

Comparison of Three ELISA Kits for the Differentiation of Foot-and-mouth Disease Virus-infected from Vaccinated Animals*

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Abstract: A study was performed to validate 3 FMDV 3ABC-I-ELISA kits developed in China for the differentiation of FMDV infected and vaccinated animals. Sets of sera from naive and vaccinated cattle as well as from cattle that had been infected were tested for antibodies against nonstructural proteins (NSPs) of FMDV by commercial diagnosis kits, Ceditest® FMDV-NS (Ceditest® kit), UBI® FMDV NON-STRUCTURAL PROTEIN ELISA DIRECTION INSERT (UBI® kit) and a FMDV 3ABC-I-ELISA kit developed at the Lanzhou Veterinary Research Institute. The test parameters (sensitivity and specificity) of the three kits were determined, and the result obtained from FMD 3ABC-I-ELISA kit was compared with that obtained from two foreign kits. The results indicated that the coincidence rate between the FMDV 3ABC-I-ELISA and Ceditest® kits was 98.05%, and the coincidence rate between the FMDV 3ABC-I-ELISA and UBI® kits was 94.4%; the sensitivity of both Ceditest® and FMDV 3ABC-I-ELISA kit was 100%. However, the sensitivity of the UBI® kit was only 81.8%. With sera from naive or vaccinated non-infected animals, the specificity of all tests exceeded 90%.

Key words: Foot-and-mouth disease virus (FMDV); Non-structural protein 3ABC; ELISA

The non-structural proteins (NSPs) of Foot-and-mouth disease virus (FMDV) have received considerable attention in recent years with the search for improved serological tests for the virus(1,5,7,13,14). The virus neutralization test (VNT) (6) and liquid phase blocking ELISA (LPBE) are currently the prescribed tests by the OIE (Office International des Epizooties). However, these tests require each serotype to be tested separately, are time consuming to perform, require virus containment facilities and cannot differentiate vac-

inated from convalescent animals. The development of a simple, quick to use test that covered all 7 serotypes as differentiating vaccinated from convalescent animals would be a major advance in the epidemiological tool for FMDV diagnosis.

The NSP s are only expressed in animals with replicating virus, therefore only animals that have been infected with live virus should develop antibodies to these proteins(1, 2, 7, 11). The currently used inactivated vaccines had been purified to remove cellular

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proteins and NSPs and should not induce antibodies to these proteins. However, in practice, vaccinated animals do produce antibodies to some of the NSPs such as 3D(13) and antibodies to others, such as 2C, were found to rapidly fall below detectable levels(10). The 3ABC proteins appear to be the most promising protein as diagnostic antigens(3, 8, 12, 15).

The following paper described a comparison of the Ceditest® kit and UBI® kit with the FMDV 3ABC-I-ELISA kit developed at the Lanzhou Veterinary Research Institute. From the results, it could be concluded that the 3ABC antibody was the most reliable single indicator of infection. Some reference indicated that the immune response to the 3ABC appeared early after infection and the antibody to 3ABC could be detected longer than antibody to any other NSPs(4). It can be concluded that the FMD 3ABC-I-ELISA kit developed in China will be a useful tool in FMD control and eradication programmes facilitating the detection of virus circulation in FMD vaccinated populations.

1. Materials and methods

1.1 Sera

In the test, three sets of sera were included: 1). 60 sera collected from naive cattle before vaccination or infection. No serum neutralization antibody to FMDV was detected for these sera. 2). 249 “post-vaccination” sera were collected from cattle 3 weeks after the third vaccination with inactivated commercial FMD vaccine. 3). 66 “post-infection” sera, collected from 5 cattle experimentally infected with FMDV China/99 and sequentially bled at regular intervals for up to 12 months post-infection. Two of them were collected for up to 15 months post-infection.

1.2 ELISA

NSPs antibody were examined using three ELISA kits: the commercially available Ceditest® kit (Netherlands), the UBI® kit (USA) and the FMDV 3ABC-I-ELISA kit developed in the Lanzhou Veterinary Research Institute, China.

