NOTE

Comparative evaluation of two ELISA kits for detecting antibodies to a nonstructural protein of foot-and-mouth disease virus using serum samples collected from naturally and experimentally infected cows

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ABSTRACT. When foot-and-mouth disease (FMD) occurs and a "vaccination-to-live" policy is adopted in a country, the country must perform serological surveillance of a nonstructural protein (NSP) of FMD virus. The NCPanaftosa kit is the only kit for detecting antibodies to NSPs that is officially recognized as the reference regent by the World Organization for Animal Health; however, it is only used in South American countries. In this study, the specificity and sensitivity of the NCPanaftosa kit were compared with those of the PrioCHECK kit sold by an international company. Results in this study suggest that the PrioCHECK kit performs similarly to the NCPanaftosa kit in detecting antibodies to the NSP in the cattle population.

KEY WORDS: cattle, ELISA, Foot-and-mouth disease virus, nonstructural protein

Foot-and-mouth disease (FMD) is one of the most contagious diseases of cloven hoofed animals such as cows, pigs, sheep and goats [10]. Its causative agent, FMD virus (FMDV), belongs to the genus *Aphthovirus* within the family *Picornaviridae*. FMDV is divided into seven serotypes: A, O, C, SAT1, SAT2, SAT3 and Asia1 [10]. Each serotype is further classified into several topotypes based on a comparison of the nucleotide sequences of the VP1 region [17].

Countries can currently adopt one of two policies after the implementation of emergency vaccination in an FMD outbreak. The two policies are known as "vaccination-to-die" and "vaccination-to-live" policies [2]. Countries that adopt the "vaccination-to-die" policy in an FMD outbreak must sacrifice all vaccinated animals. Countries that adopt the "vaccination-to-live" policy in an FMD outbreak must perform serological surveillance instead of sacrificing vaccinated animals. The objective of the serological surveillance is to find evidence that vaccinated and subsequently infected animals do not exist in the field; therefore, the surveillance includes the measurement of antibodies to nonstructural proteins (NSPs) of FMDV because a commercial vaccine does not include generally any NSPs and non-infected animals irrespective of vaccination statuses do not theoretically have antibodies to NSPs.

The specificities and sensitivities of many ELISA kits that can measure antibodies to NSPs (NSP-ELISA) were previously evaluated [5, 9, 12]. The sensitivities of the NSP-ELISA kits are generally lower than those of ELISA kits that can measure antibodies to structural proteins (SPs) of FMDV (SP-ELISA); however, the SP-ELISA kits cannot differentiate between antibodies induced by infection and those induced by vaccination. Therefore, countries that adopt the "vaccination-to-live" policy in an FMD outbreak must use NSP-ELISA kits for serological surveillance after emergency vaccination is implemented. The recent trend is to choose the adoption of the "vaccination-to-live" policy over the "vaccination-to-die" policy in an FMD outbreak from the viewpoint of animal welfare, the preservation of valuable genetic resources and limiting environmental contamination [5, 15, 19].

In the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals produced by the World Organization for Animal Health (OIE), the NCPanaftosa ELISA/EITB kit (PANAFTOSA, Rio de Janeiro, Brazil) is described one of the NSP-ELISA kits included [3], and this kit is the only NSP-ELISA kit officially recognized as the reference regent by the OIE [4]. However, the NCPanaftosa kit is basically produced only for cattle in South American countries. On the other hand, the PrioCHECK FMDV NS (Thermo Fisher Scientific, Waltham, MA, U.S.A.) kit can be obtained commercially throughout the world through its international

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Table 1. Diagnostic specificity in non-vaccinated, non-infected cows and vaccinated, non-infected cows

Animals	No. of samples	Kits		
		LPBE (%)	PrioCHECK (%)	NCPanaftosa (%)
Non-vaccinated, non-infected cows	203	99.5	99.0	100
Vaccinated, non-infected cows				
Cows administered vaccine once	116	NC [a]	100	100
Cows administered vaccine four times	28	NC	100	100
Cows administered vaccine once in the field	40	NC	95.0	97.5
Total	184	NC	98.9	99.5

a) Not calculated because the diagnostic specificity is a percentage of samples which are correctly confirmed as antibody-negative among samples which may not have antibodies induced by virus infection as well as antibodies induced by vaccination while the LPBE kit can detect antibodies induced by virus infection as well as antibodies induced by vaccination.

manufacturer. In addition, the PrioCHECK kit is thought to be the best kit for use in countries other than South American countries because it can measure antibodies of all animal species. Furthermore, the PrioCHECK kit showed the highest specificity and sensitivity among the NSP-ELISA kits evaluated in previous studies [9, 12].

