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Use of a Standardized Bovine Serum Panel To Evaluate a Multiplexed Nonstructural Protein Antibody Assay for Serological Surveillance of Foot-and-Mouth Disease▽†

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Liquid array technology has previously been used to show proof of principle of a multiplexed nonstructural protein serological assay to differentiate foot-and-mouth disease virus-infected and vaccinated animals. The current multiplexed assay consists of synthetically produced peptide signatures 3A, 3B, and 3D and the recombinant protein signature 3ABC in combination with four controls. To determine the diagnostic specificity of each signature in the multiplex, the assay was evaluated against a naive population ($n = 104$) and a vaccinated population ($n = 94$). Subsequently, the multiplexed assay was assessed by using a panel of bovine sera generated by the World Reference Laboratory for foot-and-mouth disease in Pirbright, United Kingdom. This serum panel has been used to assess the performance of other singleplex enzyme-linked immunosorbent assay (ELISA)-based nonstructural protein antibody assays. The 3ABC signature in the multiplexed assay showed performance comparable to that of a commercially available nonstructural protein 3ABC ELISA (Cedi test), and additional information pertaining to the relative diagnostic sensitivity of each signature in the multiplex was acquired in one experiment. The encouraging results of the evaluation of the multiplexed assay against a panel of diagnostically relevant samples promote further assay development and optimization to generate an assay for routine use in foot-and-mouth disease serological surveillance.

Liquid array technology allows simultaneous measurement of the relative responses of multiple signatures to a challenge sample (19). This technology has proven successful for multiple applications; antigen and nucleic acid-based biological threat agent detection (27, 28) and serological assays (3, 16, 20, 23, 35, 42, 43) are some examples. The use of such multiplexing technology has time, cost, and manpower benefits over multiple singleplex analyses, in addition to increased confidence in the results obtained. Multiple-signature evaluation provides more confidence when obtaining a conclusive result; it eliminates variations that may occur when using a series of singleplex assays to obtain a comparative result, and it allows controls in every sample. 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 by a detector reagent indirectly labeled with a fluorescent reporter. Each optically encoded and fluorescently labeled bead is then tested with a flow cytometer. A classification laser (635 nm) excites the dye molecules inside the bead and classifies the bead into its unique bead set. A reporter laser (532 nm) excites the bound fluorescent reporter and quantifies the assay at the bead surface. The flow cytometer is capable of reading hundreds of 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 and economic platform.

Foot-and-mouth disease (FMD) is a highly infectious and contagious vesicular disease affecting cloven-hoofed animals. FMD virus (FMDV) belongs to the genus *Aphthovirus* in the *Picornaviridae* family and includes seven serotypes, O, A, Asia, C, and SAT1, -2, and -3. The circulation of FMDV in an animal population imposes severe restrictions on the movement of animal products and consequently on international trade. FMD is endemic in many parts of Asia, Africa, and South America. Moreover, the disease periodically breaks out in FMD-free countries (7) and in either case can have a significant economic impact on the affected region. An outbreak of FMD can be controlled by culling infected and contact-susceptible animals or by the use of emergency ring vaccination, a so-called vaccinate-to-live policy. Vaccination is used in South America (6) as part of a continent-wide effort to eradicate the endemic disease (5). However, FMD vaccines do not provide sterile immunity and animals can become clinically or subclinically infected and ultimately become carriers of the virus, which is considered a threat to spread the disease to other susceptible animals (13–15, 17, 22, 30). Therefore, to regain FMD-free status and re-enable international trade, postvaccination surveillance is required to demonstrate the absence of persistent infection in a vaccinated population (1).

Both infection and vaccination elicit antibodies against structural antigens as FMD vaccines are chemically inactivated, semipurified virions. Therefore, only assays that measure levels of antibodies against nonstructural protein (NSP) can differentiate infected and vaccinated animals (DIVA), pro-

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viding the vaccine used is of high purity (40). Researchers have reported many assays capable of detecting antibodies against FMDV NSPs (8, 9, 18, 25, 34, 45, 47, 49). All of the reported single-signature assays are based on the enzyme-linked immunosorbent assay (ELISA) plate format. Vaccine-challenge experiments have recently been used to evaluate the performance of NSP antibody assays (13, 14, 31, 38), and these studies showed that a single NSP assay could not always detect persistent infection and therefore cannot be relied upon to declare absolute freedom of infection in vaccinated herds. Indeed, Bergmann and coworkers at the Pan American FMD Center in Rio de Janeiro, Brazil, have implemented a combination of a 3ABC ELISA and an enzyme-linked immunoelectrotransfer blot assay to gain high specificity and sensitivity for FMDV serological monitoring in South America (5, 6). An international workshop for the validation of NSP assays in Brescia, Italy (8, 40), proposed the use of at least two assays to attain ideal sensitivity and specificity. This is consistent with the theory of liquid array multiplexing technology allowing simultaneous multiple signature evaluation, providing more confidence in obtaining a conclusive result. Liquid array technology has previously been shown to be a promising platform for the development of a multiplexed NSP FMD DIVA assay (11, 41). Described here are further developments and evaluation of the multiplexed NSP antibody assay by using samples originating from two vaccine-challenge experiments and a panel of bovine serum samples assembled to test the relative sensitivities of NSP antibody assays. The serum panel used consisted of samples originating from various vaccine potency experiments conducted at the World Reference Laboratory (WRL) for FMD in Pirbright, United Kingdom, and covers four different serotypes (O, A, Asia1, and SAT2) (38).

MATERIALS AND METHODS

Multiplexed reagent preparation and assay procedures have been previously described in detail (41). Therefore, reagent preparation and assay protocols are outlined briefly except in the case of updated specifics, where a full description is reported.

General. All reagent dilutions and assays were carried out with PBS-TN (phosphate-buffered saline [pH 7.4], 0.02% [vol/vol] Tween 20, 0.02% [wt/vol] sodium azide) and filtered through Corning 0.22-μm-pore-size filter systems before use.

Bead mixture. The bead mixture consisted of eight bead sets—four assay bead sets and four control sets (28). Synthetically produced (United Biochemical Research, Seattle, WA) peptides 3A, 3B, and 3D (peptide sequences described previously) and gel-purified recombinant NSP 3ABC (12) were each covalently coupled to a unique carboxylate bead class (Luminex Corp.) by using carbodiimide activation as described previously. Peptide-protein solutions were at 1.7 μM for bead coating. Control beads were coated as described previously and consisted of an instrument control (IC), a fluorescent control (FC), an antibody control (AC), and a negative control (NC). The bead mixture was formulated in PBS-TN to a theoretical final concentration of $\sim 5.4 \times 10^6$ beads of each class/ml. This gives a 10× mixture that can be stored over time. Following formulation, the bead mixture was enumerated by taking 5 μl of the 10× bead mixture, diluting it in 95 μl of PBS-TN, and counting all of the beads in a 50-μl sample with a Bio-Plex system (Bio-Rad). If the bead count of a particular class was significantly (>30%) lower than that of the others, a compensatory amount of that bead class was added to the bead mixture. With this dilution-and-enumeration method, the number of beads of each class was approximately 500. The bead mixtures were stored at 4°C in the dark and diluted 10-fold directly before use.