1.2.1 FMDV 3ABC-I-ELISA kit

ELISA plates were coated with purified 3ABC fusion proteins in carbonate / bicarbonate buffer at pH 9.6. Plates were washed five times between stages with phosphate buffered saline (PBS) containing 0.05% Tween-20. After blocking with 5% sucrose, 10% equine sera in PBST, test or reference sera were diluted 1 : 100 in blocking buffer (PBST, 5% non-fat dry milk, 10% equine sera, 1% *E.coli* lysate) and added to the plates in duplicate. Plates were incubated for 1h at 37°C and washed 5 times with PBST, and then incubated with 1 : 1 500 dilution of anti-bovine IgG peroxidase conjugate (Sigma chromogen.) for 1h at 37°C. After the final wash, chromogen mixture was added (OPD plus 0.004% H₂O₂ in phosphate-citrate buffer, pH 5.5). Color development was stopped after 15 min by the addition of 1.25 mol/L H₂SO₄. The optical density(OD) of each well was read at 492 nm for background correction. Results were calculated as follows using the means of the pairs of samples and the median of the 4 positive and negative controls on each plate.

$$\text{Value} = (OD_{\text{sample}} - OD_{\text{neg}}) / (OD_{\text{pos}} - OD_{\text{neg}})$$

We consider values below 0.2 as negative, values between 0.2 and 0.3 as ambiguous and values above 0.3 as positive.

1.2.2 Ceditest® kit

The Ceditest® kit was performed according to the manufacturer's instructions. Briefly, ELISA plates coated with 3ABC specific monoclonal antibody were incubated with the 3ABC protein. After washing plates,

sera samples were dispensed to the wells and incubated. After wash, conjugate was diluted according to the recommendation of the manufacturer and was incubated for 1h at room temperature. After the final wash, the chromogen/substrate was allowed to react for 20 min at room temperature before addition of stop solution. Optical density (OD) was read at a wavelength of 450 nm .

The percentage inhibition (PI) of the reference sera and the test sera were calculated according to the formula below.

$$PI=100-(OD_{100} \text{ test serum}/OD_{100} \text{ maximum} \times 100)$$

The manufacturer's recommended interpretation is PI less than 50 is negative and greater than or equal to 50 is positive.

1.2.3 UBI® kit

According to the manufacturer's instructions, the serum was diluted 1 : 21 and added to 96 wells microtitre plate pre-coated with the purified, synthetic FMDV 3B NS peptide. Antibodies to FMDV 3B were bound to the antigen forming an antigen/antibody complex on the plate surface. Unbound antibody was washed away. A conjugate was added which bound to any antibody/antigen complexes. Unbound conjugate was removed by washing. After adding the TMB substrate, blue color developed in proportioned to the amount of FMDV NS-specific antibodies present, if any, in the serum samples tested. This enzyme-substrate reaction was terminated by the addition of a solution of sulfuric acid to give a yellow color. The color changes that had occurred in each well were then measured spectrophotometrically at a wavelength of 450 nm, the Cutoff value was calculated according to the formula below.

$$\text{Cutoff value}=0.23 \times OD_{pos}$$

The manufacturer's recommended interpretation is absorbance values of specimens less than the Cutoff-

value are negative, greater than or equal to the Cutoff value are positive.

2. Results

2.1 The specificity and sensitivity of the FMDV3ABC-I-ELISA kit

The specificity (Sp) of the FMDV 3ABC-I-ELISA kit was evaluated with sera collected from non-vaccinated and vaccinated animals. A total of 60 sera from non-vaccinated cattle were tested for antibodies to 3ABC, observing that 1 out of 60 sera gave a positive reaction (Table 1). In addition, 249 sera, collected from vaccinated cattle were also tested, indicating that 8 out of 249 gave a positive reaction (Table 1).

Thus, the specificity of the FMDV 3ABC-I-ELISA kit was over 96% when used to examine sera from naive and vaccinated cattle (Table 1). For the calculation of specificity, results in the "doubtful" range were counted as positive.

The sensitivity (Se) of the assay was evaluated by examining 66 serum samples from experimentally infected cattle. All sera gave a positive reaction, thus the sensitivity of the assay was 100%.

2.2 The Specificity and sensitivity of the Ceditest® and UBI® kit

The specificity and the sensitivity of the Ceditest® kit and UBI® kit were evaluated using the same sera. Sixty-seven out of 249 vaccinated sera were not tested because of limited Ceditest® kit (Table 2).

