Use of a Baculovirus-Expressed Structural Protein for the Detection of Antibodies to Foot-and-Mouth Disease Virus Type A by a Blocking Enzyme-Linked Immunosorbent Assay

Young-Joon Ko,1,4† Hyang-Sim Lee,3 Hye-Young Jeoung,1 Eun-Jeong Heo,1 Hyo-Rim Ko,1 Byung-Sik Chang,2 Hoo-Don Joo,2 U. Gerelmaa,3 B. Dashzeveg,3 S. Tserendorj,3 R. Sodnomdarjaa,3 Jong-Hyeon Park,1 Chang-Hee Kweon,1 In-Soo Cho,1 and Sang-Gi Paik1

National Veterinary Research and Quarantine Service, Anyang, Gyeonggi 430-824, South Korea1; Department of Research and Development Laboratory, Jenobiotech Inc., Chuncheon, Gangwon, South Korea2; States Central Veterinary Laboratory, Ulaanbaatar 210153, Mongolia3; and Department of Biological Science, Chungnam National University, Daejeon, South Korea4

Received 19 September 2009/Returned for modification 13 October 2009/Accepted 27 October 2009

Foot-and-mouth disease (FMD) is a highly contagious disease that affects cloven-hoofed animals such as cattle, pigs, and goats (12). The causative agent, foot-and-mouth disease virus (FMDV), belongs to the genus Aphthovirus in the family Picornaviridae (3). FMDV is a nonenveloped virus containing a single-stranded RNA genome of about 8,500 bases surrounded by an icosahedral capsid that comprises 60 copies of four structural proteins: VP1, VP2, VP3, and VP4 (10). It is classified into seven serotypes (serotypes O, A, Asia 1, C, SAT 1, SAT 2, and SAT 3), and multiple subtypes occur within each serotype (14).

Although the virus neutralization test (VNT) is recognized as a standard method for the detection of antibodies to FMDV structural proteins (21), it is laborious and time-consuming and requires a biosecurity facility (19). The liquid-phase blocking (LPB) enzyme-linked immunosorbent assay (ELISA) (11) has been widely applied as a surrogate for the VNT. However, it still has a drawback of utilizing inactivated FMDV as the diagnostic antigen. There is always a risk of virus escape from a laboratory when live FMDV is manipulated to produce diagnostic antigen. In fact, there have been several outbreaks through a laboratory when live FMDV is manipulated to produce diagnostic antigen. There is always a risk of virus escape from laboratories in Germany in 1987 and 1988, in Russia in 1993, and in England in 2007 (13, 17, 26).

To avoid exposure to the live virus, recombinant structural proteins for FMDV type O and type Asia 1 were previously described as diagnostic antigens or vaccine candidates (5, 16, 17, 20). However, a serological method based on recombinant protein antigens for FMDV type A has not yet been developed. Since FMDV type O and type A are the most prevalent throughout the world (13, 15, 27), we developed and evaluated a blocking ELISA using a baculovirus-expressed structural protein and monoclonal antibody (MAb) for the detection of antibodies to FMDV type A in this study.

