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ARTICLE *in* ANALYTICAL CHEMISTRY · SEPTEMBER 2006

Impact Factor: 5.64 · DOI: 10.1021/ac060235e · Source: PubMed

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Multiplexed Detection of Antibodies to Nonstructural Proteins of Foot-and-Mouth Disease Virus

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Liquid array technology was used to develop a multiplexed assay for the detection of antibodies to viral nonstructural proteins (NSPs), raised in cattle in response to infection with foot-and-mouth disease (FMD) virus. Two assays, one based on recombinant NSPs and the other on synthetically produced peptides, were developed and compared side-by-side. Serum samples from serial bleeds of cattle, each experimentally infected with one of the seven serotypes (C, A, O, Asia, SAT1, SAT2, SAT3) of FMD virus were analyzed. A distinct pattern in the detection of NSP antibodies and a close correlation of the recombinant protein and peptide-based assays were observed. The detection of antibodies to NSPs is a method to differentiate FMD-infected and FMD-vaccinated animals, and a high-throughput assay would be an invaluable tool in the case of an outbreak of FMD in North America, when emergency vaccination may be utilized to spare vaccinated, noninfected animals from slaughter and subsequent disposal.

Foot-and-mouth disease (FMD) is a highly contagious viral disease that affects domestic and wild ruminants and swine. The disease is endemic in parts of the Middle East, Africa, Asia, and South America but periodically spreads to countries free of the disease as demonstrated by the outbreak of FMD in the UK in 2001.[1] This outbreak severely affected the UK economy, costing the taxpayer over U.S. $5 billion,[2] and required the destruction of millions of animals and the subsequent disposal of their carcasses.[3] Travel restrictions were implemented, and travel, tourism, and other private industries were significantly affected.

Routine vaccination is widely and successfully used to minimize the impacts of FMD in countries where the disease is endemic, and long-term vaccination plans may eventually allow a country to become disease-free, thereby enabling international trade opportunities.[4] However, vaccination is not practiced in North America and the EU, primarily because FMD virus (FMDV) is not present and it is difficult to routinely distinguish between vaccinated animals and those that are vaccinated and subsequently become infected. In the event of an outbreak of FMD in North America, where emergency vaccination is employed, a high-throughput testing strategy will be required to distinguish between those animals that have been vaccinated and those that have been infected and become carriers. The diagnosis and control of FMD is dependent on both clinical recognition in the field and confirmation of the presence and type of the virus by laboratory testing.

The FMD viral genome encodes a single polyprotein. Viral proteases cleave this polyprotein into viral polypeptides, including eight different nonstructural proteins (NSPs) designated as L, 2A, 2B, 2C, 3A, 3B, 3C, and 3D. During viral replication, antibodies are raised against the structural viral coat proteins and the NSPs. FMDV vaccines consist of chemically inactivated, semipurified, whole-virus particles grown in cell culture, and therefore, vaccinated animals should only raise antibodies to structural proteins. Several research groups have used recombinant NSPs to develop the basis for differentiation of naturally infected from vaccinated animals (DIVA) serology assays using an ELISA format,[5−9] a multiplexed, His-tagged, bead-based assay[10] and an enzyme-linked immunoelectrotransfer blot assay.[11] However, because vaccine purity levels vary with manufacturers,[12,13] some NSPs are contained in some vaccine formulations, and consequently, low levels of antibodies against NSP 3D have been detected in vaccinated

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animals.[13,14] DIVA assays based on the detection of NSP 3D alone, therefore, cannot provide definitive results.

Peptides have also been used as the basis of ELISA DIVA assays.[15,16] Peptides are simple to prepare, are easy to purify and characterize, and are stable when stored as lyophilized powders. Conversely, recombinant NSPs can copurify with antigens from the expression vector. Antibodies to *Escherichia coli* are commonly found in the sera of uninfected and vaccinated animals,[6,17,18] and this complicates the interpretation of recombinant protein DIVA assay results. Additionally, it has been suggested that a number of epitopes on the complete recombinant proteins could potentially exhibit cross-reactivity to other picornaviruses (swine vesicular disease virus and bovine and porcine enterovirus are examples of closely related picornaviruses).[19]