Table 1. Specificity of the FMDV 3ABC-I-ELISA kit

Background of cattle	No. of positive/ no. of tested	Specificity (%)
Sera without vaccination	1/60	98.3%
Sera with vaccination	8/249	96.8%

Table 2. Specificity and sensitivity of the Ceditest® kit and UBI® kit

Background of cattle	Ceditest®	UBI®
	Data of positive/data tested (specificity or sensitivity)	Data of positive/data tested (specificity or sensitivity)
Non-vaccinated sera	2/60(Sp=96.7%)	1/60(Sp=98.3%)
Vaccinated sera	6/182(Sp=96.7%)	1/249(Sp=99.6%)
Experimentally infected sera	66/66(Se=100%)	54/66(Se=81.8%)

The sensitivity of Ceditest® kit and UBI® kit was evaluated by examining 66 sera from experimentally infected cattle (Table 2). As the table showed, the specificity of the Ceditest® kit exceeded 96% when used to examine vaccinated or non-vaccinated sera. By comparison, the specificity of the UBI® kit was extremely high though the sensitivity was low.

The results showed that the UBI® kit had a low sensitivity, the sensitivity was only 81.8%, 54 out of 66 sera gave positive results, 12 out of 66 sera gave negative results, most of these sera were bled after the 12th month post-infection. In contrast, the Ceditest® kit had a high degree of sensitivity.

2.3. Comparison of coincidence rate

The results obtained from the FMDV 3ABC-I-ELISA kit was compared with those of two foreign ELISA kits (Table 3 and Table 4). 302(70+232) out of 308

showed the same results as shown in Table 3, corresponding to a coincidence rate of 98.05%. In contrast, only 354(55+299) out of 375 showed the same results as that indicated in Table 4, corresponding to a coincidence rate of 94.4%. One possible reason is that the UBI® kit detects the anti-3B antibody which exists in sera of cattle not more than 364 days, so some experimentally infected sera gave negative results (Table 4). However, anti-3ABC antibodies could be detected in experimentally infected cattle up to 742 days post-infection(1).

3. Discussion

The results indicate that both the Ceditest® kit and the FMDV 3ABC-I-ELISA kit perform particularly well as compared with the UBI® kit, the sensitivity of the UBI® kit is low though the specificity is very high.

Table 3. Comparison of FMDV 3ABC-I-ELISA kit with ceditest®kit

Background of cattle	No.of tested	FMDV 3ABC-I-ELISA		Ceditest® (Coincidence No.of)	
		Positive	Negative	Positive	Negative
Non-Vaccinated	60	1	59	2(0)	58(57)
Vaccinated	182	5	177	6(4)	176(175)
Infected	66	66	0	66(66)	0
Total	308			70	232

Table 4. Comparison of FMDV 3ABC-I-ELISA kit with UBI®kit

Background of cattle	No. of tested	FMD 3ABC-I-ELISA		UBI®(No. of coincidence)	
		Positive	Negative	Positive	Negative
Non-Vaccinated	60	1	59	1(0)	59(58)
Vaccinated	249	8	241	1(1)	248(241)
Infected	66	66	0	54(54)	12(0)
Total	375			55	299

It is worthy to note that the sensitivity and the specificity are not fixed values and will vary between sub-population and between populations conditional on the distribution of influential covariates. Although the UBI® kit is less than perfect, it still may be useful depending on the circumstances in which it is used.

The 3ABC protein was the most antigenic of the NS proteins and it had been argued that antibody specific for this protein could be the most useful marker of viral replication(2). The results described here support these hypothesis, as all sera from infected cattle gave positive results in both FMDV 3ABC-I-ELISA kit and Ceditest® kit. In contrast, 12 out of 66 sera from infected cattle gave negative results in the UBI® kit. A possible reason is that the UBI® kit use a synthetic peptide 3B as the antigen for detection of antibodies to FMDV NSP. The use of this synthetic peptide minimizes the incidence of non-specific reactions originating from antibody reactivity in the specimen towards host cell antigens or expression vector antigens which are copurified with virus-derived or recombinant-derived antigens(7, 9, 12).

However, antibody to 3B falls below detectable levels before antibody to 3ABC of FMDV (which may be detectable 742 days post-infection).