The objective of the present study is to compare the specificities and sensitivities of the NCPanaftosa and PrioCHECK kits to help determine an appropriate control strategy after the implementation of emergency vaccination in an FMD outbreak in Japan and elsewhere.

The Animal Care and Use Committee of the National Institute of Animal Health (NIAH) approved all animal procedures prior to the initiation of this study (authorization numbers: 763, 812, 826, 11-033, 12-027, 13-054, 14-080). All experimental infections were performed in rooms that were approximately 14 m^2 in area in a high-containment facility at the NIAH.

A total of 203 serum samples were obtained for serological surveillance of FMD from cattle kept on 14 farms in Japan during 2010. The cattle were judged clinically not to be infected with FMDV nor administered any FMDV vaccines by veterinarians of animal hygiene service centers.

The monovalent vaccines (Aphtopor, Merial, Lyon, France) used in this study were preserved for emergency use in a cold room of an animal quarantine center by the Ministry of Agriculture, Forestry and Fisheries of Japan. Each vaccine was formulated with serotypes A, O, and Asia1, respectively, and contained six 50% protection doses (PD_{50}) per dose. In general, 2 ml of the vaccine was administered to cattle.

Six cows were administered one of the three serotype FMDV vaccines intramuscularly, and a total of 144 serum samples were collected routinely from the animals. The day when the animals were administered the vaccine was designated 0 days post-vaccination (dpv). In the case of animals administered a single dose of vaccine, the serum samples were collected daily until 10 dpv, at 3- to 4-day intervals until 21 dpv and at approximately 1- to 2-week intervals after that, and the animals were monitored for approximately 8 months. The exception was a cow administered a single dose of the vaccine and monitored for approximately 4 months. The cows administered a single dose of the vaccine are described as "cows administered vaccine once" in Table 1. In the case of animals that were administered the vaccine four times, the serum samples were collected daily until 4 dpv, at 3- to 4-day intervals until 22 dpv and at approximately 1-week intervals after that, and the animals were monitored for approximately 2 months. The cows administered the vaccine four times are described as "cows administered vaccine four times" in Table 1. In addition, 40 serum samples were collected from 40 cows administered the vaccine as a control measure in the 2010 epidemic in Japan. The cows administered the vaccine as the control measure are described as "cows administered vaccine once in the field" in Table 1.

A total of 102 serum samples were obtained from cattle kept on 66 farms in Japan during 2010. The cattle were confirmed to be infected with FMDV by RT-PCR [11], virus isolation [14] or the Liquid phase blocking immunoassay for detection of antibodies of foot-and-mouth disease virus (LPBE; Biological Diagnostic Supplies Ltd., Ayrshire, U.K.). The cattle were sacrificed immediately after they were confirmed to be infected with FMDV by one of the assays.

The full details of the experimental infections that provided serum samples for this study have already been published [13, 14, 18]. Briefly, (i) Two 6-month-old Holstein cows housed in separate rooms were inoculated with 1 ml of $10^{6.2}$ 50% of tissue culture infectious dose ($TCID_{50}$) of the FMDV O/JPN/2010-1/14 [11] on their tongues by an intradermal route. At 1 day post-infection (dpi), two additional 6-month-old Holstein cows were housed with the infected cows. They were housed in the same room for approximately 1 month [18]. (ii) Four 3-month-old Holstein cows housed in separate rooms were inoculated with 1 ml of 10^6 $TCID_{50}$ of the O/JPN/2010-1/14C on their tongues by the intradermal route. They were housed in separate rooms for approximately 2 weeks [13]. (iii) Seven 3-month-old Holstein cows were administered the FMDV vaccine intramuscularly. At 3 or 30 dpv, the vaccinated cows were inoculated with 1 ml of 10^6 $TCID_{50}$/ml of the FMDV O/JPN/2010-1/14C on their tongues by the intradermal route. They were observed for approximately 2 weeks to 1 month after the infection [14].

