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Research paper

Enhanced sensitivity in detection of antiviral antibody responses using biotinylation of foot-and-mouth disease virus (FMDV) capsids

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ABSTRACT

Analysis of the immune response to infection of livestock by foot-and-mouth disease virus (FMDV) is most often reported as the serum antibody response to the virus. While measurement of neutralizing antibody has been sensitive and specific, measurements of the quality of the antibody response are less robust. Determining the immunoglobulin (Ig) isotype of the serum antibody response provides a deeper understanding of the biology of the response and more sensitive methods for these assays will facilitate analyses of B cell mediated immunity. We tested the hypothesis that using the virus as the molecular probe could be achieved by adding tags to the surface of the FMDV capsid, and that would enhance sensitivity in assays for anti-FMDV antibody responses. The use of a FLAG-tagged virus in these assays failed to yield improvement whereas chemically biotinylating the virus capsid resulted in significant enhancement of the signal. Here we describe methods using biotinylated virus for measuring anti-viral antibody in serum and antibody secreting cells (ASCs) in blood that are sensitive and specific. Finally, we describe using the biotinylated virus in flow cytometry where such assays should greatly enhance the analysis of anti-virus antibody producing B cells, allowing the investigator to focus on only the FMDV specific B cells when analyzing the development of the B cell response to either infection or vaccination.

1. Introduction

Foot-and-mouth disease (FMD) is a viral infection of cloven hooved animals characterized by the appearance of vesicular lesions on the feet and in the mouth. The causal agent, FMD virus (FMDV), has an icosahedral capsid, enclosing a positive, single strand RNA genome. Following primary infection, FMDV rapidly replicates within epithelial tissues which precedes a marked viremia that peaks 4 days following infection. There is a rapid antibody response to the virus that can be detected in serum 2–4 days following infection (Ostrowski et al., 2007; Pega et al., 2013). In laboratory studies, viremia declines rapidly and becomes undetectable 7 days following inoculation (Burrows et al., 1981; Pacheco et al., 2010a). Following vaccination with empty capsid vaccine, anti-FMDV antibody has been detected for as long as six months, (Sitt, T., et al., manuscript in preparation) however induction of FMDV specific memory B cells has yet to be documented following either vaccination or infection.

Detection of antigen specific serum antibodies by ELISA has long been used to study the humoral response to infection. This method has the advantage of being easy and the serum collected can be stored and analyzed at later time points. The value of following the humoral antibody response to infection on a cellular level, specifically enumerating the number of plasma cells in the target tissue (blood, lymph node, spleen etc) and what Ig isotype these cells produce, would allow a deeper understanding of the anti-FMDV antibody response by B cells. To date, the main method for enumerating antibody secreting cells (ASCs) has been to use an ELISpot assay (Czerkinsky et al., 1983; Grant et al., 2012; Jahnmatz et al., 2013).

ELISpot assays have been facilitated by using biotinylated antigen. This technique has potential for greater specificity and sensitivity than traditional methods of coating ELISpot plates with antigen, where high concentrations of antigen are required and the target epitopes on the antigen can be altered by the process of binding the filter matrix. By changing the assay to use antigen to probe the plate in the final steps, the assay yields more defined and intense spots and requires much less protein than the system of coating the plate with antigen.

B cell enumeration in veterinary species is largely limited to an ELISpot assay, and to date, labelled virus particles have not been developed or used for enumeration of virus specific B cells in these species. ELISpot assays to enumerate antigen specific B cells in cattle have been recently described using a model antigen (Grant et al., 2012; Lefevre et al., 2009) and FMDV (Pega et al., 2013), all of which coat the plates with antigen. Enumeration of both ASCs and memory cells

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after both infection with and vaccination against FMDV has been recognized as essential in interrogating the mechanisms of immunity. The increase in spot definition by using biotinylated antigen as a detector is especially relevant when the antigen is not heat stable, like FMDV. Biotinylation of virus and virus like particles has been used with varying success to detect virus specific B cells (Doucett et al., 2005; Valtanen et al., 1999).