In short, by comparing with two foreign ELISA kits, we can draw the following conclusions: the FMDV 3ABC-I-ELISA kit developed in China can be used in laboratories with limited facilities for FMD diagnosis. It is well suited for use in large-scale serological survey. In addition, the high sensitivity and specificity of the test make the FMDV 3ABC-I-ELISA kit a potentially valuable diagnostic tool for FMD eradication campaigns and control programs. The study reported here demonstrates that animals that have been infected with FMDV can be differentiated from those that have been

vaccinated on the basis of the detection of antibody to 3ABC.

References

1. Berger H G, Straub O C, Ahl R, *et al.* 1990. Identification of foot-and-mouth disease virus replication in vaccinated cattle by antibodies to non-structural virus proteins[J]. *Vaccine*, 8(3): 213-216.
2. Bergmann I E, de Mello P A, Neitzert E, *et al.* 1993. Diagnosis of persistent aphthovirus infection and its differentiation from vaccination response in cattle by use of enzyme-linked immunoelectrotransfer blot analysis with bioengineered non-structural viral antigens[J]. *Am J Vet Res*, 54(6): 825-831.
3. Bergmann I E, Malirat V, Neitzert E, *et al.* 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 [J]. *Arch Virol*, 145(3): 473-489.
4. Clavijo A, Wright P, Kitching P. 2004. Developments in diagnostic techniques for differentiating infection from Vaccination in foot-and-mouth disease [J]. *The Vet J*, 167:9-22.
5. De Diego M, Brocchi E, Mackay D, *et al.* 1997. The non-structural polyprotein 3ABC of foot-and-mouth disease virus as a diagnostic antigen in ELISA to differentiate infected from vaccinated cattle[J]. *Arch Virol*, 142(10): 2021-2033.
6. Golding S M, Hedger R S, Talbot P. 1976. Radial immunodiffusion and serum-neutralisation techniques for the assay of antibodies to swine vesicular disease[J]. *Res Vet Sci*, 20(2): 142-147.
7. Lubroth J, Brown F. 1995. Identification of native foot-and-mouth disease virus non-structural protein 2C as a serological indicator to differentiate infected from vaccinated livestock [J]. *Res Vet Sci*, 59(1): 70-78.
8. Mackay D K, Forsyth M A, Davies P R, *et al.* 1998. Differentiating infection from vaccination in foot-and-mouth disease using a panel of recombinant, non-structural proteins in ELISA[J]. *Vaccine*, 16(5): 446-459.
9. Meyer R F, Babcock G D, Newman J F, *et al.* 1997. Baculovirus expressed 2C of foot-and-mouth disease virus has the potential for differentiating convalescent from vaccinated animals[J]. *J Virol Methods*, 65(1): 33-43.
10. Mezcencio J M, Babcock G D, Meyer R F, *et al.* 1998.

- Differentiating foot-and-mouth disease virus-infected from vaccinated animals with baculovirus-expressed specific proteins[J]. *Vet Q*, 20(Suppl 2)S11-13.
11. **Pinto A A, Garland A J.** 1979. Immune response to virus-infection-associated (VIA) antigen in cattle repeatedly vaccinated with foot-and-mouth disease virus inactivated by formalin or acetyleneimine[J]. *J Hyg (Lond)*, 82(1): 41-50.
 12. **Shen F, Chen P D, Walfield A M, et al.** 1999. Differentiation of convalescent animals from those vaccinated against foot-and-mouth disease by a peptide ELISA [J]. *Vaccine*, 17 (23-24): 3039-3049.
 13. **Sorensen K J, Hansen C M, Madsen E S, et al.** 1998. Blocking ELISA using the FMDV non-structural proteins 3D, 3AB and 3ABC produced in the baculovirus expression system[J]. *Vet Q*, 20(Suppl 2): 17-20.
 14. **Sorensen K J, Madekurozwa R L, Dawe P.** 1992. Foot-and-mouth disease: detection of antibodies in cattle sera by blocking ELISA[J]. *Vet Microbiol*, 32(3-4): 253-265.
 15. **Sorensen K J, Madsen K G, Madsen E S, et al.** 1998. Differentiation of infection from vaccination in foot-and-mouth disease by the detection of antibodies to the non-structural proteins 3D, 3AB and 3ABC in ELISA using antigens expressed in baculovirus[J]. *Arch Virol*, 143(8): 1461-1476.