Detector reagent. The detector (secondary) 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 immunoglobulin Y (IgG), Fc fragment specific (Jackson ImmunoResearch Laboratories), as a control at 0.2 μg/ml in PBS-TN and diluted 10-fold directly before use.

Reporter reagent. Streptavidin-R-phycoerythrin (SA-PE; Caltag Laboratories) was prepared in PBS-TN at 24 μg/ml and diluted 10-fold in PBS-TN directly before use for a working concentration of 2.4 μg/ml.

Serum sample preparation for multiplexed assays. All serum samples were stored at −20°C. Samples were thawed and diluted 1:400 in PBS-TN directly before use. All samples were used in an assay a maximum of 1 h postdilution.

Sera—general. The serum samples used were collected during the course of a series of vaccination-challenge experiments carried out in biosecurity containment at the WRL for FMD, Pirbright, United Kingdom. Typically, naive cattle were vaccinated and at 21 days postvaccination (dpv) they were challenged with either homologous or semiheterologous FMDV by intradermolingual inoculation or by direct contact with infected cattle. Sera were collected at various days postvaccination and days postchallenge (dpc). The experimental details, including virological and serological findings, have been extensively reported previously (13, 14, 36–39). These experiments were assigned two-letter identifiers (e.g., UV), and these identifiers coupled with numbers are used to designate the animals in an experiment.

Sera from naive cattle. One hundred four serum samples were collected from cattle at the beginning of vaccination-challenge experiments before any administration of vaccine or virus. For the identities of the samples, see the supplemental material.

Sera from vaccinated and infected cattle. Ninety-four serum samples from cattle were tested at 14 or 21 dpv, in some cases from the same animal at each time point, during the course of vaccination-challenge experiments. For the identities and numbers of days postvaccination of the samples from vaccinated animals, see the supplemental material. In addition, samples from two O serotype vaccine-challenge experiments (UV [14] and UY [13] series) were tested at 0, 14, and 21 dpv and 0 and 28 dpc. The details of the experiments used to generate these samples have been previously reported in full.

Bovine serum panel. Thirty-six bovine serum samples were selected from a series of vaccination-challenge experiments. The details of the experiments used to generate these samples have been previously reported in full (13, 14, 37, 38). The state of the animals (vaccination, challenge, mode and serotype of challenge, and carrier status) when the serum samples were taken is summarized in Table 1. The bovine serum panel was also tested after heat inactivation at 56°C for 2 h.

Assays. A 96-well MultiScreen-BV 1.2-μm filter plate (Millipore) was wetted with 100 μl of PBS-TN. A 100-μl diluted sample was deposited in each well. A 50-μl bead mixture was added to each sample well, and the plate was incubated in the dark for 20 min. Samples were washed twice with 100 μl of PBS-TN. The beads were resuspended in 100 μl of PBS-TN, 50 μl of detector reagent was added, and the beads were incubated in the dark for 15 min. Samples were washed with 100 μl of PBS-TN. The beads were resuspended in 100 μl of PBS-TN, 50 μl of SA-PE was added, and the beads were incubated in the dark for 5 min. The samples were washed with PBS-TN and resuspended in 100 μl of PBS-TN. Finally, the suspended beads were transferred to a Corning Costar round-bottom 96-well plate for analysis with a Bio-Plex configured to count a minimum of 100 beads per class and a 50-μl sample size. Normal bovine serum (Sigma) and a strong positive sample (UV23, 37 dpc) were used on each plate as negative and positive controls, respectively, at a 1:400 dilution in PBS-TN.

RESULTS

Figure 1 shows a schematic representation of the liquid array, bead-based, NSP antibody assay. Each antigen is covalently conjugated to a particular bead set. The covalently bound antigen captures antibodies in sera from FMDV-infected animals. A biotinylated secondary or detector antibody and streptavidin-phycoerythrin reporter quantify the assay at each bead surface, and the complex is analyzed in a flow cytometer. Previous work showed a close correlation between the responses of recombinant NSPs and synthetically produced peptides 3A, 3B, and 3D on this platform (41). Therefore, three peptides representing NSP antigens 3A, 3B, and 3D were combined with recombinant NSP antigen 3ABC and four controls—IC, FC, AC, and NC—to generate an eightplex for further development. Peptides were chosen over recombinant antigens where appropriate as peptides can be easily produced

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TABLE 1. Origins of bovine serum panel used to test relative sensitivities of NSP antibody assays[a]

Cattle group and sample identifier	Vaccination strain	Challenge serotype[b]	Challenge method[b]	Clinical signs	Time (dpc) when serum was taken
Vaccinated carriers					
UV9	O Manisa	O UKG	Contact		174
UV10	O Manisa	O UKG	Contact		174
UV11	O Manisa	O UKG	Contact		174
UV13	O Manisa	O UKG	Contact		174
UV17	O Manisa	O UKG	Contact		174
UV19	O Manisa	O UKG	Contact		174
UY83	O Manisa	O UKG	Contact		107
UY90	O Manisa	O UKG	Contact		106
UZ58	A Iran 96	A Iran 96	Contact		32
UZ59	A Iran 96	A Iran 96	Contact		32
UZ60	A Iran 96	A Iran 96	Contact		32
UZ62	A Iran 96	A Iran 96	Contact		32
VE63	Asia 1 Shamir	Asia 1 Shamir	Inoculation		42
VE64	Asia 1 Shamir	Asia 1 Shamir	Inoculation		42
VE65	Asia 1 Shamir	Asia 1 Shamir	Inoculation	Yes	42
VE66	Asia 1 Shamir	Asia 1 Shamir	Inoculation		42
VE67	Asia 1 Shamir	Asia 1 Shamir	Inoculation	Yes	42
VL83	SAT2 3218	SAT2 Eritrea	Inoculation		36
VL89	SAT2 3218	SAT2 Eritrea	Inoculation	Yes	36
VL90	SAT2 3218	SAT2 Eritrea	Inoculation		37
Unvaccinated carriers					
UZ68	NA[c]	A Iran 96	Inoculation	Yes	33
UZ69	NA	A Iran 96	Inoculation	Yes	33
UY95	NA	O UKG	Contact	Yes	107
UY96	NA	O UKG	Contact	Yes	107
UV26	NA	O UKG	Contact	Yes	174
VH44	NA	O UKG	Contact	Yes	40
VH45	NA	O UKG	Contact	Yes	40
Vaccinated noncarriers					
VE73	Asia 1 Shamir	Asia 1 Shamir	Inoculation		43
VE71	Asia 1 Shamir	Asia 1 Shamir	Inoculation		43
UZ54	A Iran 96	A Iran 96	Inoculation	Yes	32
UY79	O Manisa	O UKG	Contact		106
Unvaccinated noncarriers					
VE60	NA	Asia 1 Shamir	Inoculation	Yes	42
VE62	NA	Asia 1 Shamir	Inoculation	Yes	42
UY94	NA	O UKG	Contact	Yes	107
UV23	NA	UKG 34/01	Contact	Yes	37
UV24	NA	O UKG	Contact	Yes	174

