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

Introduction.

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 a few examples. The use of such multiplexing technology has time, cost and manpower benefits over multiple, singleplex analyses, in addition to an increased confidence in results. 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 using a detector reagent, indirectly labeled with a fluorescent reporter. 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 bound fluorescent reporter and quantifies the assay at the bead surface. The flow cytometer is capable of reading around one 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 and economic platform.

Foot-and-mouth disease (FMD) is a highly infectious and contagious vesicular disease affecting cloven-hoofed animals. Foot-and-mouth disease virus (FMDV) belongs to the genus *Aphthovirus* in the *Picornaviridae* family and includes seven serotypes; O, A, Asia, C, SAT1, 2 and 3. The circulation of foot-and-mouth disease virus (FMDV) in an animal population imparts

severe restrictions on the movement of animal products, and consequently, the international trade of the affected region. 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 sub-clinically infected and ultimately become a carrier of the virus, which is considered as a threat of spreading disease to other susceptible animals (13-15, 17, 22, 30). Therefore, to regain FMD-free status and re-enable international trade, post-vaccinal 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, semi-purified virions. Therefore, only assays that measure levels of antibodies against non-structural protein (nsp) can differentiate infected and vaccinated animals (DIVA) providing 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 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 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 in FMDV serological monitoring in South America (5, 6). An international workshop for the validation of nsp tests 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 and has previously been shown to be a promising platform for the development of a multiplexed nsp FMD DIVA assay (11, 41). Here, further development and evaluation of the multiplexed nsp antibody assay is described using samples originating from two vaccine / challenge experiments and a panel of bovine serum samples assembled to the test relative sensitivity of nsp antibody assays. The sera panel consists of samples originating from various vaccine potency experiments conducted at the World Reference Laboratory (WRL) for FMD in Pirbright, UK 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 in PBS-TN (phosphate buffered saline, pH 7.4; Tween 20 0.02 % v/v; sodium azide 0.02 % w/v) and filtered through Corning 0.22 µm filter systems before use.

Bead mixture. The bead mixture consisted of 8 beads sets – 4 assay beads and 4 controls (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.) using the 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), fluorescent control (FC), antibody control (AC) and a negative control (NC). Bead mixture was formulated in PBS-TN to a theoretical final concentration of ~ 5.4 x 10^6 of each bead class / mL. This gives a 10X mixture that can be stored over time. Following formulation, the bead mixture was enumerated by taking 5 µL of the 10X bead mixture, diluting it in 95 µL PBS-TN and counting all the beads in a 50 µL sample using a Bio-Plex system (Bio-Rad). 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. With this dilution and enumeration method, the numbers of bead in each class was approximately 500. The bead mixtures were stored at 4 ºC 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 IgY (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 ºC. Samples were thawed and diluted 1:400 for serial bleed assays in PBS-TN directly before use. All samples were used in an assay a maximum of 1 h post dilution.

Sera — General. The sera were collected during the course of a series of vaccination / challenge experiments carried out in biosecurity containment at the WRL for FMD, Pirbright, UK. Typically, naïve cattle were vaccinated, and at 21 days post vaccination (dpv), were challenged by either homologous or semiheterologous FMD virus by intradermolingual inoculation or by direct contact with infected cattle. Sera were collected at various days pre- (dpv) and post-challenge (dpc). The experimental details, including virological and serological findings, have been extensively reported (13, 14, 36-39). The experiments were designated with a two letter identifier (e.g. UV) and these identifiers coupled with a number are used to describe each animal in an experiment.

Sera from naïve cattle. 104 serum samples were collected from cattle at the beginning of vaccination / challenge experiments before any administration of vaccine or virus. The identifications of the samples are listed in the supplemental material.

Sera from vaccinated and infected cattle. 94 serum samples were tested from cattle at 14 or 21 dpv, in some cases from the same animal at the different time point, during the course of vaccination / challenge experiments. The identification and dpv of the vaccinated samples are listed in the supplemental material. In addition, samples from two O serotype vaccine / challenge experiments (UV (14) and UY series (13)) were tested at 0 dpv, 14 dpv, 21 dpv/0 dpc and 28 dpc. The details of the experiments used to generate these samples has been previously reported in full.