Luminex liquid array technology is an analyte detection system composed of polystyrene microspheres (beads) to which receptors such as antibodies, oligonucleotides, and small molecules, can be covalently conjugated. The platform enables the simultaneous detection of numerous analytes in a single assay, and there are now many examples of the creative use of this technology.[20,21] The liquid array consists of beads that are embedded with precise ratios of red and infrared fluorescent dyes yielding 100-bead sets, each with a unique spectral address. Analyte that is captured on a modified bead is detected using a secondary reagent, indirectly labeled with a fluorescent reporter (phycoerythrin). Each optically encoded and fluorescently labeled bead is then interrogated by a flow cytometer. A classification laser (635 nm) excites the dye molecules inside the bead and classifies the bead to its unique bead set. A reporter laser (532 nm) excites the fluorescent molecules bound to the bead surfaces and quantifies the assay at the bead surface. The flow cytometer is capable of reading several hundred beads per second; analysis can be completed in as little as 15 s and potentially up to 100 different analytes can be assayed simultaneously, thereby providing a high-throughput platform. It is also conducive to automation, employing high-throughput, liquid-handling robotics platforms that minimize human resources required for running routine screening.[22] Liquid array technology has been successfully applied to the detection of biothreat agents[23,24] and other infectious disease.[25−27]

Here, the development of two liquid array, multiplexed assays for the simultaneous detection of antibodies to a panel of FMDV NSPs is described. One assay employs recombinant NSPs covalently attached to beads, and the other uses synthetically produced peptides, i.e., amino acid sequences from the NSPs. These two serology assays were analyzed for their performance in detecting the level of NSP antibodies in a series of cattle serum samples taken over a 28−30-day experimental infection with FMDV. Serum samples were obtained from several cattle, each infected with one of the seven serotypes (C, A, O, Asia, SAT1, SAT2, SAT3) of FMDV.

MATERIALS AND METHODS

General Information. All reagent dilutions and assays were carried out in phosphate-buffered saline, pH 7.4; Tween 20 0.02% v/v; BSA 0.1% w/v; sodium azide 0.02% w/v (PBS-TBN) and filtered through Corning 0.45-μm filter systems before use.

Recombinant Proteins. Gel-purified recombinant NSPs 3A, 3B, 3ABC, and 3D were generated as described previously.[9] Briefly, recombinant proteins to the NSPs 3A, 3B, 3ABC, and 3D were produced by cloning PCR-amplified DNA fragments from cDNA of the FMDV strain O_1/Campos/Brazil/58.[28] The proteins were each covalently coupled to a unique carboxylate bead class (Luminex Corp.) using the carbodiimide activation as described below.

Peptides. Peptides 3A, 3B, 3D1, and 3D2 were commercially synthesized via standard Fmoc solid-phase synthesis and purified by reversed-phase HPLC (United Biochemical Research, Seattle, WA). The details of the peptides are outlined in the Supporting Information, and peptide 3D1 is the amino terminus of the 3D protein. Peptide 3D2 is an adjacent sequence. The peptide sequences of 3A and 3B were previously described,[15] (O_1/Campos/Brazil/58) and the sequences of the 3D peptides were obtained from published sequences in Genbank. The peptides were generally soluble in PBS, but residual insoluble material was removed by centrifugation. The lyophilized peptides were dissolved in PBS (pH 7.4, sodium azide 0.02% w/v) and diluted into 2-(*N*-morphilino)ethanesulfonic acid (MES; 0.1 M, pH 4.5), directly before the bead coupling procedure. The peptides 3A, 3B, 3D1, and 3D2 were each covalently coupled to a unique carboxylate bead class using the carbodiimide activation as described below.

Bead Coupling. One milliliter (1.25×10^7) of carboxylate beads, used as received in PBS, was centrifuged at 5000 rpm and the supernatant removed. The beads were resuspended in 500 μL of sodium phosphate (0.1 M, pH 6.0), vortexed, centrifuged, supernatant removed, and resuspended in 400 μL of sodium phosphate. A 25-μL aliquot of an aqueous solution (50 mg/mL) of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (Pierce Biotechnology) followed by 25 μL of an aqueous solution (50 mg/mL) of sulfo-*N*-hydroxysuccinimide (Pierce Biotechnology) was added to each tube. The tubes were vortexed and gently agitated for 20 min in the dark. The beads were centrifuged, supernatant removed, and resuspended in 500 μL of MES (0.1 M, pH 4.5), centrifuged, and resuspended in 500 μL of protein/peptide solution, vortexed, and gently agitated at 4 °C overnight

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in the dark. Beads were coated with protein/peptide solutions at several concentrations during assay development (0.85, 1.7, and 3.4 μM). Beads were centrifuged and supernatant was removed and blocked with BSA by washing twice with PBS-TBN. Finally, the coupled beads were resuspended in 1 mL of PBS-TBN for formulation and stored at 4 °C in the dark.