[a] The experiments in which the samples were obtained are described in references 13, 14, and 38.
[b] Cattle were challenged at 21 dpv.
[c] NA, not applicable.

in large quantities under strict quality control without biosafety level 3 containment. Recombinant antigen 3ABC remained in the multiplex, as this signature is commonly used in NSP ELISAs (2, 4, 6, 8–10, 24, 29, 33, 38, 44, 46). The controls have been previously described (41), but briefly, the IC (coated with R-phycoerythrin) verifies the reporter fluorescence optics of the flow analyzer. A large change in the median fluorescence intensity (MFI) indicates fluctuations in the reporter laser performance. The FC (coated with biotinylated bovine serum albumin [BSA]) tests for the addition of the reporter reagent, SA-PE, in the assay. The AC (coated with chicken IgG) gives a signal when both a biotinylated anti-chicken IgG control (a component of the detector reagent) and SA-PE have been added; lack of signal on the AC beads indicates that the detector antibody cocktail was not added. Finally, a bead coated with BSA serves as an NC to determine the extent of nonspecific binding.

The eightplex assay was tested against 104 FMD-naive serum samples to establish normal variation in a naive population and also to determine a cutoff above which a sample is deemed positive for prior infection with FMDV. During the initial development of the multiplexed assay, the response on the antigen-coated or "assay" beads during the first few days of infection was extremely low and the response on the BSA-coated NC bead remained more or less constant when analyzing samples from serial bleeds of experimentally infected cattle (41). However, upon expanded analysis of samples from a naive population of cattle (data not published to date), significant differences in the responses of the assay beads were observed. This correlated with large differences in the response of the NC bead. Therefore, the NC is used to normalize the response of the assay beads, and other researchers have used this approach to normalize liquid array-based serology assay

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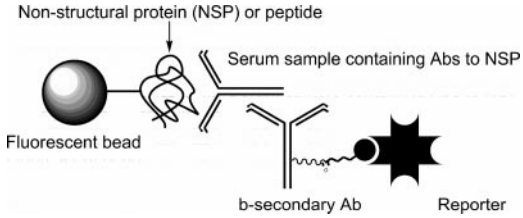

FIG. 1. Schematic of bead-based, multiplexed NSP antibody (Ab) assay. A peptide or NSP is covalently conjugated to a Luminex bead. The peptide or recombinant protein captures antibodies to NSPs in serum samples from cattle infected with FMDV. The captured antibodies are subsequently detected by a secondary biotinylated detector antibody, followed by a fluorescent reporter molecule. The complex is analyzed in a flow cytometer. The beads are tested 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 bound fluorescent reporter and quantifies the assay at the bead surface—only those beads labeled with a reporter molecule will fluoresce in yellow, and the signal is proportional to the captured-antibody concentration.

results (21). Significantly, FMDV-infected cattle generally maintain a low response on the NC and therefore normalization effectively increases the sensitivity of the multiplexed liquid array assay.

Figure 2a to d show the normalized response on each of the four assay beads in response to sera from 104 FMD-naive cattle. For the animal identification codes; the crude MFI values on all beads, including controls; and the normalized values and standard deviations, see the supplemental material. The standard deviation of the normalized responses of the naive population was, on average, ~10% of the value. The 104 samples were used to generate cutoffs of 97% specificity for antigens 3A, 3D, and 3ABC and 95% for 3B. Positive bars in Fig. 2a to d are above the cutoff. Sera from 94 vaccinated cattle were also run against the multiplexed assay, and the normalized responses of each assay bead to each sample are shown in Fig. 3a to d. The samples were taken from cattle at 14 dpv (black bars) and 21 dpv (gray bars). For the animal identification codes; the crude MFI values on all beads, including controls; and the normalized values and standard deviations, see the supplemental material. The standard deviation of the normalized responses of the naive population was, on average, ~10% of the value. With the cutoffs determined from the naive populations (positive bars are above the cutoff in Fig. 3), antigen 3A showed a specificity of 96%, antigen 3B showed a specificity of 97%, antigen 3D showed a specificity of 93%, and 3ABC showed a specificity of 97%, indicating that a vaccinated population gives a response similar to that of a naive population, provided the vaccine used is of high purity.

The multiplexed assay was used to analyze the 36 samples of a bovine serum panel assembled by the WRL for FMD (38). Table 1 lists the origins of the samples, including the details of vaccination and challenge and the times at which the sera were collected. The normalized responses of the antigens in the multiplex to the samples are listed in Table 2. The data in Table 2 are averages of two experiments, with three repeats for each sample in each experiment. For the crude MFI values on all beads, including controls, and the normalized values and standard deviations, see the supplemental material. The serum