In addition to ELISpot, and the above described advantages, a biotinylated antigen can also be used to enumerate antigen-specific B cells using flow cytometry. This technique, and conjugation of fluorochromes directly to antigens, has been used to enumerate plasmablasts and memory B cells in the peripheral blood, largely in studies on influenza virus infection and vaccination (Bardelli et al., 2013; Whittle et al., 2014), HPV infection (Scherer et al., 2014), and dengue (Woda and Mathew, 2015). Although documented, use of antigen-probes for studying ~~to~~ B cell responses to viral infection is still not widespread.

Here, we describe development of biotinylated FMDV virions (bFMDV), for use in ELISA, ELISpot and flow cytometry and detected with streptavidin coupled to enzymes or fluorophores as the probe in these assays. These techniques provided a more sensitive and consistent measure of anti-viral antibodies and ASCs. Further, using biotinylated virions plus fluorophore labelled streptavidin as the probe, analysis of virus specific B cells by flow cytometry can provide the necessary separation of B cell populations.

2. Materials and methods

Previously, we tested the use of FMD (Uddowla et al., 2012) viruses genetically engineered to include a FLAG-tag in the capsid (Lawrence et al., 2013), combined with using anti-FLAG antibody as the probe in these assays. The sensitivity was insufficient and the system required that a new virus with a FLAG insert be made for each strain under study. As such, we assessed biotinylation of the FMDV capsid as a method for enhancing detection of virus in these assays for virus specific antibody and antibody secreting B cells.

2.1. Cell lines and hybridoma cells

Baby hamster kidney (BHK-21) cells (ATCC CCL-10) and LFBK-$\alpha_V\beta_6$ cells, derived from swine kidney cells expressing the integrin receptor, were maintained as described in LaRocco et al. (2013, J. Clin. Micro.). LFBK, formally believed to be bovine in origin, has since been typed as swine. LFBK-$\alpha_V\beta_6$ were stably transfected with the genes expressing the bovine heterodimer, $\alpha_V\beta_6$, a dominant receptor for FMDV in cattle (LaRocco et al., 2013).

Mouse B cell hybridomas 6HC4.1.3 (6HC4) and 12FE9.2.1 (12FE9) were produced and characterized previously (Saiz et al., 1989; Stave et al., 1988). Hybridomas were grown in 1× Minimal Essential Medium, 10% FBS plus supplements (Patch et al., 2011) at 37 °C in an atmosphere of 5% CO_2 and 100% humidity.

2.2. Virus growth and purification

Preparations of FMDV were concentrated and purified using a continuous sucrose density gradient (10–50%). Briefly, 6×10^9 cells were infected at a multiplicity of infection of 10:1 with either FMDV A24 Cruziero (FMDV A24) or O1 Manisa (FMDV O1). The antigens were then concentrated and partially purified with 8% polyethylene glycol 8000 (Sigma-Aldrich, St. Louis, MO) (Uddowla et al., 2012) After extracting the virus from the PEG with ice-cold TNE Buffer (10 mM TRIS, pH 7.6, 150 mM NaCl, and 1 mM EDTA), the virus was then purified over a continuous sucrose density gradient, (10%–50%). Upon completion, 0.5 ml aliquot fractions of the gradient were collected and assayed for virus using absorbance at 259 nm (Elzein and Crowther, 1979). The aliquots containing the peak virus concentrations were pooled, diluted 1:4 in PBS and ultracentrifuged at 90,000 g at 4 °C overnight. The supernatant was discarded and the pellet was resuspended in PBS such that the resultant concentration was 1 mg/ml as assayed by absorbance at 259 nm (Elzein and Crowther, 1979).

2.3. Biotinylation of FMDV

FMDV O1 or FMDV A24 were prepared at a concentration of 1 mg/ml in 1× PBS for use in the biotin reaction. To biotinylate the virus, the EZ-Link™ NHS-LC-Biotin reagent (Cat# 21336, Thermoscientific, Waltham, MA) was used. The molecular weight used to determine molar excess ratios of biotin to use with FMDV was 7,690,000 Da. These calculations were performed according to the manufacturer's instructions. Based on the Poisson distribution of biotinylating virus (akin to Multiplicity of Infection), 8 molecules of biotin would need to be added for every 1 molecule of virus to achieve biotinylation of 100% of the virions. After this determination, FMDV O1 was incubated at 4 °C after adding a molar excess ratio of 16–64 biotin. FMDV A24 was incubated at 4 °C after adding 16–512 molar excess of biotin.