Bovine sera panel. 36 bovine serum samples were selected from a series of vaccination / challenge experiments. The details of the experiments used to generate these samples has been previously reported in full (13, 14, 37, 38). The state of the animal (vaccination, challenge, mode and serotype of challenge, and carrier status etc.) when the serum samples were taken is

summarized in Table 1. The bovine sera 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 PBS-TN. 100 µL of diluted sample was deposited in each well. 50 µL bead mixture to each sample well and incubated in the dark for 20 min. Samples were washed twice with 100 µL PBS-TN. The beads were re-suspended in 100 µL PBS-TN, 50 µL detector reagent was added and incubated in the dark for 15 min. Samples were washed with 100 µL PBS-TN. The beads were re-suspended in 100 µL PBS-TN, 50 µL SA-PE was added, and incubated in the dark for 5 min. The samples were washed with PBS-TN and re-suspended in 100 µL PBS-TN. 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 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 a negative and positive control respectively, at 1:400 dilution in PBS-TN.

Results.

Liquid array technology has been used to develop a multiplexed nsp-based DIVA assay for FMD (41). Figure 1 shows a schematic representation of the liquid array bead-based nsp 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 as the complex is analyzed in a flow cytometer. Previous work showed a close correlation between the response 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

155 combined with recombinant nsp antigen 3ABC and four controls—an instrument control (IC), a
156 fluorescent control (FC), an antibody control (AC) and a negative control (NC)—to generate an
157 8-plex for further development. Peptides were chosen over recombinant antigens where
158 appropriate as peptides can be easily produced in large quantities under strict QC control without
159 biosafety level 3 containment. Recombinant antigen 3ABC remained in the multiplex as this
160 signature is commonly used in ELISA nsp assays (2, 4, 6, 8-10, 24, 29, 33, 38, 44, 46).

161 The 8-plex assay was tested against 104 FMD-naïve serum samples to establish normal
162 variation in a naïve population and also to determine a cut off, above which a sample is deemed
163 positive for infection with FMDV. During initial development of the multiplexed assay, the
164 response on the antigen-coated or "assay" beads during the first few days of infection was
165 extremely low and the response on the BSA-coated NC bead remained more or less constant
166 when analyzing samples from serial bleeds of experimentally infected cattle (41). However,
167 upon expanded analysis of samples from a naïve population of cattle (data not published to date),
168 significant differences in the responses of the assay beads were observed. This correlated with
169 large differences in the response of the NC bead. Therefore, the NC is used to normalize the
170 response of the assay beads and other researchers have used this approach to normalize liquid
171 array-based serology assay results (21). Significantly, FMDV-infected cattle generally maintain
172 a low response on the NC and therefore, normalization effectively increases the sensitivity of the
173 multiplexed liquid array assay.

174 Figure 2a-d shows the normalized response on each of the four assay beads in response to
175 sera from 104 FMD naïve cattle. The animal identification codes, the crude median fluorescence
176 intensity (MFI) values on all beads including controls, the normalized values and standard
177 deviations are listed in the supplementary material. The standard deviation on the normalized

responses of the naïve population was on average ~10 % of the value. The 104 samples were used to generate a cut off of 97 % specificity for antigens 3A, 3D and 3ABC and 95 % for 3B. Positive bars in Figures 2a-d are above cut off. 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 Figure 3a-d. The samples were taken from cattle 14 dpv (black bars) and 21 dpv (gray bars). The animal identification codes, the crude MFI values on all beads including controls, the normalized values and standard deviations are listed in the supplementary material. The standard deviation on the normalized responses of the naïve population was, on average, ~10 % of the value. Using the cuts offs determined from the naïve populations (positive bars are above cut off in Figure 3) antigen 3A showed a specificity of 96 %, antigen 3B 97 %, antigen 3D 93 % and 3ABC 97 % indicating a vaccinated population gives a similar response to a naïve population provided the vaccine used is of high purity.

