Appendix 35

Synthetic peptide-based serosurveillance and vaccine system for FMD

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Introduction

We have designed a synthetic peptide-based system for the control of FMD, having synthetic peptide-based NS immunoassays and peptide-based marker vaccines. The UBI® system for FMD has two indirect ELISAs for screening animals based on 3B non-structural protein (NS) synthetic antigen, for detection of animals that may be carrying infectious virus and for differentiation of exposed from vaccinated animals (the UBI® FMDV NS EIA SWINE and CATTLE) [1,2]. The addition to the UBI® system of two peptide-based confirmatory tests, a 3B NS blocking ELISA (the UBI®FMDV NS 3B Neutralization Assay) and a 3A NS indirect ELISA (the UBI® NS 3A EIA) is reported here. We report on the initial validation studies of these tests and on a large-scale field trial showing the overall performance of the UBI® NS immunosurveillance system that includes the 3B screening test and the two confirmatory immunoassays.

The other half of the UBI peptide-based system uses VP1 G-H loop peptide immunogens in marker vaccines against FMDV. The G-H loop peptide has been optimized for cross-reactivity to FMDV and for immunogenicity by the inclusion of cyclic constraint for relevant conformation, by extending the critical G-H loop site with adjoining VP1 sequences for additional Th epitopes and more authentic conformation, and by linkage to a promiscuous UBITh® T helper epitope. The extrinsic T cell help provided by the UBITh® site overcomes a lack of responsiveness to the G-H loop domain in many swine and cattle that is due to genetic restriction. The incorporation of consensus residues into the hypervariable positions of the G-H loop domain provides for broad cross-reactivities across strains. A novel consensus O immunogen (Fig. 1) was formulated into acceptable water-in-oil vaccine formulations and successfully protected swine from infection by heterologous FMDV $O_{1\ \text{Taiwan}}$ [3]. UBI vaccines serve as marker vaccines because of the absolute absence of NS antigens, and so are complementary when used together with the UBI NS peptide immunoassays. Furthermore, peptide-vaccinated animals can be readily identified by use of a UBI serotype-specific VP1 ELISA [2]. In the present communication we report on further efforts to develop our swine vaccine, including a dose response, a vaccine stability and a vaccine duration study. And, we present a preliminary formulation study directed towards application of the peptide-based vaccine to cattle.

Materials and Methods

Peptide synthesis

The peptide antigens for immunoassays and the peptide immunogens for vaccines were produced by synthesis on a solid-phase support using an Applied Biosystems Peptide Synthesizer Model 430A, and Fmoc protection for the α-NH_2 terminus and side chain protecting groups of trifunctional amino acids. Peptides having combinatorial library Th were prepared by providing a mixture of the desired amino acids at the specified positions. Completed peptides were cleaved from the solid support and side chain protecting groups removed by 90% trifluoroacetic acid. Synthetic peptide preparations, except for the library immunogens, were characterized for correct composition by Matrix-Assisted Laser Desorption Time-of-Flight Mass Spectrometry using a PerSeptive Biosystems/Vestec LaserTec Benchtop 11 Mass Spectrometer, and by Reverse Phase HPLC. The library immunogen for the vaccine was characterized by size exclusion chromatography to a specification that requires 90% of the integrated area to exceed a mass threshold limit value, and by Edman degradation for N-terminal amino acid analysis. Liquid phase cyclization of the peptide immunogens was accomplished by dissolving the peptides in water at 0.8 mg/ml, pH 3, adding DMSO to 1% (v/v) and adjusting to pH 7.5 with NH_4OH. The solution was incubated at ambient temperature in air and checked daily for 3 days by colorimetric assay using Ellman's reagent until disulfide bond formation was at least 90% complete.