2.4. Antibody detection by ELISA

To demonstrate the biotinylation of the antigen and to see whether it can detect FMDV specific antibodies, we carried out an antibody capture assay ELISA (ACA ELISA, Fig. 1) with archived serum samples from cattle before and after infection (0 dpi and 8 dpi) with either FMDV strain A24 Cruzeiro or O1 Manisa. A 96-well, flat-bottom plate (Immulon 2 HB Plate, Thermoscientific, Waltham, MA) was coated with 1:100 mouse anti bovine IgG1-isotype (Genway, San Diego, CA) with 0.05 M sodium carbonate-bicarbonate coating buffer (pH 9.6) (Sigma, St. Louis, MO) overnight at 4 °C. The plate was washed with 1× PBS + 0.05% Tween 20 (PBST), and a 1:100 single point dilution of either 0 dpi or 8 dpi serum was incubated on the plate for 1 h at 37 °C. The plate was washed with PBST and dilutions of 3 different bFMDV generated using different excess biotin:antigen ratios (16, 32 and 64) were incubated in a checkerboard fashion. This was incubated for 1 h at 37 °C. The plate was washed as above and avidin, neutravidin®-HRP (neutravidin-HRP) (Thermofisher, Waltham, MA) was added at a 1:1000 dilution in 1× PBST, 5% non-fat dry milk to each well and incubated for 1 h at 37 °C. At this time, the plate was washed and SureBlue ™ TMB Microwell Peroxidase Substrate (Seracare, Milford, MA) was added to each well. The reaction was stopped after about 15 min with 1 M sulphuric acid. The plate was read with a plate reader at optical density at 450 nm (OD_{450}/OD_{570}). Fig. 1 illustrates the principle of this ACA ELISA. This format of ELISA was used to determine both the ability of the bFMDV to detect antibody, but also to choose the optimal ratio of biotin:FMDV out of the 3 used in the pilot biotinylation analysis.

2.5. Virus specific B cell detection by Enzyme-linked Immunospot (ELISpot) Assay

To optimize the detection concentrations of the bFMDV for use in ELISpot, a quality control method utilizing mouse hybridoma cells was carried out. Antibody secreted by the hybridomas 6HC4, IgG2b (A24 specific) (Saiz et al., 1989, Virus Res.) and 12FE9, IgG1 (O1 specific) (Stave et al., 1986) were captured on mixed cellulose ester membrane ELISpot plates (EMD Millipore, Billerica, MA). Briefly, plates were coated using sheep anti mouse Ig (H + L) (Biorad, Hercules, CA, Serotec, AAM10) diluted to 1:50 in 0.05 M carbonate-bicarbonate coating buffer (Sigma, St. Louis, MO) overnight at 4 °C. Plates were washed, and blocked for at least 20 min at room temperature with hybridoma media (1× Minimal Essential Medium, 10% FBS, plus supplements (Patch et al., 2011). The A24 specific hybridoma cells were added and incubated overnight at 37 °C, 5% CO_2, with 100% humidity. The cells were washed once, lysed with Milli-Q water for 1 min at room temperature, washed with PBST, and the presence of anti-FMDV antibody was probed with bFMDV capsid. Virus was titrated starting at 1:100 and two