FMDV type A (A22 IRQ 24/64) was obtained from the Institute for Animal Health (Pirbright Laboratory, Surrey, United Kingdom). Viral RNA was extracted from FMDV type A-infected IBRS-2 cells with an RNase extraction mini kit (Qia-gen). Complementary cDNAs for the P1 and 3C genes were produced by using random hexamers and an AccuPower reverse transcriptase premix (Bioneer, Daejeon, South Korea). The genes were amplified from cDNA by using nPfu DNA polymerase (Enzymomics, Seoul, South Korea). The following primers were designed on the basis of the sequence with GenBank accession no. AY593780: primer P1 forward (5′-GAG GATCCATGGGTGCCGGCAATCC AGCCCG-3′), primer P1 reverse (5′-AATGACTTACTTGTTGAGGTCAAT GAT-3′), primer 3C forward (5′-GATTTCGAGATGAGTGG TTGCCCTTCCGACCAGG-3′), and primer 3C reverse (5′-TAC AGCATGCTACTCGTGGTGCGGCTCAGGGTC-3′). The incorporated restriction enzyme sites are underlined. The P1 gene was amplified in a thermal cycler with an initial denaturation at 95°C for 2 min, followed by 35 cycles at 95°C for 30 s, 55°C for 1 min, and 72°C for 2 min 30 s and a final extension at 72°C for 5 min. PCR amplification of the 3C gene was conducted as described above, except that the elongation step was at 72°C for 1 min. Each of the amplified P1 and 3C genes was cloned separately into a pFastBacDual vector (Invitrogen). The P1 gene was inserted under the polyhedrin promoter by using BamHI and SpeI. The 3C gene was inserted under the polyhedrin promoter by using XhoI and SpeI. The 3C gene was inserted under the polyhedrin promoter by using XhoI and SpeI. The 3C gene was inserted under the polyhedrin promoter by using XhoI and SpeI. The 3C gene was inserted under the polyhedrin promoter by using XhoI and SpeI. The 3C gene was inserted under the polyhedrin promoter by using XhoI and SpeI.
frozen and thawed three times and clarified by centrifugation at 10,000 × g for 30 min. The supernatant fraction of the recombinant structural protein was used as the diagnostic antigen for this study.

The recombinant protein expressed in Sf9 cells was identified by immunofluorescence assay, as described previously (16), with rabbit serum raised against the FMDV VP1 peptide (GAGRRGDLGPLAARTAAQLPA)\(^{160}\), based with GenBank accession no. AF204108; A22 India 17/77 (Fig. 1A) and the 3C peptide (MLDGRAMTDSYR)\(^{70}\) based on the sequence with GenBank accession no. AY312587; O/SKR/00 (Fig. 1B).

To investigate whether the positive reactivity was derived from the P1 precursor or the VP1 subunit cleaved by the 3C protease, Western blot analysis was conducted, as described previously (16), with MAb 1G73, which is specific for FMDV type A (Fig. 1C). The band corresponding to VP1 was observed, but there was no trace of the P1 band, indicating that the fluorescent reactivity noted above was mainly due to the VP1 protein cleaved by the 3C protease and not the P1 precursor.

To examine the time course profiles of protein expression, the recombinant protein was harvested daily after recombinant baculovirus infection of Sf9 cells and was analyzed by Western blotting with MAb 1G73 (Fig. 1C). The band corresponding to VP1 was observed at 3 dpi postinfection (dpi). The band intensity peaked at 4 dpi and was maintained at the same level until 8 dpi.

Since many livestock are susceptible to FMDV, the use of an assay with a blocking format and an MAb was essential so that sera from many species could be applied in the same manner. Also, the use of an MAb ensured consistent quality compared to that which would be obtained with a polyclonal serum because of the minimization of batch-to-batch variation.

The competitor MAb in the blocking ELISA was generated by immunizing a VP1 peptide corresponding to the GH loop, which is known to be immunologically important as a cell receptor site (1, 4, 8, 22, 24). In this regard, neutralizing MABs were produced by immunizing mice with a VP1 peptide (GAGRRGDLGPLAARTAAQLPA)\(^{160}\), based on the sequence with GenBank accession no. AF204108; A22 India/17/77. Although MAb 1G73 was used to identify the recombinant structural protein by Western blotting in this study, it did not work as well as MAb 6G13 as a competitor in the present blocking ELISA (data not shown). Whereas MAb 6G13 showed reactivity for FMDV type A with an optical density (OD) above 2.0, it did not react with type O and Asia 1 with an OD below 0.1 by indirect ELISAs with peptide antigens and inactivated FMDV antigens. MAB 6G13 was purified with an ImunoPure IgG purification kit (Pierce, Rockford, IL) and was labeled with horseradish peroxidase by use of a peroxidase labeling kit (Roche Diagnostics, Basel, Switzerland).