The LPBE was performed for the detection of antibodies to SPs of FMDV according to the manufacturer's instructions. The FMDV O Manisa strain was used as the antigen of the LPBE. The PrioCHECK FMDV NS [21] and NCPanaftosa ELISA/EITB [8] kits were used to detect antibodies to the NSPs of FMDV according to the manufacturers' instructions. All of the positive results obtained by an ELISA system in the NCPanaftosa kit were reconfirmed using an enzyme-linked immunoelectrontransfer blot

Table 2. Diagnostic sensitivity in non-vaccinated, experimentally infected cows and vaccinated, experimentally infected cows

Dpi and dpc [a]	No. of samples	Kits		
		LPBE (%)	PrioCHECK (%)	NCPanaftosa (%)
Non-vaccinated, infected cows				
0–6	56	21.4	7.1	0
7–15	34	100	94.1	50.0
>15	20	100	100	100
Vaccinated, infected cows				
0–6	49	NC[b]	24.5	0
7–15	28	NC	96.4	46.4
>15	15	NC	100	100

a) Days post-infection and days post-contact. b) Not calculated because the diagnostic sensitivity is a percentage of samples which are correctly confirmed as antibody-positive among samples which may have antibodies induced by virus infection while the LPBE kit can detect antibodies induced by virus infection as well as antibodies induced by vaccination.

(EITB) test included with the system in the kit.

In this study, the specificity and sensitivity were calculated with the following formulas:

$$\text{specificity (\%)} = \frac{\text{the numbers of animals that showed negative results when tested using the NSP} - \text{ELISA kits}}{\text{the numbers of animals that have never had an infection due to the FMDV}} \times 100$$

$$\text{sensitivity (\%)} = \frac{\text{the numbers of animals that showed positive results when tested using the LPBE or NSP} - \text{ELISA kits}}{\text{the numbers of animals that had an infection associated with FMDV}} \times 100$$

Statistically analyses were conducted with the Microsoft Excel 2016 in this study.

Significant differences in specificity were not observed statistically among the LPBE and two NSP-ELISA kits in non-infected, non-vaccinated cows (Table 1). The specificities of the LPBE and two NSP-ELISA kits were between 99.0 and 100%, respectively. Similarly, the specificities of the NSP-ELISA kits were also high in non-infected, vaccinated cows, and ranged from 95.0 to 100%. In addition, antibodies to NSPs were not detected using the NSP-ELISA kits in cows administered the vaccine four times.

Antibodies were detected in 29 (28.4%) of 102 serum samples collected from the cows, which were confirmed to be infected with FMDV in the field by RT-PCR, virus isolation or LPBE, using the PrioCHECK kit. In contrast, antibodies were detected in 18 (17.7%) of the samples using the NCPanaftosa kit.

The Terrestrial Animal Health Code approves to use both of SP-ELISA, such as the LPBE, and NSP-ELISA as an assay for serological surveillance in unvaccinated population. Therefore, the sensitivity of the LPBE was compared with those of the two NSP-ELISA kits in non-vaccinated, infected cows in this study. At 0–6 dpi/dpc, the sensitivities of the two NSP-ELISA kits in non-vaccinated, infected cows showed statistically significant differences ($P<0.05$ (between the LPBE and PrioCHECK kit), $P<0.01$ (between the LPBE and NCPanaftosa kit), Table 2); although the sensitivity of the LPBE was 21.4%, those of the NSP-ELISA kits were 7.1% and 0%, respectively. At 7–15 dpi/dpc, the sensitivities of the LPBE and two NSP-ELISA kits in non-vaccinated, infected cows also showed statistically significant differences ($P<0.01$, Table 2); although the sensitivities of the LPBE and PrioCHECK kit were 100% and 94.1%, respectively, that of the NCPanaftosa kit was 50.0%. At >15 dpi/dpc, all of the sensitivities of the LPBE and two NSP-ELISA kits were 100% (Table 2).

The LPBE can detect antibodies induced by both of vaccination and infection. Therefore, only the sensitivities of the two NSP-ELISA kits were compared in vaccinated, infected cows in this study. At 0–6 dpi, the sensitivities of the two NSP-ELISA kits in vaccinated, infected cows showed a statistically significant difference ($P<0.01$, Table 2); although the sensitivity of the PrioCHECK kit was 24.5%, that of the NCPanaftosa kit was 0%. At 7–15 dpi, the sensitivities of the two NSP-ELISA kits in vaccinated, infected cows also showed a statistically significant difference ($P<0.01$, Table 2), although the sensitivity of the PrioCHECK kit was 96.4%, that of the NCPanaftosa kit was 46.4%. At >15 dpi, both the sensitivities of the two NSP-ELISA kits were 100% (Table 2).

