimum disparity between the lots compared to polyclonal anti-sera. However, a MAb recognize a single epitope, thus it should be evaluated broad intra- and inter-type reactivity of the MAbs to cover the antigenic variability of FMD viruses. In regard to this point, we have carried out making the panel of our MAbs against recent pandemic FMDV strains in preparation for antigenic varieties (data not shown). Therefore it is undetectable with decrease in virus shedding. In all samples, the peak of amounts of detected virus genome (Ct values) and virus antigens (OD values) were almost coincided. The correlation coefficient of the OD values of each ELISA and Ct values were showed as follows: the MSD-ELISA/MS (r = 0.529, p = 0.021), the MSD-ELISA/SS (r = 0.622, p = 0.004) and the IS-ELISA (r = 0.31, p = 0.240).

Evaluation of ELISAs for FMD Virus Antigen

Field samples

In addition to these samples from animal experiments, we used 178 RT-PCR-positive field samples (135 oral swab samples, 7 nasal samples, 24 oral and nasal swab samples, 12 samples of 10% emulsion of homogenized epithelial tissues) from cattle and pigs affected by the 2010 type O FMD outbreak in Japan to compare the sensitivity of the MSD-ELISAs (MS and SS/O) and IS-ELISA. In the results, the positive sample detection rate of the IS-ELISA was 8.52%, while on the other hand, those of the MSD-ELISA/MS and MSD-ELISA/SS/O were 57.30% and 64.04%, respectively (Table 2). It means that the sensitivities of both MSD-ELISAs (MS and SS) were about 7 times higher than that of the IS-ELISA against each sample (P<0.01). However, the detection rates of IS-ELISA against oral and/or nasal swabs were low, it seems to depend on the amount of antigen of each sample.

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will be possible to change or combine antigen detection MAbs according to epidemic FMDV strains as needed. To be a larger diagnostic use of the MSD-ELISAs, further validation study should be conducted using field samples of the other virus strains, which are epidemic in especially Asian countries.

Author Contributions
Conceived and designed the experiments: KM KY. Performed the experiments: KM KF KY. Analyzed the data: KM. Contributed reagents/materials/analysis tools: KM KF KY TK. Wrote the paper: KM KF KS KY TK.

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