Because FMDV type A is considered to be the most antigenically diverse among the different serotypes (15), it was necessary to examine whether MAb 6G13 from the A22 India sequence could bind to peptides from other strains of type A virus. MAb 6G13 showed reactivity against the A24 Cruz/Brazil/55 strain by the peptide ELISA and the A22 IRQ 24/64 strain virus by immunofluorescence assay (data not shown). The reactivity with A22 IRQ 24/64 was anticipated because only 3 of 22 amino acids are different between the VP1 peptides of the two strains. In contrast, the reactivity with A24 Cruz/Brazil/55 was notable because 7 of 22 amino acids are different between the VP1 peptides. Although these results may indicate that MAb 6G13 might bind to a broad spectrum of type A strains, more extensive tests need to be conducted with other strains of FMDV type A.

The blocking ELISA with the recombinant protein (the RP ELISA) was conducted as described previously, with some modifications (16). A trapping antibody of the RP ELISA, MAb 70-17, was previously shown to exhibit broad reactivity with FMDV types O, A, Asia 1, and C; but it did not have FMDV neutralizing activity (20). Because the RP ELISA is a sandwich ELISA in which MAb 70-17 is used for coating to trap the recombinant protein antigens, it was unnecessary to purify the antigens to remove other proteins inherent to the insect cells. The optimal conditions were initially determined for all components. The recombinant protein antigen and the test sera were diluted in the diluent at 1:8 and 1:5, respectively. MAb 6G13 conjugated with horseradish peroxidase was added at a concentration of 0.1 µg/ml. The OD value was converted to a percent inhibition (PI) value by the following formula: 100 × [1 − (OD\(_{test\ serum}/\)OD\(_{control}\))], where OD\(_{test\ serum}\) is the OD for the test serum sample and OD\(_{control}\) is the mean OD value for the wells containing MAb alone.

To define the cutoff level of PI to differentiate positive from
negative sera by the RP ELISA, naive sera (n = 1,760) derived from domestic cattle, pigs, and goats were tested (Table 1). The cutoff level of PI was established at 53% inhibition by calculating the mean PI value plus three times the standard deviation (14% + 3 x 13% = 53%). With this cutoff level, the RP ELISA exhibited specificities of 98.5%, 99.5%, and 99.1% with sera from cattle, pigs, and goats, respectively. In total, 19 of 1,840 serum samples were nonspecific positive, resulting in a specificity of 99% for the RP ELISA.

Hyperimmune sera were used to determine the detection limit of the RP ELISA and were collected from one cow and one pig at 2 weeks after the fourth vaccination with the type A22 IRQ vaccine (Bayer, Germany). Another hyperimmune caprine serum sample was collected at 2 weeks after the second vaccination with the same vaccine. The sera were serially diluted with the respective naive sera and were tested by the RP ELISA and the VNT (Fig. 2A to C), which was performed with A22 IRQ 24/64 and IBRS-2 cells, and a virus neutralization (VN) titer equal to or greater than 45 was considered a positive result, as described previously (21). The results were comparatively analyzed by identifying the last serum dilution defined as positive by each assay on the basis of the cutoff of 53% inhibition for the RP ELISA and a VN titer of 45 (5.5 log$_2$) for the VNT. The RP ELISA exhibited positive reactions for the serially diluted sera, and the VN titers were 22, 22, and less than 11 for the sera from the pig, goat, and cow, respectively.