Sera

A total of 14 positive sera (Table 1) from animals infected with various FMDV serotypes were obtained from the United States Department of Agriculture (USDA) and used in the study. To test the specificity of the confirmatory tests, 1612 normal swine sera and 1200 ruminant (800 cattle, 200 sheep, and 200 goats) sera, originally collected from US slaughterhouses, were purchased from Covance and Valley Biomedical and used in the study. In addition, 10,169 field sera samples from pigs vaccinated with commercial FMDV O vaccines, which were collected from pig farms in Taiwan in a nationwide serological survey in 2001, were also tested with the confirmatory tests.

ELISAs

All ELISA assays, including the NS EIA confirmatory tests, were performed as described in the Direction Inserts of the test kits. Briefly, 100 µl of diluted samples are added to the microtiter wells of the UBI® FMDV NS assay and allowed to react at 37˚C for I hour. The sample wells are then washed six times with UBI® Wash Buffer. A standardized preparation of HRP-conjugated ImmunoPure® Protein A/G (Pierce Chemical Co., Rockford IL, USA) is added to each well and incubated for 30 minutes at 37˚C. The wells are again washed six times and then reacted with 3,3',5,5'-tetramethylbenzidine (TMB) for 15 minutes. Reactions are stopped by the addition of 1.0 M H_2SO_4 and the A_{450} determined. For the FMDV NS 3B Neutralization Assay, serum samples were individually prediluted in duplicate with Specimen Diluent and with NS 3B Neutralization Buffer, before adding to NS Reaction Microplate coated with the NS 3B peptide [1]. The NS 3B Neutralization Buffer is equivalent in composition to the Specimen Diluent except that the 3B peptide was added to the buffer at a final concentration of 50 □g/ml. The rest of the assay procedure was identical to that of the

UBI FMDV NS EIAs. The neutralization or inhibition rate of the NS 3B Neutralization Buffer to each serum sample was calculated by the following formula:

$$\textbf{\% NEUTRALIZATION RATE (NR)} = \frac{(A_{450} \text{ of SD}) - (A_{450} \text{ of NB})}{(A_{450} \text{ of SD})} \times 100\%$$

Where "**A_{450} of SD**" is the absorbance of sample mixed with the Specimen Diluent; "**A_{450} of NB**" is the absorbance of sample mixed with the NS 3B Neutralization Buffer. Based on preliminary studies, the Cutoff for the NS 3B Neutralization Assay was set at 60% Neutralization Rate (NR); i.e. sera giving NR values equal or greater than 60% are considered to be true positive, those giving NR values less than 60% are considered to be false positive.

The procedure of the FMDV NS 3A EIA was identical to that of the FMDV NS (3B) EIA.

Neutralizing Antibody Assay

The quantitative N.A. assay for antibodies that neutralize FMDV is performed with BHK-21 cells in flat-bottomed microtiter plates. The test is an equal volume test in 50 µl. Starting from a 1:4 dilution, sera are diluted two-fold across the plate. Diluted sera are mixed with equal volumes of FMDV $O_{1\ Taiwan}$ (200 $TCID_{50}$/50 µl) and incubated at one hour at 37˚C. Cells at 1 x 10^6 cells/mL are added in medium containing 10% bovine serum. Microscopic examination is feasible after 48 hours. Titers are expressed as the reciprocal of the final dilution of serum at the 50% endpoint.

NS Enzyme-linked Immunoassays (EIAs) background

False-positive samples can impose a serious problem for immunoassays for detection of FMD. Because non-specific reactivity and cross reactivity can both give false-positive results in enzyme-linked immunoassays (EIAs), no EIA test can give a specificity of 100.0%. The UBI® FMDV NS EIAs are no exception to the rule. Despite their excellent specificities in detecting antibodies to the non-structural (NS) protein 3B in normal and vaccinated swine and cattle samples, which ranges from 97.5% to 99.3%, false positives still occur among samples from non-infected animals [1,2]. False-positive test results can be ominous uncertainties:

1. In the early phase of an outbreak, before official confirmation of FMDV infection, uncertainties to the accuracy of a positive test result can be devastating, as any delays to the final confirmation of FMD outbreak will lead to wider spread of the disease.
2. After the first case of FMD has been confirmed in an outbreak, false-positive test results can be severely damaging to farmers and economies. Quick detection is critical to effectively control this extremely contagious disease, and in many cases no time will be allowed for laborious confirmation tests. Consequently, all suspicious animals will be slaughtered. Farms and agricultural areas can be wrongly classified

as “infection zones” or “infection centers”, and emergency response resources will be wasted and local economies will be severely affected by this classification.