Controls. A set of four internal controls built into every sample monitors and reports every step of the assay.[23] The details and concentrations at which the control beads were prepared are outlined in the Supporting Information. The instrument control (IC) verifies the reporter fluorescence optics of the flow analyzer. A change in the median fluorescent intensity (MFI) indicates fluctuations in the reporter laser performance. The fluorescent control (FC) tests for the addition of the fluorescent reporter streptavidin−R-phycoerythrin (SA-PE) in the assay. An antibody control (AC) gives a signal when both a biotinylated control (a component of the detector antibody cocktail) and SA-PE have been added; lack of signal on the AC bead indicates that the detector antibody cocktail was not added. Finally, a bead coated with BSA serves as the negative control (NC). BSA does not specifically bind to the target analytes, and consequently, the MFI of the NC should always be low. A high MFI on the NC bead obtained in the presence of serum sample would indicate nonspecific binding.

Bead Mixture Formulation. The bead mixtures were formulated in PBS-TBN to a final concentration of ~7 × 10^5 of each bead class/mL. This gives a 10× mixture that can be stored over time. The long-term stability of these peptides and NSPs coupled to the bead has not been determined at this time. Following formulation, 10 μL of the 10× bead mixture diluted in 90 μL of PBS-TBN was run in duplicate in a Bio-Plex (Bio-Rad) to 10 000 events/bead class with a 50-μL sample size to determine whether the bead counts in each class were approximately equal. If the bead count of a particular class was significantly (>30%) lower than the others, a compensatory amount of that bead was added to the bead mixture. The bead mixtures were stored at 4 °C in the dark and diluted 10-fold directly before use.

Biotinylated Antibody Cocktail (b-Abc). For bovine serum sample assays, the secondary (detector) antibody cocktail was prepared as a mixture of biotin-SP-conjugated Affinipure Goat anti-bovine (Jackson ImmunoResearch Laboratories) at 30 μg/mL and biotin-SP conjugated Affinipure rabbit anti-chicken IgY (IgG), Fc fragment specific (Jackson ImmunoResearch Laboratories) as a control at 0.2 μg/mL in PBS-TBN and diluted 10-fold directly before use.

Reporter Reagent. SA-PE (Caltag Laboratories) was prepared in PBS-TBN at 24 μg/mL and diluted 10-fold directly before use for a working concentration of 2.4 μg/mL.

Serum Samples. Serial bleed samples from animals infected with one of each of the seven serotypes (C_1 Noville, A_{24} Cruzerio, O_1 UKG 11/2001, Asia1 Shamir, SAT1 Bot 1/68, SAT2 SAU 1/2000, SAT3 Zim 4/81) of FMDV were obtained as described previously.[10] Briefly, Holstein calves weighing 150−350 kg were infected with FMDV of a particular serotype. Cattle were inoculated intradermally on the dorsal aspect of the tongue with a total virus dose of 10^6 $TCID_{50}$ distributed over five sites. Animals were sampled at 0 days postinfection (dpi), and then consecutive blood samples were taken at certain time points over 28−30 days. All animals were maintained in a cubicle within a biocontainment facility BSL-3. A veterinarian observed animals daily for any clinical signs of the disease. The local animal care committee approved all animal procedures prior to initiation of the studies. All serum samples were stored at − 20 °C. Samples were thawed and diluted 1:400 for serial bleed assays in PBS-TBN directly before use. All samples were used in an assay a maximum of 1-h postdilution. Normal bovine serum (Sigma) was used in each assay as a negative control at 1:400 dilution in PBS-TBN.