In the non-vaccinated, infected cows, antibodies were detected initially between 4 and 9 dpi with the LPBE and antibody titers were ranged from 32 to 724 (Table 3). With the PrioCHECK kit, antibodies were detected initially between 5 and 12 dpi. With the NCPanaftosa kit, antibodies were detected initially between 9 and 12 dpi, although antibodies were not detected in cow 152 during the experimental periods. In the cows in which antibodies were detected using the LPBE and both the NSP-ELISA kits, the day when the antibodies were initially detected with the NSP-ELISA kits was delayed between 1 and 8 days compared to the LPBE.

In infected cows vaccinated at 30 days before virus infection (dbv), antibodies were detected initially from 23 dbv with the LPBE and antibody titers were ranged from 90 to 362 before the virus infection and from 181 to 5,792 after the infection (Table 4). With the PrioCHECK kit, antibodies were detected initially between 3 and 7 dpi. With the NCPanaftosa kit, antibodies were detected initially from 8 dpi in one of three cows administered the vaccine at 30 dbv; however, antibodies were not detected during

Table 3. Detection of antibodies in non-vaccinated, infected cows by the three kits

Cow Nos.	Kits	Dpi and dpc [a]																	Clinical signs
		0	1	2	3	4	5	6	7	8	9 [b]	12 [c]	15 [d]	19 [e]	23 [f]	27 [g]	30 [h]	33 [i]	
121 [j]	LPBE	<32	<32	<32	<32	<32	45 [k]	181	90	NT [l]	362	724	362	362	181	362	362	362	+
	PrioCHECK	-	-	-	-	-	-	-	+ [m]	NT	+	+	+	+	+	+	+	+	
	NCPanaftosa	-	-	-	-	-	-	-	-	NT	+ [n]	+	+	+	+	+	+	+	
122 [j]	LPBE	<32	<32	<32	<32	<32	90	362	724	NT	512	512	724	512	362	724	724	362	+
	PrioCHECK	-	-	-	-	-	-	-	+	NT	+	+	+	+	+	+	+	+	
	NCPanaftosa	-	-	-	-	-	-	-	-	NT	+	+	+	+	+	+	+	+	
123 [j]	LPBE	<32	<32	<32	<32	<32	<32	<32	NT	NT	90	181	362	512	362	362	362	256	+
	PrioCHECK	-	-	-	-	-	-	-	NT	NT	-	+	+	+	+	+	+	+	
	NCPanaftosa	-	-	-	-	-	-	-	NT	NT	-	+	+	+	+	+	+	+	
124 [j]	LPBE	<32	<32	<32	<32	<32	<32	<32	NT	NT	181	362	724	362	362	362	181	181	+
	PrioCHECK	-	-	-	-	-	-	-	NT	NT	-	+	+	+	+	+	+	+	
	NCPanaftosa	-	-	-	-	-	-	-	NT	NT	-	+	+	+	+	+	+	+	
143	LPBE	<32	<32	<32	<32	<32	<32	181	181	362	362	362	724	NT	NT	NT	NT	NT	+
	PrioCHECK	-	-	-	-	-	-	+	+	+	+	+	+	NT	NT	NT	NT	NT	
	NCPanaftosa	-	-	-	-	-	-	-	-	-	+	+	+	NT	NT	NT	NT	NT	
147	LPBE	<32	<32	<32	<32	<32	<32	181	362	724	724	724	724	NT	NT	NT	NT	NT	+
	PrioCHECK	-	-	-	-	-	-	+	+	+	+	+	+	NT	NT	NT	NT	NT	
	NCPanaftosa	-	-	-	-	-	-	-	-	-	-	+	+	NT	NT	NT	NT	NT	
152	LPBE	<32	<32	<32	<32	45	181	724	1448	1024	1024	724	724	NT	NT	NT	NT	NT	+
	PrioCHECK	-	-	-	-	-	-	+	+	+	+	+	+	NT	NT	NT	NT	NT	
	NCPanaftosa	-	-	-	-	-	-	-	-	-	-	-	-	NT	NT	NT	NT	NT	
154	LPBE	<32	<32	<32	<32	32	128	362	362	512	512	362	724	NT	NT	NT	NT	NT	+
	PrioCHECK	-	-	-	-	-	-	+	+	+	+	+	+	NT	NT	NT	NT	NT	
	NCPanaftosa	-	-	-	-	-	-	-	-	-	-	+	+	NT	NT	NT	NT	NT	