There are several OIE-recognized FMD diagnostic methods that can be used for confirmation of the NS EIA results. However, for the following reasons, those tests described in the OIE Manual of Standards for Diagnostic Tests and Vaccines (O.I.E. 2000) cannot be readily conducted or completed within a short period of time (a few hours).

1. Two non-immunological virus identification methods are described in the OIE Manual, virus isolation by tissue culture and reverse-transcription polymerase chain reaction (RT-PCR). Both methods require tissue samples isolated from the epithelial cells of the suspected animals. However, in many cases, it is very inconvenient, time-consuming, or impossible (due to termination) to isolate epithelial samples from suspected animals. It often requires veterinarians to go back to the farms that the sera samples were originally collected to obtain the epithelium samples of the suspected animals. Moreover, the tissue culture procedures for viral amplification and detection requires at least 1-2 days. This 1-2 days delay will be very costly in a real FMD outbreak situation, particularly in the early stage of an outbreak when no infection has been confirmed and no measures have been put into place to prevent or control its spread. RT-PCR is a highly specialized technique that requires unusual care and skill for accurate results.

2. There are also several enzyme-linked immunosorbent assays (ELISAs) described in the OIE Manual that can be used to confirm the NS EIA results. However, these serological methods use either antisera to the whole viral particle or to a major structural component of the virus (the 146S antigen), which can only detect FMDV infection in cases of high systemic viremia. Therefore, these methods are often not very sensitive. In addition, because polyclonal antisera against many viral proteins are used as the capturing and detecting antibodies, the specificity of these assays tends not to be very good.

3. The viral neutralization (VN) test is also a method for detection of antibodies to FMDV. This method requires growing cells by tissue culture, and therefore often takes at least 1-2 days. Furthermore, the sensitivity of this method is often not good enough because that in many cases FMDV infections do not trigger high titers of neutralizing antibodies, and that the virus serotype used in the VN test may not be of sufficient serological cross-reactivity to the infecting virus for high sensitivity. In addition, sera from vaccinated, but not infected, animals also contain neutralizing antibodies, which can complicate the interpretation of the result of the VN test.

4. The Western blot method for detection of anti-NS antibodies is laborious and time consuming, and requires a high degree of skill and experience, so cannot be considered an easy, fast, and convenient confirmation method.

Therefore, there is a need for an FMD confirmatory test that is sensitive and specific, easy to perform, and quick to finish and generate results. This confirmatory test, once developed will be extremely useful for confirmation of positive test results of FMDV NS EIAs. Fast and reliable confirmation of the NS EIA diagnostic results will be critical to FMD management and control, especially in the early phase of an outbreak and in the later eradication phases.

We have developed two confirmatory tests that are designed to rapidly confirm positive results by UBI® FMDV NS EIAs. The UBI® NS confirmatory tests, which consist of the UBI® FMDV NS 3B Neutralization Assay and UBI® FMDV NS 3A EIA, use the same indirect ELISA format that allows the assays to be easily performed and finished within two hours.

The UBI® FMDV NS 3B Neutralization Assay (NA) is a blocking ELISA that has been designed to confirm whether a positive seroreactivity identified by a UBI® FMDV NS EIA results from the specific reaction of serum antibodies to the NS 3B peptide. A 3B seroreactivity specifically directed to FMDV will be markedly reduced by mixing the serum with NS 3B Neutralization Buffer containing the soluble NS 3B peptide. In contrast, a non-specific 3B seroreactivity will be largely unaffected by mixing the sample with NS 3B Neutralization Buffer.