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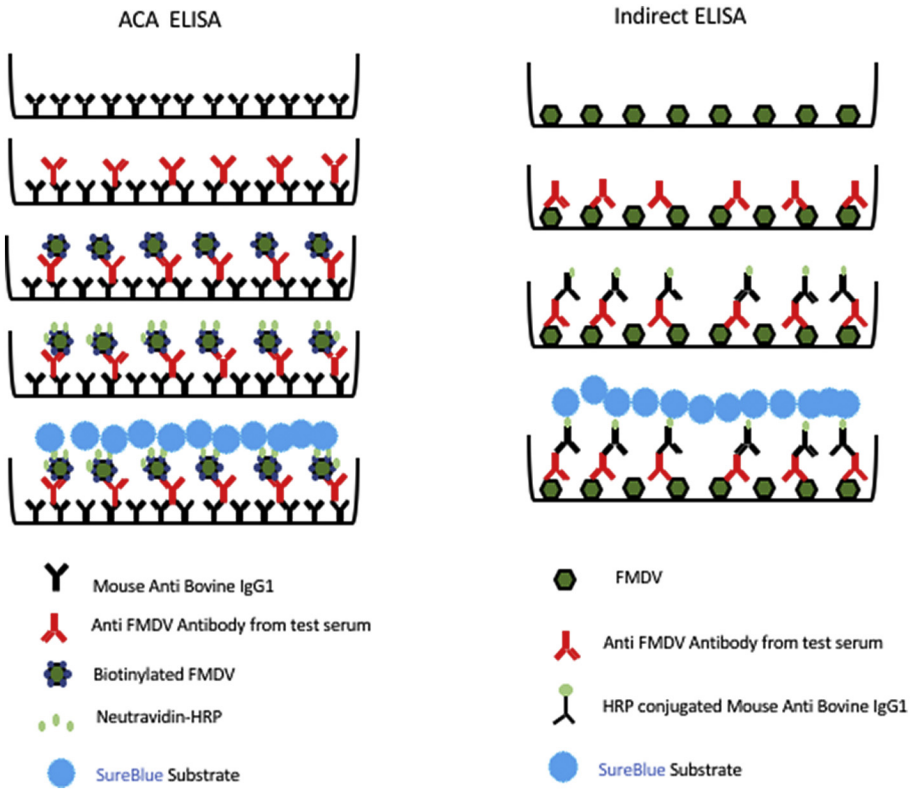

Fig. 1. Diagrammatic representation of the classical indirect ELISA assay for anti-FMDV antibody and the Antibody Capture Assay (ACA) ELISA using biotinylated FMDV.

fold, serially diluted across the plate 1:2. Fig. 2 shows two different lots of bFMDV A24. After washing, 1:1000 Neutravidin-HRP (Invitrogen, Carlsbad, CA) was then added and plate was incubated at room temperature for 1 h. After washing with PBST followed by Milli-Q water (the use of tween in wash buffers can be a source of increased background on ELISpot membranes), TrueBlue™ peroxidase substrate (Seracare, Milford, MA) was added to each well and the reaction was stopped after spot appearance (about 15 min) with Milli-Q water. Plates were read on an ELISpot reader (Cell Technologies Limited, Shaker Heights, OH).

2.6. Identification of anti-FMDV specific B cells by flow cytometry

Hybridoma cells producing murine monoclonal antibodies against FMDV A24 (6HC4; IgG2b) (Saiz et al., 1989) and bovine MHC-II (IL-A21; IgG2a) (Taylor et al., 1993) were mixed together in a 1:1 ratio, such that the final concentration of cells was 1×10^7/ml. 100 μl of these cells were added to an appropriate number of wells in a 96 well plate with an equal volume of 1x PBS and washed. Each wash consisted of three cycles of resuspending the cells in the 200 μl of the indicated wash buffer and centrifuged at 1200 rpm (334 g) at 4 °C for 6 min unless otherwise noted, Cells were re-suspended in 100 μl of 1% LIVE/DEAD® Fixable Yellow Dead Cell Stain (Life Technologies, Frederick, MD), diluted 1:1000 in 1× PBS (v/v) and incubated in the dark at room temperature for 15 min. At this time, an equal volume of FACS buffer (PBS, 0.3% bovine serum albumin, 0.1% sodium azide) was added to each well and then washed. Cells were then resuspended in 100 μl of FACS buffer containing bFMDV A24 at a concentration of 1:200, goat anti mouse IgG2b-PE-Cy7 (Southern Biotech, Birmingham, AL) at 1:500, and goat anti mouse IgG2a-Alexa Fluor® 647 (Thermofisher, Waltham, MA) at 1:2000 - and incubated on ice for 30 min in the dark. Cells were washed and re-suspended in Brilliant Violet 421™ (BV421) streptavidin (Biolegend, San Diego, CA) at a concentration of 1:1000 in FACS buffer. This was incubated for 30 min at 4 °C in the dark. Cells were then washed and resuspended in 200 μl FACS buffer.