a) Days post-infection and days post-contact. b) The serum samples were collected from cows 123 and 124 at 8 dpc. c) The serum samples were collected from cows 123 and 124 at 11 dpc, and from cows 143, 147, 152 and 154 at 10 dpi. d) The serum samples were collected from cows 123 and 124 at 14 dpc, from cows 147 and 154 at 14 dpi, and from cow 152 at 13 dpi. e) The serum samples were collected from cows 123 and 124 at 18 dpc. f) The serum samples were collected from cows 123 and 124 at 22 dpc. g) The serum samples were collected from cows 123 and 124 at 26 dpc. h) The serum samples were collected from cows 123 and 124 at 29 dpc. i) The serum samples were collected from cow 122 at 34 dpi, and from cow 123 at 32 dpc. j) The results of the LPBE and PrioCHECK kit in cows 121, 122, 123 and 124 have already been reported in a previous report [14]. k) Days when the antibodies were detected by the LPBE are colored orange. l) Not tested. m) Days when the antibodies were detected by the PrioCHECK kit are colored green. n) Days when the antibodies were detected by the NCPanaftosa kit are colored pink.

the experimental period in the others.

In infected cows vaccinated at 3 dbv, antibodies were detected initially from 2 dpi with the LPBE and antibody titers were ranged from 45 to 1,448 (Table 4). With the PrioCHECK kit, antibodies were detected initially between 5 and 6 dpi; however, in cow 142, no antibody was detected at 14 dpi in the kit. In the NCPanaftosa kit, antibodies were detected initially between 7 and 8 dpi in three of four cows administered the vaccine at 3 dbv; however, antibodies were not detected during the experimental period in cow 142.

In our previous study, the specificity of the PrioCHECK kit was already confirmed to be as high as that of the LPBE [12]. In addition, the specificity of the NCPanaftosa kit was confirmed to be as high as those of the LPBE and PrioCHECK kit in this study (Table 1). Previously, the specificity of the NCPanaftosa kit has mainly been analyzed using serum samples collected from South American countries and has been reported to be high in several reports [6–8, 16]. In South American countries, active surveillances to confirm that live viruses are not circulating are performed widely using the NCPanaftosa kit [1]. In general, the specificity of an NSP-ELISA kit depends on the purity of the vaccine applied to tested animals as well as the kit's own performance, such as the quality of the antigen [20]. Vaccines produced by manufacturers located in South American countries satisfy a requirement for the purity of the vaccine that was established by the OIE [3]. In addition, all positive results are reconfirmed using the EITB test included with the ELISA system in the NCPanaftosa kit. Therefore, non-vaccinated and vaccinated cows that have never been infected with FMDV are likely to be judged precisely as negative in serological surveillance performed in South American countries.

Detection of NSP antibodies with the PrioCHECK kit took longer than detection of SP antibodies with the LPBE in our previous study [13]. In the present study, the detection of NSP antibodies with the PrioCHECK and NCPanaftosa kits also took longer than that with the LPBE (Tables 3 and 4). In addition, there were several cows in which antibodies were not detected with the NCPanaftosa kit during the experimental period. In general, NSP-ELISA kits are recommended to be applied at a herd level [19]. As mentioned above, serological surveillance is performed using the NCPanaftosa kit in South American countries [1]; however, cases that infected cows are not detected with the NCPanaftosa kit may be present in the field as shown by the results of this