The multiplexed assay was used to analyze the 36 samples of a bovine sera panel assembled by the WRL for FMD (38). Table 1 lists the origin of each sample including the details of vaccination and challenge and the time at which the sera were collected. The normalized responses of each antigen in the multiplex to each sample are listed in Table 2. The data in Table 2 are an average of two experiments, three repeats for each sample in each experiment. The crude MFI values on all beads including controls, the normalized values and standard deviations are listed in the supplementary material. The serum 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 as a comparison. The results of the Cedi® test and other non-structural antibody assay results for each sample in the

panel have been previously reported (38). The cut off for each antigen generated from the naïve population was applied to the results obtained for bovine serum panel. The 3ABC antigen in the multiplexed assay shows good correlation with the Cedi® test results with the exception of sample UV83, which proved negative with the multiplexed assay. However, two carrier cattle (UZ59 and UZ62, carrier status confirmed by RT-PCR / virus isolation) were also detected positive by the 3A, 3B and 3ABC signature in the multiplexed assay, whereas they were missed by the Cedi® test. Moreover, vaccinated, non-carrier animal UZ54 was clearly positive according to 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 in 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. The crude MFI values on all beads including controls, the normalized values and standard deviations are listed in the supplementary material. Heat inactivation reduces the likelihood that the serum samples are contaminated with live FMDV and allows the samples to be analyzed outside BSL-3 containment. Therefore, it is important to determine if the performance of nsp antibody assays are not 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 the response fell below the cut off, 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 28 dpc. The serum samples were taken at 0 dpv, 14 dpv, 21 dpv/0 dpc, 28 dpc. Again, the cattle are grouped according to their serological status, which has previously been determined. Table 4 shows results of 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, non-carrier cattle are negative for antibodies to nsps pre- and post-challenge with a few exceptions. UV15 (3A and 3ABC) and UV20 (3ABC) rose above cut off post challenge and this is contrary to the results of the Cedi® test. UV6 and UV7 are false positives on one signature 0 dpv. UV12 shows a large degree of non-specific binding, particularly on 3A, 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 28 dpc and again, the other antigens in the multiplex show different degrees of response. The unvaccinated control cattle 28 dpc show large responses against all 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 vaccine, resulting in fewer cattle with a carrier status. Again, there is good correlation in the results obtained against samples taken 28 dpc with the 3ABC antigen in the multiplex and the results of the Cedi® test with the exception of UY79. Samples UY77 and UY87 have large false positive results on the 3D signature on samples pre-challenge. Sample UY81 also shows false positive results on the 3ABC signature pre-challenge. The unvaccinated control cattle 28 dpc show large responses against all antigens in the multiplex, with the exception of the 3D signature with UY94, UY96 and UY97.

Discussion.

When assessing the performance of a novel assay platform or signatures, it is essential to compare performance with standard samples against a current 'gold standard' assay. To compare relative sensitivities of nsp antibody assays, the World Reference Laboratory (WRL) for FMD in Pirbright, UK generated a bovine sera panel comprised of thirty-six samples (38). The sera 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 3–5 months post challenge. 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 using 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 of end users inadvertently using different vendors for BSA in the assay buffer and consequently achieving anomalous results, BSA was removed from the assay buffer in this further development.

Samples from FMD naïve cattle were used to generate a cut off for each signature in the multiplexed assay. Figure 2 shows the responses of each signature to this naïve population. The cut off was determined individually for each signature to give high specificity; 97 % for 3A, 3D and 3ABC, 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 naïve population. It appears there is a large degree of non-specific binding on the 3D signature in naïve sera and this variation increases the

cut off of the 3D signature. The false positives on the 3D signature were also large responses. The responses of the vaccinated population shown in Figure 3 are very similar to the naïve 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 naïve population.

Against the sera panel (Table 2), the 3ABC signature in the multiplex showed comparable performance to the Cedi® test, a 3ABC ELISA, where all samples except UY83 exhibited a positive response, i.e. equal to or above cut off. Peptide signatures 3A and 3B peptide signatures exhibited a positive response to many of the samples, the exceptions being in the UV and UY series samples at 3-5 months post challenge. 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 in the multiplexed assay reports the relative response of each epitope. The 3D signature generally performed poorly against weak positive samples 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.