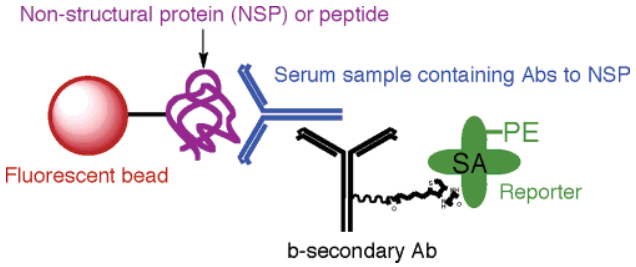

Figure 1. Representation of a liquid array serology assay. A NSP or peptide is covalently attached to a Luminex bead. The NSP or peptide captures antibodies to NSPs in serum samples obtained from cattle infected with FMDV. The captured NSP antibodies are subsequently detected by a biotinylated secondary (detector) antibody, followed by a fluorescent reporter molecule (SA-PE). The complex is analyzed in a flow cytometer. The beads are interrogated one at a time. A classification laser (635 nm) excites the dye molecules inside the bead and classifies the bead to its unique bead set. A reporter laser (532 nm) excites the fluorescent molecules bound to the bead surfaces and quantifies the assay at the bead surface—only those beads labeled with a reporter molecule will fluoresce in the yellow, and the signal is proportional to antibody concentration.

Assays. All assays were performed using reagents diluted directly before use. A 96-well MultiScreen-BV 1.2-μm filter plate (Millipore) was wetted with 100 μL of PBS-TBN and vacuum aspirated. A 100-μL aliquot of sample was deposited in each well and a 50-μL bead mixture to each sample well and the resultant mixture was incubated in the dark for 20 min. Samples were aspirated and washed twice with 100 μL of PBS-TBN. The beads were resuspended in 100 μL of PBS-TBN; 50 μL of b-Abc was added and the resultant mixture incubated in the dark for 15 min. Samples were aspirated and washed with 100 μL of PBS-TBN. The beads were resuspended in 100 μL of PBS-TBN, 50 μL of SA-PE was added, and the resultant mixture was incubated in the dark for 5 min. The samples were aspirated, washed with PBS-TBN, and resuspended in 100 μL of PBS-TBN. Finally, the suspended beads were transferred to a Corning Costar round-bottomed 96-well plate for analysis with a Bio-Plex configured to count a minimum of 100 beads/class and a 50-μL sample size.

RESULTS AND DISCUSSION

The liquid array serology assay format is depicted in Figure 1. The NSP or peptide, covalently attached to a unique bead class, captures NSP antibodies in the serum of animals infected with FMDV. Subsequently, a biotinylated secondary or detector antibody is added, followed by the reporter SA-PE. The MFI of the reporter is used to quantify the amount of antibody captured on each NSP or peptide bead.

NSP Assay Optimization. Beads were initially covalently coupled to NSPs using protein solutions at 0.85 μM. Each NSP-coupled bead was analyzed individually for response to a 28-dpi

Table 1. Assay Development—MFI Values of Each NSP-Coated Bead in a Simplex (i.e., One NSP Bead Plus Four Controls)[a]

	MFI			
	3A	3B	3ABC	3D
buffer blank	5	5	5	16
normal serum	75	110	102	166
28 dpi (1:200)	1830	717	5308	3265

[a] Tested against buffer blank, commercially available normal serum, and FMD-infected serum (A_{24} Cruzerio) 28 dpi at 1:200 dilution in PBS-TBN. The NSP beads clearly captured the antibodies present in the 28-dpi serum sample, evidenced by a large increase in the MFI. Data are an average of two repeats.

Table 2. Assay Development—MFI Values for the Peptide-Coated Beads in a Multiplex (Four Peptide plus Four Controls)[a]

	MFI			
	3A	3B	3D1	3D2
buffer blank	6	17	19	43
normal serum	40	49	62	64
14 dpi (1:400)	400	1605	422	56
28 dpi (1:400)	354	1655	300	63

[a] Tested against buffer blank, normal serum and FMD-infected serum (O_1 UKG 11/2001) 14 and 28 dpi at 1:400 dilution in PBS-TBN. The peptide beads clearly captured the antibodies present in the 14- and 28-dpi serum samples, evidenced by a large increase in the MFI with the exception of 3D2. Data are an average of two repeats.