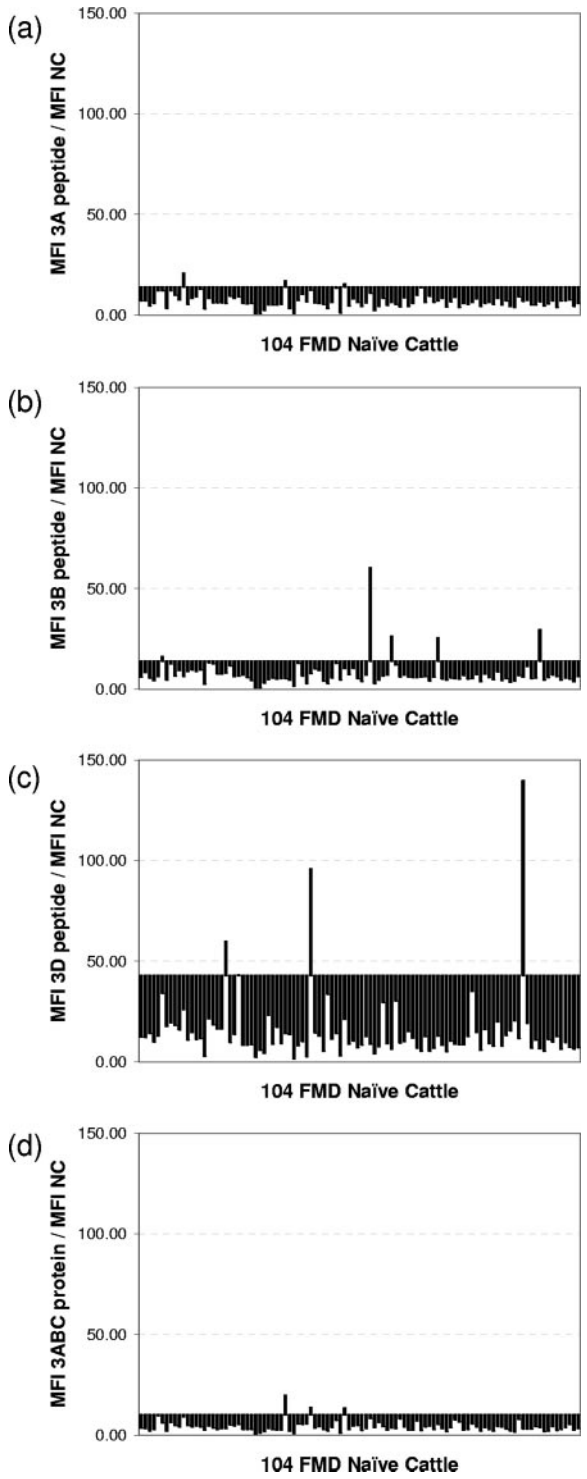

FIG. 2. Responses of each signature in a multiplexed assay to a challenge with sera from 104 FMD-naive cattle to illustrate the expected variation in a naive population. Responses of assay beads are reported as MFI of the assay bead normalized by using the MFI of the NC BSA-coated bead in each sample. All signatures are shown on the same scale for visual comparison. Positive bars indicate a normalized MFI value above the cutoff. Cutoffs were determined from this naive population to give 95 to 97% specificity. Negative bars indicate a normalized MFI value below the cutoff. (a) 3A peptide, 97% specificity cutoff. (b) 3B peptide, 95% specificity cutoff. (c) 3D peptide, 97% specificity cutoff. (d) 3ABC protein, 95% specificity cutoff.

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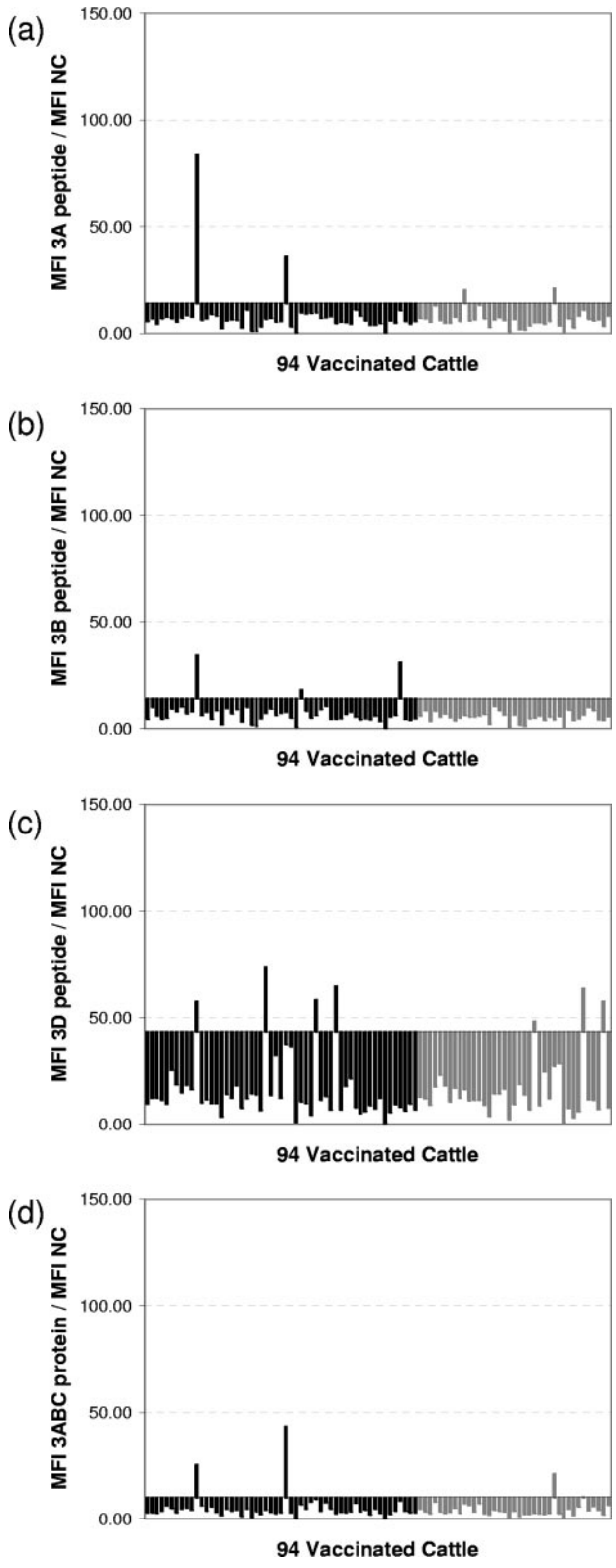

FIG. 3. Responses of each signature in a multiplexed assay to a challenge with sera from 94 vaccinated cattle to illustrate the expected response of a vaccinated population. Black bars are responses to sera from cattle at 14 dpv; gray bars are responses to sera from cattle at 21 dpv. For a list of the vaccine strains used, see the supplemental material. Responses of assay beads are reported as the MFI of the assay bead normalized by using the MFI of the NC BSA-coated bead in each sample. All signatures are shown on the same scale for visual comparison. Positive bars indicate a normalized MFI value above the cutoff. Cutoffs were determined from a naive population to give 95 to 97% specificity. Negative bars indicate a normalized MFI value below the cutoff. (a) 3A peptide, 97% specificity cutoff. (b) 3B peptide, 95% specificity cutoff. (c) 3D peptide, 97% specificity cutoff. (d) 3ABC protein, 95% specificity cutoff.

samples are divided into groups, depending on vaccination-carrier status in accordance with previously published material (13, 14, 36, 37). In addition, the qualitative results obtained when using the Cedi test (Cedi Diagnostics, Lelystad, The Netherlands), a 3ABC ELISA, are listed for comparison. The results of the Cedi test and other nonstructural antibody assay results for each sample in the panel have been previously reported (38). The cutoff for each antigen generated from the naive population was applied to the results obtained for the bovine serum panel. The 3ABC antigen in the multiplexed assay shows a good correlation with the Cedi test results, with the exception of sample UY83, which proved negative by the multiplexed assay. However, two carrier cattle (UZ59 and UZ62, carrier status confirmed by reverse transcription PCR-virus isolation) were positive by the 3A, 3B, and 3ABC signature in the multiplexed assay, whereas they were missed by the Cedi test. Moreover, vaccinated noncarrier animal UZ54 was clearly positive on three signatures in the multiplexed assay but gave positive and negative results in the Cedi test. The other signatures, 3A, 3B, and particularly 3D, showed a large range of responses to each sample, and the values depended heavily on the serotype of the vaccination-challenge and the time point at which the serum sample was taken. The significance of these differences is discussed more fully below.