293 The large dynamic range of the responses in the multiplexed assay is also of note. Normalized
294 responses range from just above cut off for the samples expected to have low levels of antibodies
295 i.e. 3-5 months post challenge to 20-30 times cut off for strong positives. The liquid array
296 technology with fluorescent detection is extremely sensitive considering the serum samples are at
297 a final dilution of 1:600 in the assay. This dilution factor is required for the bead-based serology
298 assay; with significantly lower dilution factors, the non-specific binding on the antigen-coated
299 beads overwhelms the specific interaction. There have been attempts to reduce the non-specific
300 binding in liquid array serology assays (48), including the production of commercially available
301 serology beads (Luminex Corp), however it remains a challenge to reduce background in bead-
302 based serological assays.

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

309 The overall results of the O Manisa vaccination / O UKG challenge (UV and UY series)
310 experiments have been previously reported (13, 14). The analysis of the samples with the
311 multiplexed assay again allowed comparison with the results of the Cedi® test. In general, the
312 results of the 3ABC signature in the multiplexed assay correlated with the results of the Cedi®
313 test and the results on signatures 3A, 3B and 3D were variable. Once again, a large dynamic
314 range is observed. The results from the analysis of samples from unvaccinated, control cattle
315 show much larger response on all signatures than the vaccinated carrier cattle correlating with

316 observations in many singleplex nsp ELISA experiments (37). This may be attributed to the
317 limited replication of FMDV in vaccinated animals where the levels of neutralizing antibodies
318 are significantly higher than in unvaccinated animals. A few samples from vaccinated, non-
319 carrier cattle show some non-specific binding in various time points in the experiment.
320 Including the responses of cattle at 28 dpc shows the responses at the beginning of the time
321 frame that is particularly significant when using serological surveillance for FMDV post
322 outbreak. Future experiments will include analysis of these samples 1-3 months post challenge
323 as this is the time period that sero-surveillance would take place post-outbreak to declare a
324 disease-free status.

325 In conclusion, the liquid array, multiplexed nsp antibody assay shows good performance
326 against a panel of sera designed to assess the relative sensitivity of nsp antibody assays with
327 diagnostically relevant samples. The 3ABC signature in the multiplex shows comparable
328 performance to a widely used commercially available assay, and in addition, the multiplexed
329 assay provides a large amount of extra information about the relative diagnostic sensitivity of
330 each signature in one experiment. This feature of the multiplexed assay is particularly attractive
331 when considering the potential use of the assay in vaccine development and assessing vaccine
332 purity. It is trivial to prepare antigen-coated beads for serological applications, once capture
333 agents have been generated and the assay is completed in 1 h. The multiplexed assay is rapid,
334 conducive to automation and a crude cost evaluation of reagents and consumables comes to US
335 50 c per assay, which is not cost-prohibitive. Following this encouraging evaluation of the
336 sensitivity and specificity of the multiplexed assay, experiments to evaluate field performance,
337 stability of reagents and reagent lot-to-lot repeatability and possibly expanding the multiplex to
338 cover all FMDV nsp signatures will be carried out. Moreover, following experiments to test an

expanded range of signatures and improvement / understanding of the performance of the 3D signature, data evaluation will allow the generation of more standardized cut offs 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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Figure legends.

Figure 1. Schematic of bead-based, multiplexed non-structural protein antibody assay. A peptide or non-structural protein is covalently conjugated to a Luminex™ bead. The peptide or recombinant protein captures antibodies to non-structural proteins 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 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 bound fluorescent reporter 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 captured antibody concentration.