During the course of the 3B NA, each specimen is diluted in duplicate; in one well, the specimen is mixed and diluted with the Specimen Diluent of the UBI® FMDV NS EIA (SWINE) or (CATTLE), and in the other well, it is mixed with the NS 3B Neutralization Buffer. The NS 3B Neutralization Buffer is basically the Specimen Diluent plus the 3B peptide. The diluted samples are then added to the Reaction Microplate wells. FMDV NS 3B-specific antibodies, if present, bind with the liquid-phase 3B peptide antigen during mixing with the Neutralization Buffer, and most of them are no longer available for binding to the solid-phase 3B peptide coated on the Reaction Microplate. Non-specific antibodies do not bind to the solution-phase peptide antigen after mixing with the Neutralization Buffer, and remain capable of binding to the Reaction Microplate surface. Any specimen showing a reduction of A_{450nm} of certain percentage or greater in the FMDV NS EIA following mixing with 3B Neutralization Buffer will be considered seropositive (i.e., contains 3B-specific antibodies). Any specimen showing a reduction of the A_{450nm} of less than that percentage will be considered negative for antibodies to FMDV.

The UBI® FMDV NS 3A EIA employs for its sensitizing agent a 3A non-structural (NS) protein peptide that contains antigenic determinants taken from the immunoreactive domains of the NS 3A protein [1]. NS 3A is the first protein of the NS 3ABC polyprotein which has been most often used as the solid phase antigen in NS EIAs developed elsewhere. The NS 3A EIA had been tested by UBI® during initial research on selection of an NS antigen for development of a commercial NS EIA test. It was found in our previous study that the NS 3A polypeptide imparts the assay with excellent sensitivity that is as good as the NS 3B EIA [1]. However, in those studies on vaccinee samples, the FMDV NS 3A EIA had poorer specificity than that of the NS 3B EIA. That

finding was the basis for choosing NS 3B as the solid phase antigen for UBI® FMDV NS EIAs. The 3A peptide antigen may nevertheless be used for a confirmatory test. Having a 3A antigen as the solid-phase immunosorbent provides for an EIA that is serologically independent to the 3B-based UBI FMDV NS EIA. Thus, the FMDV NS 3A EIA (CATTLE) or (SWINE) provides an assay that is very similar to the UBI® FMDV NS EIA (CATTLE) or (SWINE) in principle and procedure, but capable of independent confirmation of the repeatably reactive samples identified by the 3B-based NS EIAs.

We describe in this report the results of initial validation studies of the UBI® NS EIA confirmatory tests.

EIA Results

Validation of FMDV NS confirmatory tests with FMDV positive sera

The confirmatory tests were tested against a panel of FMDV-infected animal sera obtained from the USDA (Table 1). It was found that both tests correctly identified all sera samples in the panel as positive. The NR of each sample was greater than 80% in the NS 3B Neutralization Assay, and the Signal/Cutoff ratio of each sample in NS 3A EIA was greater than 1.0 (Tables 2 and 3).

Validation of FMDV NS confirmatory tests with normal animal sera

The 1612 normal swine samples from the U.S. were first screened with UBI® FMDV NS (3B) EIA (SWINE). A total of 11 repeatably reactive samples were identified. These presumably false positive samples were then tested with FMDV NS 3B Neutralization Assay. Nine of the 11 repeatably reactive samples were identified as false positive by the Neutralization Assay. The remaining 2 positive samples were both identified as negative after testing with the FMDV NS 3A EIA (SWINE) (Table 4). Thus, a specificity of 99.87 % (1610/1612) was achieved when only the NS 3B NA was performed, and a combined specificity of 100.00% was achieved when both confirmatory tests were applied.

For the 1200 normal ruminant samples from the U.S., a total of 9 repeatably reactive samples were identified by UBI® FMDV NS (3B) EIA (CATTLE). These 9 false positive samples were tested by both NS 3B Neutralization Assay and FMDV NS 3A EIA (CATTLE). Two out of the 9 samples were identified as false-positive by the Neutralization Assay, and 6 out of the remaining 7 samples were found non-reactive by NS 3A EIA (CATTLE) (Table 5). Thus, a specificity of 99.42 % (1193/1200) was achieved when only the NS 3B Neutralization Assay was performed, and a combined specificity of 99.92% (1199/1200) was achieved when both confirmatory tests were applied.