serum sample from an animal infected with FMDV serotype A_{24} Cruzerio at different dilutions. Experimental infections lasted 28−30 days and the 28-dpi samples were known to contain a high level of antibodies against the NSPs. Therefore, these samples were used in the reagent/assay optimization. For individual NSP-coupled bead analysis, a bead mixture contained one NSP bead and four controls beads (simplex). The NSPs captured the antibodies in the 28-dpi serum sample as evidenced by a large increase in the MFI (Table 1, responses at different serum dilutions were not greatly different and are therefore not shown). Significantly, the buffer blank and normal serum elicited very low responses from each NSP-coated bead compared to the 28-dpi serial bleed serum sample. Therefore, the NSP beads were combined to form a multiplex mixture (four NSP beads and four controls), and the mixture was tested against serial bleed serum samples. Response from each NSP in the simplex assay differed little from the response of each NSP in the multiplex mixture (Supporting Information). As part of assay optimization, beads were also coated with NSPs at two higher concentrations (1.7 and 3.4 μM) and were used to analyze serial bleed serum samples (data not shown). The control beads in these optimization experiments remained constant, and the NC remained low, indicating consistency in assay performance and a lack of nonspecific binding.

Peptide Assay Optimization. Beads were covalently coupled to four synthetically produced peptides derived from the NSP amino acid sequences. Peptides 3A and 3B, and two peptides from the 3D NSP, 3D1, and 3D2 (adjacent sequences), were coupled to carboxylate beads at three different concentrations (~0.85, ~1.7, and ~3.4 μM) using carbodiimide and *N*-hydroxysuccinimide activation. The peptides were dissolved in PBS immediately before use, and concentrations are approximate as a consequence of some insoluble material in the peptide sample that was removed by centrifugation. The peptide beads at each concentration were directly combined to form a multiplex mixture (4 peptide beads and 4 control beads), and Table 2 shows the response of each peptide bead (coated at 1.7 μM) when run against buffer blanks, normal serum, and two serial bleed serum samples (14- and 28-dpi samples from an animal infected with FMDV serotype O_1 UKG 11/2001). Similarly to the NSP assay, the control beads in these optimization experiments remained constant, and the NC remained low (data not shown), indicating consistency in assay performance and a lack of nonspecific binding.

Peptides 3A, 3B, and 3D1 captured antibodies in the serum samples at 14 and 28 dpi as evidenced by a large increase in the MFI (Table 2). Conversely, buffer blanks and normal serum elicited a very low response from each peptide bead. Peptide 3D2 however, did not show any response to the serum samples and was therefore removed from future peptide bead mixtures.

b-Abc Optimization. In an effort to maximize signal intensity, different concentrations of biotinylated secondary antibody were investigated. This was not successful as increased backgrounds on blank samples and increased nonspecific binding (as evidenced by an increase in the response of the NC) were observed (data not shown). Therefore, subsequent assays were conducted with a biotinylated secondary antibody concentration of 3 μg/mL. Biotinylated rabbit anti-bovine antibody was assessed as a potential alternative detector reagent, but showed poor performance compared to b-goat anti-bovine antibody in this assay format. In addition, biotinylated protein A was assessed as a potential universal secondary antibody for the analysis of serum samples from any animal, but yielded poor performance in this assay format.

FMD Infected Serial Bleed Experiments. Following analysis of the reagent and serum sample variables, standard conditions were selected to analyze serial bleed serum samples from animals experimentally infected with one of the seven serotypes (C, A, O, Asia, SAT1, SAT2, SAT3) of FMDV. A serial bleed consisted of 13 samples taken over a 28−30-day experimental infection. The first 28−30 days are expected to elicit the production of the highest level of antibodies produced during an infection. In addition, sampling the initial days of infection establishes the point at which the antibodies against the NSPs can first be detected. These samples are ideal for assay development, and once conditions for maximum sensitivity are established, the assay can be tested against samples from convalescent, vaccinated, and disease-free animals to determine the results expected from a larger population of animals. The four NSPs, 3A, 3B, 3ABC, and 3D, were combined in a multiplexed NSP-based assay and directly compared with a three-membered 3A, 3B, and 3D1 multiplexed peptide-based assay. Beads were coated at several concentrations during assay development; however, NSP and peptide beads coated with solutions at 1.7 μM showed the highest response in both the NSP and peptide assay development and having both capture agents coated at the same molar concentration allowed more direct comparison of the results. The assays were run against buffer