To definitely evaluate the ability of the RP ELISA to detect antibodies to FMDV type A, international reference sera (Institute for Animal Health, Pirbright, Surrey, United Kingdom) comprising sera with strongly, weakly, and cutoff positive reactivities for FMDV type A22 IRQ, O1 Manisa, and Asia 1 Shamir were employed. The RP ELISA correctly scored the weakly positive serum of type A (Table 2). It has been suggested that the weakly positive serum be used as the minimum standard for the detection of antibodies in any test applied for herd-based serosurveillance (19). Although the RP ELISA did not score the cutoff positive serum sample as positive, this was consistent with previous results, which indicated that the OIE cutoff serum has been recognized as negative by ELISA methods for FMDV type O and type A (18, 19). While the Asia 1 strongly positive serum sample was positive by the RP ELISA, the type O strongly positive serum sample was negative. Although MAb 6G13 did not react with the peptide or inactivated FMDV from type Asia 1, the RP ELISA was found to cross-react with the strongly positive serum of type Asia 1. This

![FIG. 2. Defining the detection limit of the RP ELISA for bovine, swine, and caprine sera (A to C) and the early antibody response of the RP ELISA, the LPB ELISA, and the virus neutralization test for three goats (goats 93, 95, and 96) immunized with FMDV type A monovalent vaccine (D to F). Virus neutralizing titers below 11 are represented as 3 log$_2$ for simplicity.](image-url)
seemingly contradictory result may be explained by the notion that epitopes on the antigen surface may be blocked by the steric hindrance or the conformational changes induced by antibodies binding to other epitopes (25). However, the cross-reactivity among FMDV serotypes was also reported in a previous study in which the authors presumed that the cross-serotype reactivity could be an advantage rather than a disadvantage in the case of a simultaneous outbreak caused by different serotypes by focusing attention on animals suspected of being infected (6). Therefore, it was assumed that some degree of cross-reactivity among serotypes could be inherent to assays for the detection of antibodies to FMDV structural proteins. The RP ELISA remains to be evaluated with more serum samples derived from other serotypes to determine the degree of cross-reactivity among serotypes.

To investigate the time course of detection of early antibodies in FMDV-susceptible species, goats were immunized intramuscularly with the FMDV strain A22 IRQ 24/64 vaccine (Indian Immunologicals, Hyderabad, India). The caprine sera were collected at 4, 7, 10, 14, 21, and 28 days postvaccination (dpv). The RP ELISA detected early antibodies at 7 dpv, and the titers above the cutoff were maintained throughout the experiment (Fig. 2D to F). This result was in accordance with that of the VNT. The LPB ELISA, in which a titer above 45 was considered positive with a cutoff of 50% inhibition (21), also resulted in the cleavage of a larger amount of VP1 from P1 than an equal ratio of P1 to 3CD (7). Similar strategies have already been employed for FMDV type O and type Asia 1 (5, 7, 16, 20).

Although an empty capsid, such as pentamer-like particles for type O (20), was not observed, it was apparent that the recombinant structural protein preserves the epitopes of authentic FMDV to some degree because the results of the RP ELISA were comparable to those of the VNT and the LPB ELISA with sera from vaccinated animals. These results were not achieved by a blocking ELISA with the VP1 peptide, the VP1 protein, or P1 (data not shown).

Currently, the potencies of FMD vaccines are assessed in vivo by challenging fully susceptible vaccinated cattle. However, many studies have discussed alternative FMD vaccine potency tests that are amenable from an animal welfare perspective and show better statistical reliability (1, 2, 9, 23). In this regard, the RP ELISA could be employed as a simple and reliable assay to replace the LPB ELISA or the VNT. Most of all, the diagnostic antigen could easily be produced without any biosecurity concerns, which is an advantage of the RP ELISA over the other current assays.

Besides its utility as a diagnostic antigen, the recombinant structural protein may shed light on the development of a recombinant vaccine to replace the current inactivated FMDV vaccine for type A in the future. Some prospects have already been reported for FMDV type Asia 1 (5, 17).

In summary, this is the first report that a blocking ELISA with a baculovirus-expressed structural protein and an MAb has the potential to be an alternative to the current assay for the detection of antibodies to FMDV type A. This RP ELISA should be a useful tool that will meet the needs for epidemiological analysis at the time of an outbreak, along with nationwide surveillance.