Validation of FMDV NS confirmatory tests with sera from vaccinated swine

The samples tested were from a recent Taiwanese survey in which a total of 10,169 swine samples were collected from 85 farms, with 120 animals (60 sows and 60 growth pigs) randomly screened per farm. All animals had received at least two doses of FMDV O vaccines. The infection status of these animals was unknown since FMD is endemic in

Taiwan. From the serological screening by the UBI® FMDV NS EIA (SWINE), a total of 413 repeatably reactive (RR) samples were identified. Of the 413 RR samples, 330 were found to be false positive, i.e., the reactivities were not specific to the NS 3B peptide antigen, when tested by the FMDV NS 3B Neutralization Assay. The remaining 83 samples were subjected to the FMDV NS 3A EIA (SWINE), and 58 of them remained positive after the second confirmatory test. To analyze the true infection status of these 58 triple-positive samples, a herd analysis was applied. FMDV is highly contagious and thus multiple infections within a herd would be expected. Therefore, it was assumed that for herds where multiple triple-positive samples were identified, they are most likely to be true positives. Conversely, if a triple-positive sample was from a farm where only a single triple-positive sample was identified, it is likely that this sample was false positive. By these criteria, 50 out of the 58 triple-positive samples were found to be true positive, and the other 8 to be false positive. Thus, based on this analysis, the specificity on vaccinee sera for UBI® FMDV NS EIA (SWINE) plus the NS 3B NA was 99.18 % (10036/10119); the specificity for UBI® FMDV NS EIA (SWINE) plus both the confirmatory tests was 99.92% (10111/10119) (Table 6).

Vaccine results

Swine

For the vaccine dose response and stability trial shown in Table 7, the synthetic FMDV VP1 immunogen shown in Fig. 1 was dissolved into water and formulated into water-in-oil vaccines at the doses shown in the Table, with Seppic Montanide ISA 50v as the oil phase. The final volumes of peptide vaccines and placebo were kept constant at 1.0 ml, the commercial vaccine was in 2.0 ml. Pigs were immunized on weeks 0 and 4 by intramuscular injection and challenged with FMDV $O_{1\ Taiwan}$ on week 8 by injection of $10^{4.5}$ $TCID_{50}$ of pig-passaged virus. Swine were monitored for clinical signs of FMD. These included body temperatures elevated to ≥40º for three successive days, lameness and vesicular lesions on the snout and coronary bands of the legs. Groups 1-3 were a dose response experiment using a four-fold range from 3.12 to 50 µg. The vaccine was protective at all doses, so we were unable to estimate a PD_{50}. The neutralizing antibody titers by day of challenge were correlated to dose. Group 3, the group given the lowest dose, also had the lowest neutralizing responses.

Group 4 was given 50 µg doses of freshly prepared peptide vaccine. Groups 5 and 6 received comparable doses that had been aged for 6 months and one year, respectively, to evaluate vaccine stability. The vaccine remained fully stable and effective for at least six months as shown by the protected animals, while retaining significant potency for one year. The placebo controls all experienced disease, while the positive control group given a commercial FMDV $O_{1\ Taiwan}$ vaccine was fully protected.

Table 8 shows the results of a vaccine duration study. At the start of the study, pigs were given a single 25 µg I.M. dose of the FMDV O consensus immunogen in ISA 50v. At monthly intervals, groups of five were challenged by an FMDV PanAsia isolate. The duration of protective immunity lasted for at least six months.