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

559 Figure 3. Responses of each signature in multiplexed assay to challenge with sera from 94
560 vaccinated cattle to illustrate expected response of a vaccinated population. Black bars are
561 responses to sera from cattle 14 dpv; gray bars are responses to sera from cattle 21 dpv. Vaccine
562 strains are listed in the supplemental material. Responses of assay beads are reported as median
563 fluorescence intensity (MFI) of the assay bead normalized using the MFI of the negative control
564 (NC) BSA-coated bead in each sample. All signatures are on shown on the same scale for visual
565 comparison. Positive bars indicate a normalized MFI value above cut off. Cuts offs are
566 determined from naïve population to give 95-97 % specificity. Negative bars indicate a
567 normalized MFI value below cut off. (a) 3A peptide, 97 % specificity cut off. (b) 3B peptide, 95
568 % cut off. (c) 3D peptide, 97 % cut off. (d) 3ABC protein 95 % cut off.
569

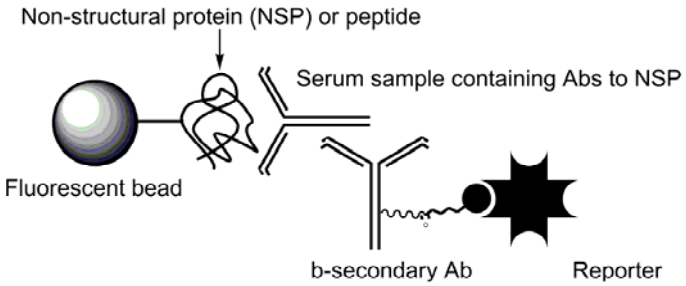
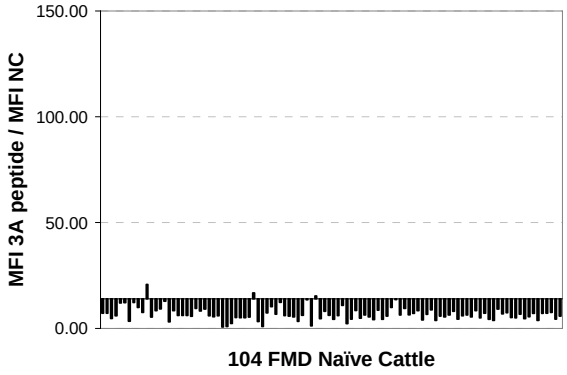

Non-structural protein (NSP) or peptide
Serum sample containing Abs to NSP
Fluorescent bead
b-secondary Ab
Reporter

Figure 1

(a)



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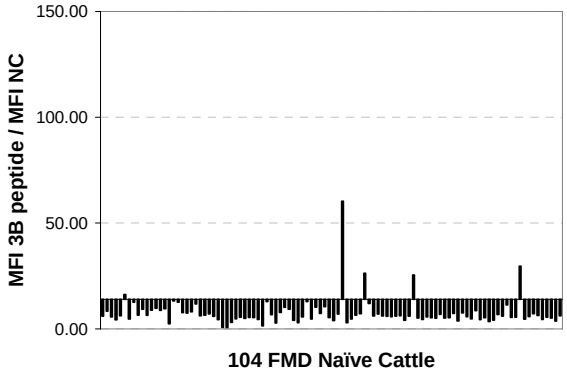

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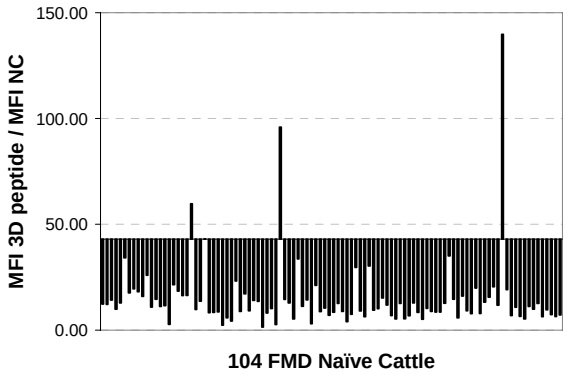