We thank V. A. Srinivasan of the Research and Development Center from Indian Immunologicals for the generous provision of a monovalent vaccine for FMDV type A.
This work was supported by a grant from the National Veterinary Research and Quarantine Service, Ministry for Food, Agriculture, Forestry and Fisheries, South Korea.

REFERENCES

1. Acharya, R. E., Fry, D., Stuart, G., Fox, D., Rowlands, and F. Brown. 1989. The three-dimensional structure of foot-and-mouth disease virus at 2.9 Å resolution. Nature 337:769–771.

2. Barnett, P. V., R. J. Statham, W. Vosloo, and D. T. Haydon. 2003. Foot-and-mouth disease vaccine potency testing: determination and statistical validation of a model using a serological approach. Vaccine 21:3240–3248.

3. Belsham, G. J. 1993. Distinctive features of foot-and-mouth disease virus, a member of the picornavirus family; aspects of virus protein synthesis, protein processing and structure. Prog. Biophys. Mol. Biol. 60:241–260.

4. Bittle, J. L., R. A. Houghten, H. Alexander, T. M. Shinnick, J. G. Sutcliffe, R. A. Lerner, D. J. Rowlands, and F. Brown. 1982. Protection against foot-and-mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. Nature 298:30–33.

5. Cao, Y., Z. Lu, J. Sun, X. Bai, P. Sun, H. Bao, Y. Chen, J. Guo, D. Li, X. Liu, and Z. Liu. 2009. Synthesis of empty capsid-like particles of Asia I foot-and-mouth disease virus in insect cells and their immunogenicity in guinea pigs. Vet. Microbiol. 137:10–17.

6. Chenard, G., K. Miedema, P. Moonen, R. S. Schrijver, and A. Dekker. 2003. A solid-phase blocking ELISA for detection of type O foot-and-mouth disease virus antibodies suitable for mass serology. J. Virol. Methods 107:89–98.

7. Chung, Y. C., J. H. Huang, C. W. Lai, H. C. Sheng, S. R. Shih, M. S. Ho, and Y. C. Hu. 2006. Expression, purification and characterization of enterovirus-71 virus-like particles. World J. Gastroenterol. 12:921–927.

8. Cooke, J. N., and K. M. Westover. 2008. Serotype-specific differences in antigenic regions of foot-and-mouth disease virus (FMDV): a comprehensive statistical analysis. Infect. Genet. Evol. 8:855–863.

9. Goris, N., E. Maradit, R. D’Alia, N. Fontevila, N. Mattion, A. Perez, E. Smitsaart, H. J. Nauwynck, J. La Torre, E. Palma, and K. De Clercq. 2008. Foot-and-mouth disease vaccine potency testing in cattle using homologous and heterologous challenge strains: precision of the “Protection against Podal Generalisation” test. Vaccine 26:3432–3437.

10. Grubman, M. J., and B. Baxt. 2004. Foot-and-mouth disease. Clin. Microbiol. Rev. 17:465–493.

11. Hamblin, C. L. T. Barnett, and R. S. Hedger. 1986. A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus. I. Development and method of ELISA. J. Immunol. Methods 93:115–121.

12. Kitching, P., J. Hammond, M. Jeggo, B. Charleston, D. Paton, L. Rodriguez, and R. Heckert. 2007. Global FMD control—is it an option? Vaccine 25:5660–5664.

13. Klein, J. 2009. Understanding the molecular epidemiology of foot-and-mouth-disease virus. Infect. Genet. Evol. 9:153–161.

14. Knowles, N. J., M. H. Nazem Shirazi, J. Wadsworth, K. G. Swabey, J. M. Stirling, R. J. Statham, Y. Li, G. H. Hutchings, N. P. Ferris, U. Parlak, F. Ozoruk, K. J. Sumption, D. P. King, and D. J. Paton. 2009. Recent spread of a new strain (A-Iran-05) of foot-and-mouth disease virus type A in the Middle East. Transbound. Emerg. Dis. 56:157–169.