Cattle

A synthetic serotype O peptide immunogen was prepared as water-in-oil vaccine formulations in ISA 50v, at a dose of 200 μg (Table 9). One group of three cattle was given that emulsion, which is equivalent to a higher dose of the vaccine that was fully protective in swine. A second group was given the emulsion augmented by a proprietary peptide stabilizer. These synthetic peptide vaccines were given I.M. on days 0 and 21. A control group was given placebo (ISA 50v only). The animals were challenged intradermolingually with 10^4 BID_{50} of a PanAsia O virus on day 42. The neutralizing antibody titers at day 35 were predictive of protection for the groups given either of the two vaccine formulations. However, only the group that received the vaccine formulation with peptide stabilizer was protected. The augmented formulation was needed to achieve protection in cattle.

The neutralizing antibody titers in both swine and cattle did not relate to protective immune responses. N.A. titers were low in the low dose group (Group 3) of swine who nevertheless were protected (Table 7), and high and predictive of protection in the cattle given the unaugmented peptide emulsion vaccine who nevertheless were not protected (Table 9). A lack of correlation between neutralizing antibodies and protective immunity suggests that other correlates of protection are involved.

Discussion

The peptide-based FMDV NS confirmatory tests provide a system that allows rapid confirmation of positive results of UBI® FMDV NS EIAs. In addition, because these confirmatory tests can screen out false-positive samples, they also allow end users to lower the Cutoffs of the UBI® FMDV NS EIAs, to raise assay sensitivity without severely degrading overall specificity. Although lowering the assay Cutoff for higher sensitivity will inevitably create more false positive results on the screening tests, the majority of those samples will be identified as false positive by the confirmatory tests.

In a recent study designed to test whether false positive samples created by lowered assay Cutoffs can indeed be eliminated by the confirmatory tests, we have confirmed the prediction that the confirmatory tests can improve the specificity of UBI® FMDV NS EIAs even though the Cutoffs of the assays were significantly reduced (results not shown). Thus, while the lowered Cutoffs increased the assay sensitivity, the subsequent application of the NS confirmatory tests more than compensated by increasing overall assay specificity. The combination of lowering assay Cutoffs and using confirmatory tests to sort out the positive samples allow net gains in both sensitivity and specificity.

An effective synthetic peptide-based vaccine for FMD has been developed for pigs and is now being developed for cattle. The UBI vaccine formulations are 1) effective without adverse local reactions, 2) they have stability beyond six months (the peptide antigens themselves are stable for years), 3) the consensus antigen approach provides for activity against a broad range of viruses in a given serotype, and 4) the peptide vaccines can be economically produced and sold at acceptable cost because of the small peptide doses. Moreover, as peptide-based vaccines, they have the unique advantages of absolute

biosafety in both manufacturing and use, high flexibility to quickly confront newly emerging subtypes because new peptide antigens can be rapidly manufactured. As subunit vaccines they have the advantage of being antigenic marker vaccines.

The peptide-based vaccine presented here reproducibly elicited protective immune responses in natural host species. The antigen was designed to accurately mimic the critical VP1 G-H loop epitope for cross-reactivity to FMDV. That it does so was demonstrated by the capability of the vaccine to produce neutralizing antibodies. However, protective immunity here was not well-correlated to neutralizing antibody titers, suggesting involvement of other correlates of immunity. The antigen was designed for enhanced immunogenicity by the addition of promiscuous T cell help and immunogenicity was further enhanced by oil adjuvant and a peptide stabilizer. We believe that these additional vaccine components contributed to other critical factors such as cytokine responses for innate immunity, and for altering the balance between Th1 and Th2 immune pathways. These issues are presently being investigated.

A peptide-based system for differential enzyme-linked immunoassays in combination with synthetic peptide vaccines is an integrated system for FMD control. The chemically defined immunogen of UBI vaccines are site-specific antigenic markers whose effects can be readily detected by the use of UBI® VP1 EIAs, useful for monitoring the effectiveness of vaccination campaigns. And, the VP1-specific responses to the peptide vaccines can be readily distinguished from the immune response to virus by the UBI® FMDV NS EIAs.

Literature Cited

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Figure 1

UBITh®1 FMDV O consensus immunogen for swine and cattle. T cell help is provided by the combinatorial library UBITh®1 site shown, linked through an ε-lysine to the consensus 0 VP1 G-H loop site [3]