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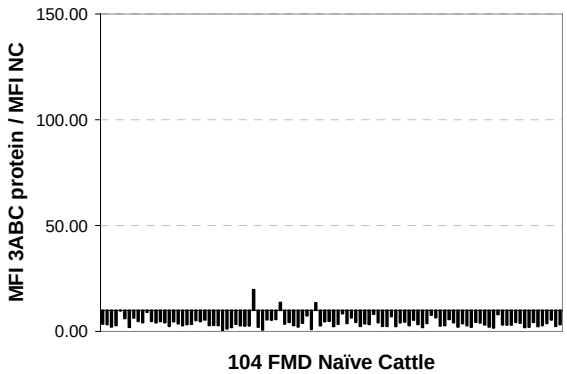
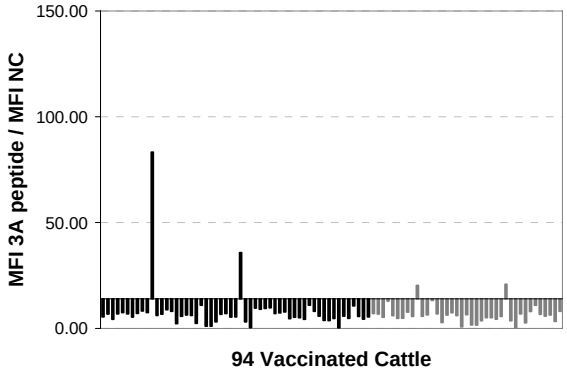
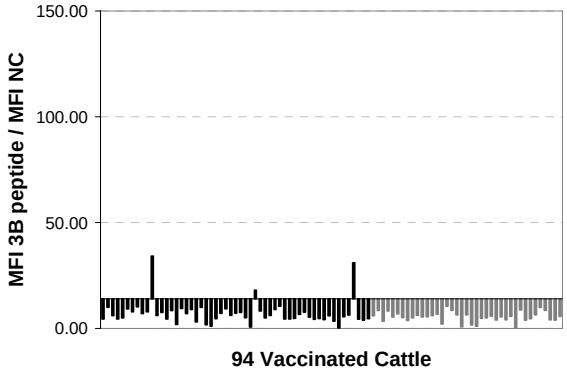


Figure 2

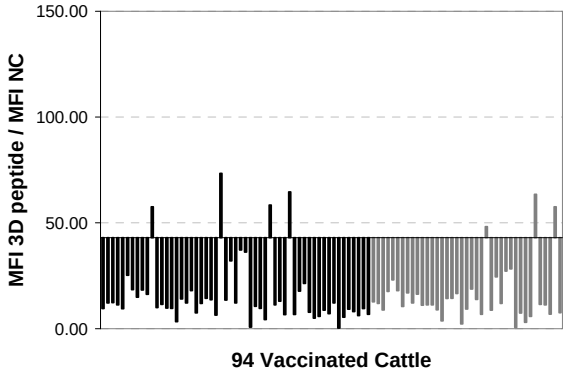
(a)



(b)



(c)



(d)

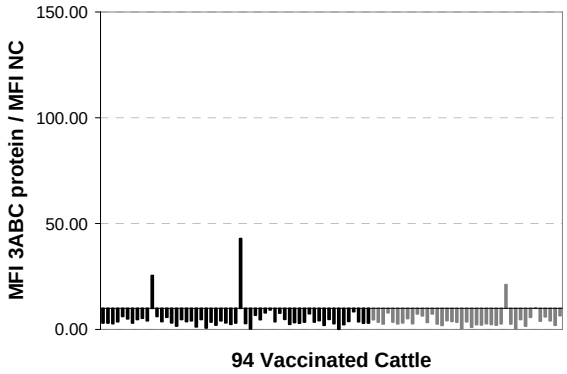


Figure 3

TABLE 1. Origin of bovine serum panel to test relative sensitivity of nsp antibody assay assays (13, 14, 38).

Sample Identifier	Vaccination strain	Challenge serotype[a]	Challenge method[a]	Clinical signs	dpc sera taken
Vaccinated, carrier cattle					
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	Innoculation		42
VE64	Asia 1 Shamir	Asia 1 Shamir	Innoculation		42
VE65	Asia 1 Shamir	Asia 1 Shamir	Innoculation	Yes	42
VE66	Asia 1 Shamir	Asia 1 Shamir	Innoculation		42
VE67	Asia 1 Shamir	Asia 1 Shamir	Innoculation	Yes	42
VL83	SAT2 3218	SAT2 Eritrea	Innoculation		36