Table 4. Detection of antibodies in vaccinated, infected cows by the three kits

Cow Nos.	Kits	-30	-29	-28	-27	-23	-20	-17	-10	-3	-2	-1	0	1	2	3	4	5	6	7	8	11	14	19	22	25	28	32[b]	Clinical signs
Vaccine administered at 30 dbv[c]																													
130[d]	LPBE	<32	<32	<32	<32	90[e]	90	362	362	362	362	362	362	362	362	362	362	724	2,896	5,792	5,792	5,792	5,792	5,792	2,048	2,896	2,896	2,896	-[f]
	PrioCHECK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+[g]	+	+	+	+	+	+	+	+	+	+	
	NCPanaftosa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+[h]	+	+	+	+	+	+	+	
141[d]	LPBE	<32	<32	<32	<32	90	256	256	256	362	181	181	181	181	181	181	90	90	128	181	362	724	724	NT[k]	NT	NT	NT	NT	-[f]
	PrioCHECK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	NT	NT	NT	NT	NT	
	NCPanaftosa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT	NT	NT	NT	NT	
146[d]	LPBE	<32	<32	<32	<32	90	181	362	362	362	362	362	362	362	362	362	512	1,448	2,896	2,896	4,096	5,792	5,792	NT	NT	NT	NT	NT	-[f]
	PrioCHECK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	NT	NT	NT	NT	NT	
	NCPanaftosa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT	NT	NT	NT	NT	
Vaccine administered at 3 dbv																													
133[d]	LPBE	NT	NT	NT	NT	NT	NT	NT	NT	<32	<32	<32	<32	<32	<32	45	256	362	362	1448	256	362	256	256	512	362	512	362	+
	PrioCHECK	NT	NT	NT	NT	NT	NT	NT	NT	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	
	NCPanaftosa	NT	NT	NT	NT	NT	NT	NT	NT	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	
137[d]	LPBE	NT	NT	NT	NT	NT	NT	NT	NT	<32	<32	<32	<32	<32	<32	45	181	362	362	724	1,448	1,024	1,448	724	724	724	512	1,024	+
	PrioCHECK	NT	NT	NT	NT	NT	NT	NT	NT	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	
	NCPanaftosa	NT	NT	NT	NT	NT	NT	NT	NT	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	
142[d]	LPBE	NT	NT	NT	NT	NT	NT	NT	NT	<32	<32	<32	<32	<32	45	90	362	724	724	724	724	512	512	NT	NT	NT	NT	NT	+
	PrioCHECK	NT	NT	NT	NT	NT	NT	NT	NT	-	-	-	-	-	-	-	-	+	+	+	+	+	-	NT	NT	NT	NT	NT	
	NCPanaftosa	NT	NT	NT	NT	NT	NT	NT	NT	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NT	NT	NT	NT	NT	
149[d]	LPBE	NT	NT	NT	NT	NT	NT	NT	NT	<32	<32	<32	<32	<32	64	256	1,024	1,448	1,448	1,448	1,448	1,448	1,024	NT	NT	NT	NT	NT	+
	PrioCHECK	NT	NT	NT	NT	NT	NT	NT	NT	-	-	-	-	-	-	-	-	+	+	+	+	+	+	NT	NT	NT	NT	NT	
	NCPanaftosa	NT	NT	NT	NT	NT	NT	NT	NT	-	-	-	-	-	-	-	-	-	-	-	+	+	-	NT	NT	NT	NT	NT	

a) Days post-infection. b) The serum samples were collected from cows 133 and 137 at 33 dpi. c) Days before virus infection. d) The results of the LPBE and PrioCHECK kit in cows 130, 141, 146, 133, 137, 142 and 149 have already been reported in a previous study [14]. e) Days when the antibodies were detected by the LPBE are colored orange. f) Except for the development of lesions on sites of virus inoculation. g) Days when the antibodies were detected by the PrioCHECK kit are colored green. h) Days when the antibodies were detected by the NCPanaftosa kit are colored pink. k) Not tested.

study. In particular, antibody responses to NSPs may be weak in vaccinated and subsequently infected animals [9, 13]. Therefore, serological surveillance using an NSP-ELISA kit in countries where routine vaccination is practiced should be performed in statistically sufficient numbers of animals, and the results of the surveillance should be judged at a herd level.