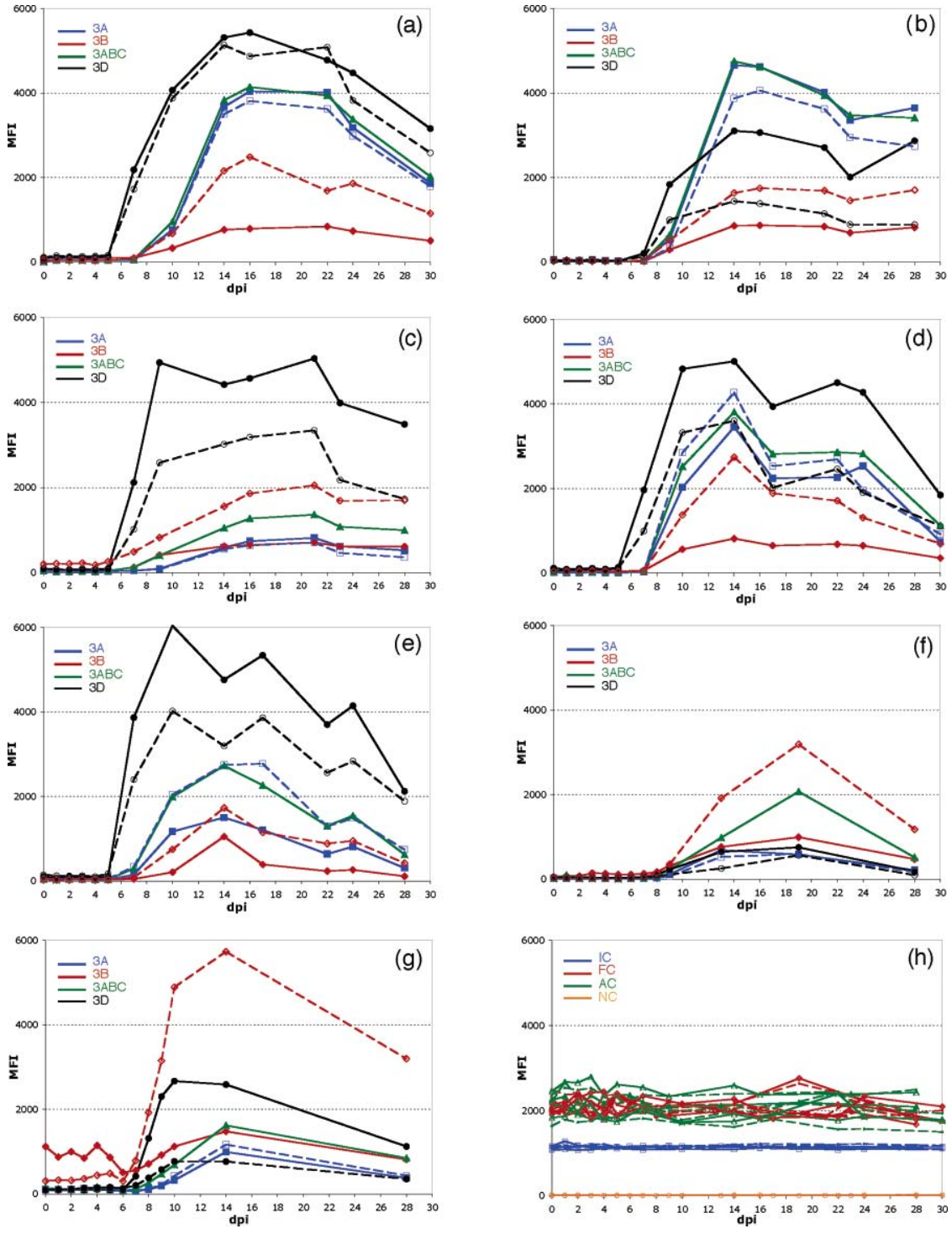

Figure 2. Assay responses (given as MFI; *y*-axis) of FMD-infected cattle serum samples (1:400 dilution) from serial bleeds over a 28−30-day period (dpi; *x*-axis). Solid lines indicate NSP-coated beads; dashed lines indicate peptide-coated beads. Blue 3A; red 3B; green 3ABC; black 3D1. For each viral serotype, serum samples were obtained from single animals, each infected with the particular viral serotype. Serotypes: (a) C_1 Noville, (b) A_{24} Cruzerio, (c) O_1 UKG 11/2001, (d) Asia1 Shamir, (e) SAT1 Bot 1/68, (f) SAT2 SAU 1/2000, and (g) SAT3 Zim 4/81. The assay response to serum from cattle infected with FMDV serotypes C, A, O, Asia, and SAT1 (a−e) are quite different from the response from SAT2 and SAT3 (f,g). Although SAT2 and SAT3 are different in profile, the NSP antibody levels at 7−9 dpi are clearly substantially higher than in the early part (0−6 dpi) of infection. The patterns of immune response to infection by FMDV are dependent on a number of factors including viral serotype, animal age, type, and general health. (h) Four representative examples of MFI values for each of the four internal controls when tested against serial bleed samples. Solid lines indicate controls from NSP assays; dashed lines indicate control beads from peptide assays. Blue IC; red FC; green AC; orange NC. The MFI values remain constant from sample to sample as expected and indicate consistency in assay performance. The low (and constant) MFI values of the NC indicate a lack of nonspecific binding, even in the complex sample matrix. Each point was performed in triplicate. Standard deviations are omitted for clarity. Standard deviation was typically <10% of value.

blanks, commercially available normal bovine serum, and the serial bleed serum samples. All serial bleed serum sample assays were carried out within 48 h of one another, and NSP and peptide assays for serum from a particular animal within 1 h, using the same reagents for highly comparable results. During assay development, a serum dilution of 1:400 consistently showed the highest responses and therefore was used in these comparative serial bleed experiments. Each point was performed in triplicate; buffer (i.e., blank) samples were replicated six times to provide good background statistics. Buffer blank data are not shown but consistently gave a low response while performing all assays (MFI 5−20).

Figure 2a−g shows the results obtained from the analysis of the serial bleed samples, directly comparing NSP and peptide assay responses. For each serial bleed assay presented in Figure 2, serum samples were obtained from single animals, each infected with a particular viral serotype. Further examples are shown in the Supporting Information. Although each assay also contained the four controls, only the bead responses for the NSPs and