Table 3 shows the response of the multiplexed assay when run against the heat-inactivated serum panel to determine if heat inactivation affected the results of the multiplexed assay. For the crude MFI values on all beads, including controls, and the normalized values and standard deviations, see the supplemental material. Heat inactivation reduces the likelihood that the serum samples are contaminated with live FMDV and allows the samples to be analyzed outside biosafety level 3 containment. Therefore, it is important to determine if the performance of NSP antibody assays is adversely affected by heat inactivation of the sera. Heat inactivation of the serum samples generally lowered the response obtained on each antigen, but only in a few cases did the response fall below the cutoff, changing the status of the sample.

Tables 4 and 5 show the results from the analysis of four samples from each of 25 cattle in two vaccination-challenge experiments (13, 14) and compare the results of the multiplexed assay with the previously reported results of the Cedi test for the samples at 28 dpc. The serum samples were taken at 0, 14, and 21 dpv and 0 and 28 dpc. Again, the cattle are grouped according to their serological status, which was previously determined. Table 4 shows results for the herd of cattle following vaccination with O_1 Manisa oil adjuvant vaccine and challenge by direct contact with O UKG 34/2001. Generally, the vaccinated noncarrier cattle are negative for antibodies to

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TABLE 2. Normalized MFI values for each signature in multiplexed NSP antibody assay in response to bovine serum panel and comparison with results of Cedi test[a]

Cattle group and sample identifier	Normalized MFI[b]				Qualitative result for Cedi test[g]
	3A[c]	3B[d]	3D[e]	3ABC[f]	
Vaccinated carriers					
UV9	10	**14**	11	**13**	+
UV10	8	**20**	11	**12**	+
UV11	11	13	16	**16**	+
UV13	**17**	13	11	**28**	+
UV17	9	11	5	**12**	+
UV19	**19**	12	13	**26**	+
UY83	4	7	4	8	+
UY90	**14**	**22**	11	**19**	+
UZ58	**52**	**32**	11	**72**	+
UZ59	**15**	**15**	16	**26**	−
UZ60	**30**	**29**	16	**53**	+
UZ62	**21**	**15**	21	**28**	−
VE63	**35**	7	16	**36**	+
VE64	**94**	**26**	12	**103**	+
VE65	**102**	**56**	**104**	**129**	+
VE66	**130**	**69**	19	**126**	+
VE67	**344**	**69**	**104**	**342**	+
VL83	**70**	**127**	**147**	**136**	+
VL89	**91**	**56**	**124**	**99**	+
VL90	**85**	**20**	18	**91**	+
Unvaccinated carriers					
UZ68	**138**	**55**	**72**	**174**	+
UZ69	**205**	**192**	**208**	**265**	+
UY95	**41**	**49**	**49**	**100**	+
UY96	13	12	4	**20**	+
UV26	**24**	**16**	28	**36**	+
VH44	**29**	**32**	**61**	**53**	+
VH45	**14**	**16**	9	**38**	+
Vaccinated noncarriers					
VE73	**21**	**31**	18	**91**	+
VE71	**207**	**76**	41	**261**	+
UZ54	**49**	**74**	11	**109**	±
UY79	9	7	20	**10**	+
Unvaccinated noncarriers					
VE60	**220**	**61**	**132**	**228**	+
VE62	**144**	**95**	**81**	**221**	+
UY94	**20**	13	5	**27**	+
UV23	**127**	**69**	**126**	**181**	+
UV24	**45**	**15**	10	**42**	+

[a] The experiments in which the samples were obtained are described in reference 38.
[b] Normalized MFI = assay bead MFI/NC MFI. Data are averages of two experiments, with three repeats for each sample in each experiment. For the standard deviations of all values, see the supplemental material; they are typically ~10% of the values shown here. Bold values are above the cutoff and therefore positive for antibodies against the NSPs. Cutoffs were determined from a naive population to give 95 to 97% specificity as follows: 3A peptide, 97%; 3B peptide, 95%; 3D peptide, 97%; 3ABC protein, 95%.
[c] MFI cutoff, 14; assay response to normal serum commercially available from Sigma (average of 95 repeats as an untreated-serum standard control), 3.
[d] MFI cutoff, 14; assay response to normal serum from Sigma, 4.
[e] MFI cutoff, 43; assay response to normal serum from Sigma, 6.
[f] MFI cutoff, 10; assay response to normal serum from Sigma, 3.
[g] Data are from Parida et al. (38). The Cedi test is a 3ABC ELISA. +, positive in all tests; −, negative in all tests; ±, some tests positive and some tests negative.

NSPs pre- and postchallenge, with a few exceptions. UV15 (3A and 3ABC) and UV20 (3ABC) rose above the cutoff postchallenge, and this is contrary to the results of the Cedi test. UV6 and UV7 are false positives on one signature at 0 dpv. UV12

TABLE 3. Comparison of normalized MFI values for each signature in a multiplexed FMD DIVA assay in response to a bovine serum panel when sera were heat inactivated and untreated