In this study, antibodies were detected in 29 (28.4%) of 102 serum samples, which were collected from infected cattle in the field, with the PrioCHECK kit, while they were detected in 18 (17.7%) of the samples with the NCPanaftosa kit. In addition, the sensitivity of the PrioCHECK kit was higher than that of the NCPanaftosa kit at 0–6 and 7–15 dpi/dpc (Table 2). Furthermore, antibodies were detected earlier with the PrioCHECK kit than with the NCPanaftosa kit (Tables 3 and 4). According to those protocols, serum samples are subjected initially to 1:5 and 1:20 dilutions by buffers in the PrioCHECK and NCPanaftosa kits, respectively. In addition, the PrioCHECK kit involves a competitive method while the NCPanaftosa kit involves an indirect method. Therefore, the difference in the dilution ratios of the serum samples and in the methods of the kits may influence the difference in the sensitivities and the initial detection of antibodies of the kits. However, the NCPanaftosa kit may not indicate the performance seen in South American countries where the kit was developed. Therefore, at least, the performance of the PrioCHECK kits is thought to be comparable with that of the NCPanaftosa kit based on the results obtained in this study.

In conclusion, the NCPanaftosa kit is the only NSP-ELISA kit recognized officially as the reference regent by the OIE and described in the OIE manual [3, 4]; however, the specificity and sensitivity of the PrioCHECK kit was confirmed to be comparable with those of the NCPanaftosa kit in this study. Therefore, the PrioCHECK kit was thought to have similar performance as the NCPanaftosa kit for detecting antibodies to an NSP of FMDV in cattle population. Taken together, the results obtained in this study will be valuable for rational decision-making in terms of an appropriate control strategy after implementation of emergency vaccination in an FMD outbreak. In contrast, our previous report showed that the PrioCHECK may not be able to detect antibodies in infected pigs with vaccination [13]. Therefore, studies need to evaluate further performance of the PrioCHECK kit in naturally and experimentally infected pigs.

REFERENCES

1. Anonymous 2015. Activities in 2015. OIE Reference Laboratory Reports Activities. http://www.oie.int/fileadmin/Home/eng/Our_scientific_expertise/reflabreports/2015/report_18_2015_Foot_and_mouth_disease_BRAZIL.pdf [accessed on June 11, 2018].
2. Anonymous 2016. Chapter 8.8. Infection with foot and mouth disease virus. Terrestrial Animal Health Code 2016. http://www.oie.int/index.php?id=169&L=0&htmfile=chapitre_fmd.htm [accessed on June 11, 2018].
3. Anonymous 2016. Chapter 2.1.8. Foot and mouth disease (infection with foot and mouth disease virus). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2016. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.08_FMD.pdf [accessed on June 11, 2018].
4. Anonymous 2016. OIE-approved International Standard Sera. 2016. http://www.oie.int/en/our-scientific-expertise/veterinary-products/reference-reagents/ [accessed on June 11, 2018].
5. Barnett, P. V., Geale, D. W., Clarke, G., Davis, J. and Kasari, T. R. 2015. A review of OIE country status recovery using vaccinate-to-live versus vaccinate-to-die foot-and-mouth disease response policies I: benefits of higher potency vaccines and associated NSP DIVA test systems in post-outbreak surveillance. *Transbound. Emerg. Dis.* **62**: 367–387. [Medline] [CrossRef]
6. Bergmann, I. E., Malirat, V., Neitzert, E., Beck, E., Panizzutti, N., Sánchez, C. and Falczuk, A. 2000. Improvement of a serodiagnostic strategy for foot-and-mouth disease virus surveillance in cattle under systematic vaccination: a combined system of an indirect ELISA-3ABC with an enzyme-linked immunoelectrotransfer blot assay. *Arch. Virol.* **145**: 473–489. [Medline] [CrossRef]
7. Bergmann, I. E., Neitzert, E., Malirat, V., Ortiz, S., Colling, A., Sánchez, C. and Correa Melo, E. 2003. Rapid serological profiling by enzyme-linked immunosorbent assay and its use as an epidemiological indicator of foot-and-mouth disease viral activity. *Arch. Virol.* **148**: 891–901. [Medline] [CrossRef]
8. Bergmann, I. E., Malirat, V. and Neitzert, E. 2005. Non-capsid proteins to identify foot-and-mouth disease viral circulation in cattle irrespective of vaccination. *Biologicals* **33**: 235–239. [Medline] [CrossRef]
9. Brocchi, E., Bergmann, I. E., Dekker, A., Paton, D. J., Sammin, D. J., Greiner, M., Grazioli, S., De Simone, F., Yadin, H., Haas, B., Bulut, N., Malirat, V., Neitzert, E., Goris, N., Parida, S., Sørensen, K. and De Clercq, K. 2006. Comparative evaluation of six ELISAs for the detection of antibodies to the non-structural proteins of foot-and-mouth disease virus. *Vaccine* **24**: 6966–6979. [Medline] [CrossRef]
10. Brown, F. 2003. The history of research in foot-and-mouth disease. *Virus Res.* **91**: 3–7. [Medline] [CrossRef]
11. Fukai, K., Morioka, K. and Yoshida, K. 2011. An experimental infection in pigs using a foot-and-mouth disease virus isolated from the 2010 epidemic in Japan. *J. Vet. Med. Sci.* **73**: 1207–1210. [Medline] [CrossRef]
12. Fukai, K., Morioka, K., Onozato, H., Yoshida, K. and Sakamoto, K. 2013. Comparative evaluation of three commercial ELISA kits for detection of antibodies to a nonstructural protein of foot-and-mouth disease virus. *J. Vet. Med. Sci.* **75**: 693–699. [Medline] [CrossRef]
13. Fukai, K., Nishi, T., Morioka, K., Yamada, M., Yoshida, K., Kitano, R., Yamazoe, R. and Kanno, T. 2016. Further evaluation of an ELISA kit for detection of antibodies to a nonstructural protein of foot-and-mouth disease virus. *J. Vet. Med. Sci.* **78**: 365–373. [Medline] [CrossRef]
14. Fukai, K., Nishi, T., Shimada, N., Morioka, K., Yamada, M., Yoshida, K., Sakamoto, K., Kitano, R., Yamazoe, R. and Yamakawa, M. 2017. Experimental infections using the foot-and-mouth disease virus O/JPN/2010 in animals administered a vaccine preserved for emergency use in