peptides are shown for clarity. The responses of the control beads IC, FC, AC, and NC were monitored throughout all experiments. Figure 2h shows four representative examples of the MFI values for each of the four internal controls when tested against serial bleed samples. MFI values for each of the internal controls are expected to remain constant, irrespective of the sample. The consistent MFI values from the control beads when analyzing all samples from a serial bleed demonstrate consistency in assay procedure. The low and constant MFI values of the NC indicate a lack of nonspecific binding, even in the complex sample matrix.

In general, the NSP and peptide assays showed similar patterns of response against serum from cattle infected with FMD serotypes C, A, O, Asia, and SAT1.The patterns of immune response to FMD infection are dependent on a number of factors—viral serotype and age, type, and general health of the animal. Generally, the first detectable response to the 3D protein and peptide was observed 5−7 dpi, with responses to the other proteins and peptides apparent 7−9 dpi. These patterns in the NSP expression profile over the course of infection with FMDV are consistent with previously published material in classical ELISA formats and other platforms.[6−10,15,16] The responses against serum from cattle infected with SAT2 and SAT3 are quite different, with the 3B peptide showing the earliest (7−9 dpi) and highest response. In these cattle, there was also an elevated response to the 3B protein 0−6 dpi. The 3B protein was expressed as a GST fusion[10] in *E. coli* for expression efficiency, and antibodies to *E. coli* are commonly found in animal sera.[6,17,18]

In all but one case (3B), the NSP showed a higher response to the serum samples than the corresponding peptide. This may be explained by slight differences in protein/peptide solution concentration when coating the beads, due to inherent inaccuracy in measuring protein/peptide concentrations, and the different reactivity of proteins and peptides with the carbodiimide-activated carboxylate beads. In addition, hydrophobic contact surface area is a contributing factor in protein−protein/ligand interactions. An intact protein has a greater hydrophobic contact surface area than a peptide, contributing to a higher binding affinity.[29,30] In the case of 3B, the peptide MFI was consistently higher than the 3B protein in all samples. The 3B peptide represents almost the entire sequence, and therefore, the size difference between the protein and the peptide is not significant in this case. Also, the 3B protein was expressed as a GST fusion,[10] potentially creating an inhibitory effect on binding in this assay format, resulting in the lower response. Both factors could contribute to the differences observed with relative responses of the 3B peptide and protein.