Cattle group and sample identifier	Normalized MFI[a]							
	3A		3B		3D		3ABC	
	HI[b,c]	Δ[d]	HI[e]	Δ	HI[f]	Δ	HI[g]	Δ
Vaccinated carriers								
UV9	13	+3	**18**	+4	14	+4	**14**	+1
UV10	8	0	**17**	−3	12	−1	**10**	−2
UV11	12	+1	13	0	19	+3	**15**	−1
UV13	11	−6	10	−3	10	−1	**20**	−8
UV17	9	0	12	−1	7	−2	**11**	−1
UV19	**20**	−1	11	−1	15	−2	**25**	−1
UY83	4	0	7	0	5	+1	7	−1
UY90	12	−2	**20**	−2	11	0	**18**	−1
UZ58	**47**	−5	**30**	−2	11	0	**64**	−8
UZ59	**14**	−1	**14**	−1	18	+2	**25**	−1
UZ60	**31**	+1	**29**	0	18	+2	**54**	+1
UZ62	**18**	−3	11	−4	19	−3	**21**	−7
VE63	**37**	+2	5	−2	13	−3	**30**	−6
VE64	**89**	−5	**21**	−5	12	0	**93**	−10
VE65	**96**	−6	**49**	−7	**89**	−15	**116**	−13
VE66	**123**	−7	**62**	−7	19	0	**124**	−2
VE67	**409**	+65	**77**	+8	**126**	+22	**427**	+85
VL83	**55**	−15	**93**	−34	**103**	−44	**105**	−31
VL89	**76**	−15	**51**	−5	**113**	−11	**80**	−19
VL90	**67**	−18	**17**	−3	17	−1	**70**	−21
Unvaccinated carriers								
UZ68	**103**	−35	**41**	−14	**61**	−11	**140**	−34
UZ69	**227**	+22	**209**	+17	**256**	+28	**296**	+31
UY95	**46**	+5	**51**	+2	**64**	+15	**100**	0
UY96	10	−3	10	−2	4	0	**16**	−4
UV26	**17**	−7	12	−4	22	−6	**24**	−12
VH44	**25**	−4	**24**	−8	**47**	−14	**41**	−12
VH45	12	−2	13	−3	8	−1	**30**	−8
Vaccinated noncarriers								
VE73	12	−9	10	−21	16	−2	**11**	−80
VE71	**109**	−98	**42**	−34	31	−10	**135**	−126
UZ54	**37**	−12	**51**	−23	9	−2	**65**	−44
UY79	6	−3	5	−2	15	−5	7	−3
Unvaccinated noncarriers								
VE60	**140**	−80	**39**	−22	**87**	−45	**146**	−82
VE62	**73**	−71	**46**	−49	**45**	−36	**112**	−109
UY94	**17**	−3	**11**	−2	5	0	**25**	−2
UV23	**62**	−65	**34**	−35	42	−84	**89**	−92
UV24	**31**	−34	9	−6	9	−1	**33**	−9

[a] Normalized MFI = assay bead MFI/NC MFI. Data are averages of two experiments, with three repeats for each sample in each experiment. For the standard deviations of all values, see the supplemental material; they are typically ~10% of the values shown here. Bold values are above the cutoff and therefore positive for antibodies against the NSPs. Underlined values indicate a change in status (positive to negative) after the serum was heat treated compared to the untreated serum. Cutoffs were determined by analysis of untreated sera from a naive population to give 95 to 97% specificity as follows: 3A peptide, 97%; 3B peptide, 95%; 3D peptide, 97%; 3ABC protein, 95%.
[b] HI, heat inactivated.
[c] MFI cutoff, 14; assay response to normal serum commercially available from Sigma (average of 95 repeats as an untreated-serum standard control), 3.
[d] Δ, response with heat-inactivated serum minus response with untreated serum (reported in Table 2). A negative result indicates that the response with heat-inactivated serum was lower.
[e] MFI cutoff, 14; assay response to normal serum from Sigma, 4.
[f] MFI cutoff, 43; assay response to normal serum from Sigma, 6.
[g] MFI cutoff, 10; assay response to normal serum from Sigma, 3.

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TABLE 4. Normalized MFI values for each signature in a multiplexed NSP antibody assay in response to serum samples taken over a vaccination-challenge experiments with the O serotype[a]

Cattle group and sample identifier[c]	Normalized MFI[b]																
	3A				3B				3D				3ABC				
	0 dpv	14 dpv	21[d] dpv	28 dpc	0 dpv	14 dpv	21[d] dpv	28 dpc	0 dpv	14 dpv	21[d] dpv	28 dpc	0 dpv	14 dpv	21[d] dpv	28 dpc	28[e] dpc
Vaccinated noncarriers																	
UV3	7	7	7	X[f]	8	10	9	X	12	12	12	X	3	3	4	X	−
UV4	5	4	X	3	6	6	X	3	14	12	X	6	2	3	X	2	−
UV6	12	7	13	6	6	5	8	6	13	9	18	11	**10**	6	8	5	−
UV7	12	7	6	6	**16**	9	5	4	34	25	23	21	6	5	4	4	−
UV8	3	5	5	6	5	8	7	8	18	19	18	15	2	3	3	3	−
UV12	**21**	**83**	**20**	**18**	6	**34**	6	4	26	**58**	16	13	9	**25**	7	8	−
UV15	9	9	13	**21**	8	5	6	6	11	10	11	10	5	6	7	**13**	−
UV16	13	8	7	9	10	8	7	8	12	10	9	13	4	3	2	6	−
UV18	8	6	6	4	13	9	11	8	22	14	14	13	5	5	4	4	−
UV20	6	6	6	12	13	9	6	10	19	18	17	40	4	4	3	**11**	−
UV21	6	2	1	1	8	3	1	1	16	8	2	2	3	1	0	1	−
Vaccinated carriers																	
UV2	7	6	7	4	6	5	6	3	12	10	13	6	4	3	5	3	−
UV5	6	7	5	9	4	5	3	6	10	11	9	9	3	4	3	9	+
UV9	12	7	5	**23**	13	10	5	**16**	20	15	11	15	6	5	3	**26**	+
UV10	10	8	8	12	7	7	4	11	18	18	17	17	5	5	5	**15**	+
UV11	8	8	6	**22**	9	8	5	11	16	16	12	19	4	4	3	**26**	+
UV13	5	6	6	**23**	9	6	5	12	11	10	11	17	5	6	7	**30**	+
UV14	8	7	6	9	10	8	6	11	15	11	11	13	4	4	3	6	−
UV17	3	2	3	7	2	2	2	6	3	3	4	4	3	2	2	**10**	+
UV19	X	6	7	**22**	X	7	9	**16**	X	12	14	16	X	4	3	**23**	+
Unvaccinated control cattle																	
UV22	6	7	5	**123**	8	9	7	**51**	16	16	13	**98**	3	4	3	**145**	+
UV23	6	5	6	**85**	8	6	6	**40**	**60**	38	33	**79**	3	4	3	**105**	+
UV24	9	6	6	**262**	12	10	9	**89**	10	9	9	**58**	5	4	4	**219**	+
UV25	8	6	7	**155**	6	5	5	**31**	14	10	12	15	5	4	4	**162**	+
UV26	9	8	7	**90**	7	6	5	**53**	**43**	26	20	**97**	5	6	5	**116**	+