15. Knowles, N. J., and A. R. Samuel. 2003. Molecular epidemiology of foot-and-mouth disease virus. Virus Res. 91:65–80.

16. Ko, Y. J., H. Y. Jeoung, H. S. Lee, B. S. Chang, S. M. Hong, E. J. Heo, K. N. Lee, H. D. Joo, S. M. Kim, J. H. Park, and C. H. Kwon. 2009. A recombinant protein-based ELISA for detecting antibodies to foot-and-mouth disease virus serotype Asia 1. J. Virol. Methods 159:112–118.

17. Li, Z., Y. Yi, X. Yin, Z. Zhang, and J. Liu. 2008. Expression of foot-and-mouth disease virus capsid proteins in silkworm-baculovirus expression system and its utilization as a subunit vaccine. PLoS One 3:e2273.

18. Mackay, D. K., A. N. Bulat, T. Rendle, F. Davidson, and N. P. Ferris. 2001. A solid-phase competition ELISA for measuring antibody to foot-and-mouth disease virus. J. Virol. Methods 97:33–48.

19. Niedbalski, W. 2004. Comparison of different ELISA methods for the detection of antibodies against foot-and-mouth disease virus (FMDV) type O. Bull. Vet. Inst. Pulawy 48:5–9.

20. Oem, J. K., J. H. Park, K. N. Lee, Y. J. Kim, S. J. Kye, J. Y. Park, and H. J. Song. 2007. Characterization of recombinant foot-and-mouth disease virus pentamer-like structures expressed by baculovirus and their use as diagnostic antigens in a blocking ELISA. Vaccine 25:4112–4121.

21. OIE. 2008. Foot-and-mouth disease, p. 198–199. In OIE manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees). OIE, Paris, France.

22. Pfaff, E., M. Mussgay, H. O. Bohm, G. E. Schulz, and H. Schaller. 1982. Antibodies against a preselected peptide recognize and neutralize foot and mouth disease virus. EMBO J. 1:689–694.

23. Robiolio, B., P. R. Grigera, O. H. Periolo, C. Seki, T. Bianchi, E. Maradei, and J. L. La Torre. 1995. Assessment of foot and mouth disease vaccine potency by liquid-phase blocking ELISA: a proposal for an alternative to the challenge procedure in Argentina. Vaccine 13:1346–1352.

24. Strohmaier, K., R. Franz, and K. H. Adam. 1982. Location and characterization of the antigenic portion of the FMDV immunizing protein. J. Gen. Virol. 59:295–306.

25. Sugiyama, M., R. Yoshiki, Y. Tatsuno, S. Hiraga, O. Itoh, K. Gamo, and N. Minamoto. 1997. A new competitive enzyme-linked immunosorbent assay demonstrates adequate immune levels to rabies virus in compulsorily vaccinated Japanese domestic dogs. Clin. Diag. Lab. Immunol. 4:727–730.

26. Valarcher, J. F., N. J. Knowles, V. Zakharov, A. Scherbakov, Z. Zhang, Y. J. Wang, Z. X. Liu, X. T. Liu, A. Sanyal, D. Hemadri, C. Toth, T. J. Rasool, B. Pattnaik, K. R. Schumann, T. R. Beckham, W. Linchongsubongkoch, N. P. Ferris, P. L. Roeder, and D. J. Paton. 2009. Multiple origins of foot-and-mouth disease virus serotype Asia 1 outbreaks, 2003–2007. Emerg. Infect. Dis. 15:1046–1051.

27. Valarcher, J. F., Y. Leforban, M. Rweyemamu, P. L. Roeder, G. Gerbier, D. K. Mackay, K. J. Sumption, D. J. Paton, and N. J. Knowles, 2008. Incursions of foot-and-mouth disease virus into Europe between 1985 and 2006. Transbound. Emerg. Dis. 55:14–34.