Commercially available normal bovine serum consistently gave a slightly elevated (2−3 times) level of response relative to the FMDV serial bleed serum samples from 0 to 5 dpi (data not shown). Commercially available bovine serum is an undocumented mixture of serum from many cattle. Therefore, a more satisfactory baseline of responses to be expected from normal cattle would be established by the analysis of serum taken from individual cattle. Two vaccinated cattle serum samples analyzed with the NSP assay gave responses in the same range as the commercially available normal bovine serum. Although the normal serum and vaccinated serum sample responses were very low in these assays compared to responses 5−9 dpi, a large number of normal serum samples obtained from individual animals and vaccinated serum samples must be analyzed to establish the distribution of responses to be expected in a normal and vaccinated animal population.

Serum samples from serial bleeds of a porcine and ovine infected with FMDV (A_{24} Cruzerio) were also analyzed using the NSP and peptide-based assays (data not shown). In these cases, anti-swine and anti-sheep secondary antibodies, respectively, were used with the control in the b-Abc. The porcine sample exhibited a pattern of results consistent with the SAT1 and SAT2 infected bovine samples; response to the 3B peptide was apparent 6−7 dpi. The ovine sample showed a pattern similar to that of the bovine samples, but with elevated responses in the early part (0−5 dpi) of the infection. However, the commercially available secondary anti-sheep antibody used in the b-Abc cross-reacted with BSA. An increased response to the NC was also observed, and as BSA was used to block the beads, this cross-reactivity significantly affected the results obtained. The initial results with porcine and ovine samples were encouraging and suggest that, with further optimization, the NSP and peptide multiplexed liquid array assay format will be suitable for analyzing the immune response to infection in a variety of species susceptible to FMD infection.

CONCLUSIONS

Two assays, one based on NSPs, and the other on synthetically produced peptides, were developed and compared as the basis for a multiplexed FMD DIVA assay. There is a large increase in the response of all NSPs and peptides in the assay 5−9 dpi with serum from cattle, each infected with one of the seven serotypes (C, A, O, Asia, SAT1, SAT2, SAT3) of FMDV. The simultaneous detection of antibodies raised against multiple NSPs in a single assay with internal controls has the potential to produce a DIVA assay with high diagnostic accuracy. This is particularly important if vaccines of low purity have been used, where false positives for infection are likely if using an assay based on the detection of antibodies against only one NSP/peptide. In addition, the expression profile of antibodies against NSPs can vary from animal to animal, and in the case of experimental infection with serotypes SAT2 and SAT3, the level of some antibodies was quite low. In

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this case, the simultaneous detection of antibodies to multiple NSPs will increase diagnostic accuracy by decreasing the likelihood of false negatives. The reagents, particularly the peptide reagents, are easy to prepare and the assay and analysis can be carried out in 90 min. Commercial partners will be used to manufacture highly reproducible reagents (peptides are particularly amenable to standard GLP/GMP practices for commercialization) for large-scale assay optimization. The international community will be engaged to test a large population of vaccinated and naturally infected animals and optimize and validate the liquid array, multiplexed DIVA assay for use in the field.

ACKNOWLEDGMENT

This work was carried out under the auspices of the U.S. Department of Energy by the University of California, Lawrence Livermore National Laboratory under Contract W-7405-Eng-48 with funding (J.P., B.J.H., R.J.L., M.T.M.) from the U.S. Department of Homeland Security Agricultural Domestic Demonstration and Application Program (AgDDAP). This work (A.C.) was supported through funds of the Laboratories Directorate of the Canadian Food Inspection Agency and the Chemical, Biological, Radiological and Nuclear Research and Technology Initiative (CRTI) No 0196RD.

SUPPORTING INFORMATION AVAILABLE

Table 1. Specifications of synthetically produced peptides. Table 2. Details of control beads. Figure 1. NSP simplex and multiplex responses to buffer blank, normal serum and 28 dpi at several dilutions. Figure 2. Further examples of responses in serial bleed experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review February 3, 2006. Accepted May 18, 2006.

AC060235E