[a] Vaccination, O_1 Manisa; challenge, O UKG 34/2001. Results obtained at 28 dpc were compared with results of the Cedi test. (14, 36, 37).
[b] Normalized MFI = assay bead MFI/NC MFI. Data are averages of three repeats for each sample. For the standard deviations of all values, see the supplemental material; they are typically ~10% of values shown here. Bold values are above the cutoff and therefore positive for antibodies against the NSP signature. Cutoffs are listed in Tables 2 and 3 and were determined from a naive population to give 95 to 97% specificity as follows: 3A peptide, 97%; 3B peptide, 95%; 3D peptide, 97%; 3ABC protein, 95%. Cutoffs were determined from analysis of untreated sera.
[c] Samples were derived from vaccination-challenge experiments and taken from cattle at 0, 14,and 21 dpv and 0 and 28 dpc. Full experimental details of the vaccination-challenge experiments and classification of cattle as carriers or noncarriers have been previously reported (14).
[d] 21 dpv = 0 dpc.
[e] 28 dpc quantitative results of the Cedi test, a 3ABC ELISA (14).
[f] X, sample missing. Samples UV3 at 28 dpc, UV4 at 21 dpv, and UV19 at 0 dpv were missing.

shows a large degree of nonspecific binding, particularly on 3B, throughout the experiment. There is a good correlation between the results obtained with the 3ABC signature in the multiplexed assay and the Cedi test on the samples from vaccinated carrier cattle at 28 dpc, and again, the other antigens in the multiplex show different degrees of response. The unvaccinated control cattle at 28 dpc show large responses against all of the antigens in the multiplex, with the exception of the 3D antigen with sample UV25. Table 5 shows the results of a herd of cattle following vaccination with a high-potency dose of O_1 Manisa oil adjuvant vaccine, resulting in fewer cattle with a carrier status. Again, there is a good correlation between the results obtained with samples taken at 28 dpc with the 3ABC antigen in the multiplex and the results of the Cedi test, with the exception of sample UY79. Samples UY77 and UY87 have large false-positive results on the 3D signature of samples prechallenge. Sample UY81 also shows false-positive results on the 3ABC signature prechallenge. The unvaccinated control cattle at 28 dpc show large responses against all of the antigens in the multiplex, with the exception of the 3D signature with UY94 and UY96.

DISCUSSION

When assessing the performance of a novel assay platform or signatures, it is essential to compare the performance obtained with standard samples against those obtained with a current "gold standard" assay. To compare the relative sensitivities of NSP antibody assays, the WRL for FMD in Pirbright, United Kingdom, generated a bovine serum panel composed of 36 samples (38). The serum panel was carefully selected to test relative assay sensitivity and reagent batch-to-batch reproducibility and contains diagnostically significant samples from vaccination-challenge experiments, in addition to sera from directly infected cattle as strong positives. The panel also contains sera from cattle defined as carriers and from cattle 3 to 5

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TABLE 5. Normalized MFI values for each signature in a multiplexed NSP antibody assay in response to serum samples taken over a vaccination-challenge experiments with O serotype[a]

Cattle group and sample identifier[c]	Normalized MFI[b]																
	3A				3B				3D				3ABC				
	0 dpv	14 dpv	21[d] dpv	28 dpc	0 dpv	14 dpv	21[d] dpv	28 dpc	0 dpv	14 dpv	21[d] dpv	28 dpc	0 dpv	14 dpv	21[d] dpv	28 dpc	28[e] dpc
Vaccinated noncarriers																	
UY72	6	11	7	**23**	4	10	6	**26**	9	12	9	12	3	5	4	**17**	+
UY73	1	1	2	4	1	2	2	4	2	14	19	21	1	1	1	3	−
UY74	1	1	2	6	1	1	1	4	6	14	14	26	1	4	2	5	−
UY77	5	7	5	6	5	7	5	8	23	**73**	**48**	**47**	3	4	3	3	−
UY78	5	7	5	5	6	9	6	7	9	14	9	9	3	3	2	3	−
UY79	5	5	4	3	5	6	4	4	17	32	25	14	3	2	2	5	+
UY80	5	5	6	**34**	5	7	5	6	9	12	12	11	3	3	3	**30**	−
UY81	**17**	**36**	21	**15**	5	8	4	4	14	37	27	19	**20**	**42**	**21**	**16**	−
UY82	3	3	4	5	5	5	6	6	24	36	28	24	2	3	3	3	−
UY83	1	0	0	2	1	1	0	2	1	1	1	3	1	0	0	2	−
UY84	7	10	7	6	13	**18**	9	10	8	11	7	7	6	7	5	4	−
UY85	10	9	3	4	7	8	4	5	10	10	3	5	5	5	2	2	−
UY86	7	9	8	10	3	5	5	9	3	4	6	16	6	8	6	9	−
UY87	12	10	11	**21**	8	6	6	**14**	**96**	**58**	**64**	40	**14**	9	**10**	**27**	+
UY88	6	7	7	8	10	9	10	11	15	11	11	13	4	4	4	4	−
UY89	6	7	6	7	9	11	9	9	13	13	11	11	4	8	6	4	−
UY91	3	5	3	6	3	4	4	5	34	**65**	**58**	41	2	2	2	3	−
UY92	6	5	8	1	6	5	6	0	11	7	8	1	4	3	7	0	−
Vaccinated carriers																	
UY76	2	3	4	**53**	3	**5**	5	11	4	6	7	13	2	2	2	**53**	+
UY90	6	8	6	**15**	4	**5**	4	11	5	6	7	13	3	5	4	**+**	+
Unvaccinated, control cattle																	
UY93	**14**	7	7	**115**	13	11	9	**77**	14	8	8	**48**	7	4	5	**193**	+
UY94	1	2	3	**138**	5	**14**	11	**51**	3	5	5	13	1	2	2	**163**	+
UV95	**15**	**16**	11	**81**	10	8	8	**48**	21	15	14	**85**	**14**	9	6	**124**	+
UY96	5	2	2	**86**	7	3	4	**47**	9	4	4	20	3	1	2	**197**	+
UY97	8	8	7	**146**	11	9	8	**51**	10	9	10	**63**	5	5	5	**158**	+

[a] Vaccination was with O_1 Manisa, and challenge was with O UKG 34/2001. The vaccination dose was increased 10-fold compared to that in experiments used to generate serum samples reported in Table 4 and comparing results obtained at 28 dpc with results of the Cedi test (13, 36, 37).

[b] Normalized MFI = assay bead MFI/NC MFI. Data are averages of three repeats for each sample. For the standard deviations of all values, see the supplemental material; they are typically ~10% of the values shown here. Bold values are above the cutoff and therefore positive for antibodies against the NSP signature. Cutoffs are listed in Tables 2 and 3 and were determined from a naive population to give 95 to 97% specificity as follows: 3A peptide, 97%; 3B peptide, 95%; 3D peptide, 97%; 3ABC protein, 95%. Cutoffs were determined from analysis of untreated sera.