VL89	SAT2 3218	SAT2 Eritrea	Innoculation	Yes	36
VL90	SAT2 3218	SAT2 Eritrea	Innoculation		37
Unvaccinated, carrier cattle					
UZ68	n/a	A Iran 96	Innoculation	Yes	33
UZ69	n/a	A Iran 96	Innoculation	Yes	33
UY95	n/a	O UKG	Contact	Yes	107
UY96	n/a	O UKG	Contact	Yes	107
UV26	n/a	O UKG	Contact	Yes	174
VH44	n/a	O UKG	Contact	Yes	40
VH45	n/a	O UKG	Contact	Yes	40
Vaccinated, non-carrier cattle					
VE73		Asia 1 Shamir	Innoculation		43
VE71		Asia 1 Shamir	Innoculation		43
UZ54		A Iran 96	Innoculation	Yes	32
UY79	O Manisa	O UKG	Contact		106
Unvaccinated, non-carrier cattle					
VE60	n/a	Asia 1 Shamir	Innoculation	Yes	42
VE62	n/a	Asia 1 Shamir	Innoculation	Yes	42
UY94	n/a	O UKG	Contact	Yes	107
UV23	n/a	UKG 34/01	Contact	Yes	37
UV24	n/a	O UKG	Contact	Yes	174

[a] Cattle were challenged 21 dpv.

TABLE 2. Normalized MFI values for each signature in multiplexed non structural protein antibody assay in response to bovine serum panel and comparison with results of Cedi® test (38).

Sample Identifier	Normalized MFI[a] 3A	Normalized MFI[a] 3B	Normalized MFI[a] 3D	Normalized MFI[a] 3ABC	Qualitative result for Cedi® test[b]
Cut off[c]	14	14	43	10	n/a
NSS[d]	3	4	6	3	n/a
Vaccinated, carrier cattle					
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, carrier cattle					
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, non-carrier cattle					
VE73	**21**	**31**	18	**91**	+
VE71	**207**	**76**	41	**261**	+
UZ54	**49**	**74**	11	**109**	+/-
UY79	9	7	20	**10**	+
Unvaccinated, non-carrier cattle					
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] Normalized Median Fluorescence Intensity (MFI): Assay bead MFI / Negative control (NC) MFI. Data are an average of two experiments, three repeats for each sample in each experiment. SD's for all values are listed in the supplemental material and are typically ~10 % of value. Bolded, shaded values are above the cut off and therefore positive for antibodies against the non-structural proteins.

[b] Data taken from Parida *et al* (38); Cedi® test is a 3ABC ELISA; + positive in all test, - negative in all tests, +/- some tests positive and some tests negative.

[c] Cuts offs are determined from naïve population to give 95-97 % specificity: 3A peptide, 97 %; 3B peptide, 95 %; 3D peptide, 97%; 3ABC protein 95 %.

[d] NSS: Normal Sigma Serum. Assay response to normal serum commercially available from Sigma; average of 95 repeats as an untreated sera standard control.

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

Sample Identifier	Normalized MFI[a] 3A		Normalized MFI[a] 3B		Normalized MFI[a] 3D		Normalized MFI[a] 3ABC	
	HI[b]	Δ[c]	HI[b]	Δ[c]	HI[b]	Δ[c]	HI[b]	Δ[c]
Cut off[d]	14	n/a	14	n/a	43	n/a	10	n/a
NSS[e]	3	n/a	4	n/a	6	n/a	3	n/a
Vaccinated, carrier cattle								
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, carrier cattle								
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, non-carrier cattle								
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, non-carrier cattle								
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 Median Fluorescence Intensity (MFI): Assay bead MFI / Negative control (NC) MFI. Data are an average of three repeats for each sample. SD's for all values are listed in the supplemental material and are typically ~40 % of value. Bolded, shaded values are above the cut off and therefore positive for antibodies against the non-structural proteins. Boxed values indicate a change in status (positive to negative) after the sera are heat treated when compared to the untreated sera.

[b] HI: Heat inactivated.

[c] Δ: Response with untreated sera (reported in Table 2) minus response with heat inactivated sera. A negative result indicates response with heat inactivated sera is lower.