Japan. *J. Vet. Med. Sci.* **79**: 128–136. [Medline] [CrossRef]

15. Geale, D. W., Barnett, P. V., Clarke, G. W., Davis, J. and Kasari, T. R. 2015. A review of OIE country status recovery using vaccinate-to-live versus vaccinate-to-die foot-and-mouth disease response policies II: waiting periods after emergency vaccination in FMD free countries. *Transbound. Emerg. Dis.* **62**: 388–406. [Medline] [CrossRef]

16. Gonzales, J. L., Barrientos, M. A., Quiroga, J. L., Ardaya, D., Daza, O., Martinez, C., Orozco, C., Crowther, J. and Paton, D. J. 2014. Within herd transmission and evaluation of the performance of clinical and serological diagnosis of foot-and-mouth disease in partially immune cattle herds. *Vaccine* **32**: 6193–6198. [Medline] [CrossRef]

17. Knowles, N. J. and Samuel, A. R. 2003. Molecular epidemiology of foot-and-mouth disease virus. *Virus Res.* **91**: 65–80. [Medline] [CrossRef]

18. Onozato, H., Fukai, K., Kitano, R., Yamazoe, R., Morioka, K., Yamada, M., Ohashi, S., Yoshida, K. and Kanno, T. 2014. Experimental infection of cattle and goats with a foot-and-mouth disease virus isolate from the 2010 epidemic in Japan. *Arch. Virol.* **159**: 2901–2908. [Medline] [CrossRef]

19. Paton, D. J., Füssel, A. E., Vosloo, W., Dekker, A. and De Clercq, K. 2014. The use of serosurveys following emergency vaccination, to recover the status of "foot-and-mouth disease free where vaccination is not practised". *Vaccine* **32**: 7050–7056. [Medline] [CrossRef]

20. Smitsaart, E., Espinoza, A. M., Maradei, E., Cosentino, B., Guinzburg, M., Madonni, G., Cadenazzi, G., Bottini, R., Filippi, J. and Bergmann, I. 2015. Importance of foot and mouth disease vaccine purity in interpreting serological surveys. *Rev. - Off. Int. Epizoot.* **34**: 755–766, 741–754. [Medline]

21. Sørensen, K. J., de Stricker, K., Dyrting, K. C., Grazioli, S. and Haas, B. 2005. Differentiation of foot-and-mouth disease virus infected animals from vaccinated animals using a blocking ELISA based on baculovirus expressed FMDV 3ABC antigen and a 3ABC monoclonal antibody. *Arch. Virol.* **150**: 805–814. [Medline] [CrossRef]