[c] Samples were derived from vaccination-challenge experiments and taken from cattle at 0, 14, and 21 dpv and 0 and 28 dpc. Vaccines were administered at 10 times the dosage used to generate serum samples reported in Table 4. Full experimental details of the vaccination-challenge experiments and classification of cattle as carriers or noncarriers have been previously reported (13).

[d] 21 dpv = 0 dpc.

[e] 28 dpc quantitative results of the Cedi test, a 3ABC ELISA (13).

months postchallenge. Significantly, the panel contains samples from cattle infected by contact, mimicking the mode of infection in the case of an outbreak.

Previously, the multiplexed assay was carried out with PBS, Tween 20, BSA, and sodium azide (41) as an assay buffer. However, during the initial course of the experiments reported here, the product number and type of BSA appeared to have an effect on the assay, severely reducing binding to the beads. Therefore, to eliminate the possibility that end users will inadvertently use different vendors for the BSA in the assay buffer and consequently achieve anomalous results, BSA was removed from the assay buffer in this further development.

Samples from FMD-naive cattle were used to generate a cutoff for each signature in the multiplexed assay. Figure 2 shows the responses of each signature to this naive population. The cutoff was determined individually for each signature to give high specificity, i.e., 97% for 3A, 3D, and 3ABC and 95% for 3B. Signatures 3A and 3ABC gave the lowest values on the false positives, and 3D showed the largest variation in response in a naive population. It appears that there is a large degree of nonspecific binding on the 3D signature in naive sera, and this variation increases the cutoff of the 3D signature. The false positives on the 3D signature were also large responses. The responses of the vaccinated population shown in Fig. 3 are very similar to those of the naive population, with similar specificities on each signature. It should be noted that the vaccines used to vaccinate this population were inactivated virus of high purity and not contaminated with NSPs and therefore these samples were expected to have a similar response to a naive population.

Against the serum panel (Table 2), the 3ABC signature in the multiplex showed performance comparable to that of the Cedi test, a 3ABC ELISA, where all of the samples except UY83 exhibited a positive response, i.e., equal to or above the

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cutoff. Peptide signatures 3A and 3B exhibited a positive response to many of the samples, the exceptions being in the UV and UY series samples at 3 to 6 months postchallenge. It should be noted that the 3ABC protein signature contains both the 3A and 3B epitopes, and the individual assessment of the 3A and 3B epitopes in the multiplexed assay reports the relative response of each epitope. The 3D signature generally performed poorly against weak positive samples in the panel. The responses were "all or nothing," giving a large positive response to some samples and a very negative response to others. The 3D signature is a peptide representing the immunogenic amino terminus of the whole 3D NSP, and the 3D protein is regarded as the most immunogenic of the NSPs. Furthermore, the 3D signature alone is not considered a DIVA marker, as NSP 3D has been shown to be a contaminant in vaccine formulations that may be due to the presence of one copy of the 3D polymerase enzyme in the virion (32), and consequently, it is possible for vaccinated animals to raise antibodies against the 3D signature (26, 47). However, simultaneous multiple-signature evaluation allows the consideration of the response of the 3D signature in the context of the responses of the other signatures to give increased confidence in calling a result. Further investigation of the performance of the 3D peptide signature is included in future development. The large dynamic range of the responses in the multiplexed assay is also of note. Normalized responses range from just above the cutoff for the samples expected to have low levels of antibodies, i.e., 3 to 6 months postchallenge to 20 to 30 times the cutoff for strong positives. The liquid array technology with fluorescence detection is extremely sensitive considering that the serum samples are at a final dilution of 1:600 in the assay. This dilution factor is required for the bead-based serology assay; with significantly lower dilution factors, the nonspecific binding on the antigen-coated beads overwhelms the specific interaction. There have been attempts to reduce the nonspecific binding in liquid array serology assays (48), including the production of commercially available serology beads (Luminex Corp.); however, it remains a challenge to reduce the background in bead-based serological assays.

While heat inactivation of the serum samples generally lowered the responses of each signature (Table 3), it only changed the final results of a few signatures on a few samples. In addition, this analysis was using a cutoff determined from the analysis on untreated sera. It is likely that the cutoffs generated from the analysis of heat-treated naive sera would also be lower, and therefore, heat inactivation has little overall effect on the performance of the liquid array multiplexed assay.

Extensive prior characterization of the samples from the O_1 Manisa oil adjuvant vaccination-O UKG challenge (UV and UY series) experiments has been previously reported (13, 14). The analysis of the samples with the multiplexed assay again allowed comparison with the results of the Cedi test. In general, the results of the 3ABC signature in the multiplexed assay correlated with the results of the Cedi test and the results obtained with signatures 3A, 3B, and 3D were variable. Once again, a large dynamic range was observed. The results of the analysis of samples from unvaccinated control cattle show much larger responses on all of the signatures than the vaccinated carrier cattle, correlating with observations in many singleplex NSP ELISA experiments (37). This may be attributed to the limited replication of FMDV in vaccinated animals, where the levels of neutralizing antibodies are significantly higher than in unvaccinated animals. A few samples from vaccinated noncarrier cattle show some nonspecific binding at various time points in the experiment. Including the responses of cattle at 28 dpc shows the responses at the beginning of the time frame that is particularly significant when using serological surveillance for FMDV postoutbreak. Future experiments will include analysis of these samples at 1 to 3 months postchallenge, as this is the time period during which serosurveillance would take place postoutbreak to declare a disease-free status.

In conclusion, the liquid array multiplexed NSP antibody assay shows good performance against a panel of sera designed to assess the relative sensitivity of NSP antibody assays with diagnostically relevant samples. The 3ABC signature in the multiplex shows performance comparable to that of a widely used commercially available assay, and in addition, the multiplexed assay provides a large amount of extra information about the relative diagnostic sensitivity of each signature in one experiment. This feature of the multiplexed assay is particularly attractive when considering the potential use of the assay in vaccine development and assessing vaccine purity. It is trivial to prepare antigen-coated beads for serological applications once capture agents have been generated, and the assay is completed in 1 h. The multiplexed assay is rapid and conducive to automation, and a crude cost evaluation of reagents and consumables comes to U.S. $0.50 per assay, which is not cost prohibitive. Following this encouraging evaluation of the sensitivity and specificity of the multiplexed assay, experiments to evaluate field performance, stability of reagents, and reagent lot-to-lot repeatability and possibly expanding the multiplex to cover all FMDV NSP signatures will be carried out. Moreover, following experiments to test an expanded range of signatures and improvement and understanding of the performance of the 3D signature, data evaluation will allow the generation of more standardized cutoffs for each signature and a determination of the number of positive signatures in the multiplex required to actually call a sample positive. This further work will likely generate a robust and reliable FMD DIVA assay for validation and use in the field.

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