[d] Cuts offs are determined from naïve population to give 95-97 % specificity: 3A peptide, 97 % ; 3B peptide, 95 %; 3D peptide, 97 % ; 3ABC protein 95 %. Cut offs were determined from analysis of untreated sera.

[e] NSS: Normal Sigma Serum. Assay response to normal serum commercially available from Sigma; average of 95 repeats as an untreated sera standard control.

TABLE 4. Normalized MFI values for each signature in multiplexed non structural protein antibody assay in response to serum samples taken over a vaccination / challenge experiments with the O serotype — Vaccination O_1 Manisa, Challenge OUKG 34/2001 and comparing 28 dpi with results of the Cedi® test (14, 36, 37).

	Normalized MFI[a] 3A				Normalized MFI[a] 3B				Normalized MFI[a] 3D				Normalized MFI[a] 3ABC				
Sample	0	14	21[c]	28	0	14	21[c]	28	0	14	21[c]	28	0	14	21[c]	28	28[d]
Identifier[b]	dpv	dpv	dpv	dpc	dpv	dpv	dpv	dpc	dpv	dpv	dpv	dpc	dpv	dpv	dpv	dpc	dpc
Vaccinated, non-carrier cattle																	
UV3	7	7	7	x	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, carrier cattle																	
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] Normalized Median Fluorescence Intensity (MFI): Assay bead MFI / Negative control (NC) MFI. Data are an average of three repeats for each sample. SD's for all values are listed in the supplemental material and are typically ~10 % of value. Bolded, shaded values are above the cut off and therefore positive for antibodies against the non-structural protein signature. Cuts offs are listed in Tables 2 and 3 and are determined from naïve population to give 95-97 % specificity: 3A peptide, 97 %; 3B peptide, 95 %; 3D peptide, 97 %; 3ABC protein 95 %. Cut offs were determined from analysis of untreated sera.

[b] Samples were derived from vaccination / challenge experiments and taken from cattle 0 dpv, 14 dpv, 21 dpv/0 dpc, and 28 dpc. Full experimental details of the vaccination / challenge experiments and classification of cattle as carriers / non-carriers has been previously reported (14).

[c] 21 dpv = 0 dpc.

[d] 28 dpc quantitative results of the Cedi® test, a 3ABC ELISA (14).

x : Sample missing; Samples UV3, 28 dpc; UV4, 21 dpv and UV19, 0 dpv were missing.

TABLE 5. Normalized MFI values for each signature in multiplexed non structural protein antibody assay in response to serum samples taken over a vaccination / challenge experiments with O serotype — Vaccination O_1 Manisa, Challenge OUKG 34/2001. The vaccination dose was increased ten-fold compared to experiments used to generate serum samples reported in Table 4 and comparing 28 dpi with results of the Cedi® test (13, 36, 37).

	Normalized MFI[a] 3A				Normalized MFI[a] 3B				Normalized MFI[a] 3D				Normalized MFI[a] 3ABC				
Sample	0	14	21[c]	28	0	14	21[c]	28	0	14	21[c]	28	0	14	21[c]	28	28[d]
Identifier[b]	dpv	dpv	dpv	dpc	dpv	dpv	dpv	dpc	dpv	dpv	dpv	dpc	dpv	dpv	dpv	dpc	dpc
Vaccinated, non-carrier cattle																	
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, carrier cattle																	
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	**16**	+
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] Normalized Median Fluorescence Intensity (MFI): Assay bead MFI / Negative control (NC) MFI. Data are an average of three repeats for each sample. SD's for all values are listed in the supplemental material and are typically ~10 % of value. Bolded, shaded values are above the cut off and therefore positive for antibodies against the non-structural protein signature. Cuts offs are listed in Tables 2 and 3 and are determined from naïve population to give 95-97 % specificity: 3A peptide, 97 %; 3B peptide, 95 %; 3D peptide, 97 %; 3ABC protein 95 %. Cut offs were determined from analysis of untreated sera.

[b] Samples were derived from vaccination / challenge experiments and taken fro cattle 0 dpv, 14 dpv, 21 dpv/0 dpc, and 28 dpc. Vaccines were administered at ten 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 / non-carriers has been previously reported (13).

[c] 21 dpv = 0 dpc.

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