For analysis of O1 Manisa, hybridoma cells producing murine monoclonal antibodies against FMDV, serotype O (12FE9; IgG1) (Stave et al., 1988) and bovine MHC-II (IL-A21; IgG2a) (Taylor et al., 1993) were

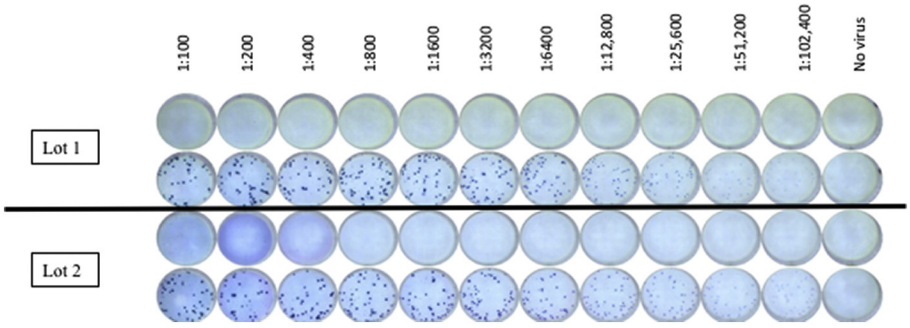

Fig. 2. ELISpot quality control assay results for two different preparations of bFMDV A24. Antibodies secreted by FMDV A24-specific hybridoma cells during an overnight incubation were captured on an anti-mouse Ig (H + L) coated ELISpot plate and then probed with increasing dilutions of two different lots of biotinylated FMDV A24. The two lots produced comparable data at all dilutions tested.

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mixed as described above. Sample preparation was the same as just described except that the secondary antibody for the MAb expressed by the 12FE9 hybridoma was goat anti-mouse IgG1 conjugated to PE at a concentration of 1:800. As described above, cells were washed 3 times and resuspended in Brilliant Violet 421™ (BV421) streptavidin (Biolegend, San Diego, CA) at a concentration of 1:1000 in FACS buffer. This was incubated for 30 min at 4 °C in the dark. Cells were then washed and resuspended in 200 μl FACS buffer.

The cells were analyzed with a BD™ LSRII flow cytometer (BD Biosciences, San Jose, CA), with data collected on the violet 405 nm laser filters: 560/40 (556LP) for LIVE/DEAD® yellow, and 450/50 for BV421. On the Blue 488 nm laser filters: 585/42 (550LP) for PE, and 780/60 (735LP) for PE-Cy7. On the Red 633 nm laser filter 660/20 for Alexa Fluor® 647 (represented as APC on flow cytometry analysis graphs). For analysis, a gating strategy that included only live, single cells was selected for downstream analysis. 200,000 events were collected. Data was analyzed with BD FACSDiva™ software, version 6.

3. Results

3.1. Analysis of anti-FMDV antibody by ELISA

The challenge in redesigning assays for detecting anti-viral antibodies using biotinylated virus as antigen is determining whether coupling biotin to the virus capsid alters capsid structure and antibody reactivity. While we predicted the sensitivity of the assays would be greatly enhanced by using avidin-biotin binding, we also needed to test the effect of biotinylation on the virus. After reviewing the calculations for optimal biotin to virus ratios described above, we tested selected preparations of biotinylated virus. In analysis of FMDV O1 we found that 128 molar excess resulted in a decrease in the titer of the virus when measuring the $TCID_{50}$ in LFBK-$\alpha_V\beta_6$ cells as compared to virus that had not been biotinylated (data not shown). This decrease was not observed at the 64 molar excess so that ratio of biotin to virus resulting in 64 molar excess being maximum concentration considered in all subsequent experiments.

The results of the initial ACA ELISA (Pacheco et al., 2010b) are summarized in Fig. 3. The viable molar excess predicted to biotinylate all virions with at least 4 biotin molecules (1:16 molar excess), the lowest biotin molar excess ratio tested. A biotin molar excess:virus ratio of 32:1 gave the best signal:noise ratio in the ACA ELISA at all virus dilutions tested when using FMDV A24. Increasing the biotinylation ratio within the ranges tested did not appear to detrimentally affect antigen:antibody binding, with an increasing signal for every increase in biotin concentration. There was a small increase in background signal correlating with an increasing biotin:virus ratio, as evidenced by an increase in optical density at 450 nm (OD_{450}/OD_{570}) values of day 0 serum. Based on these results, the 1:8 ratio (32 molar excess) was selected as the optimal concentration for biotinylating this virus. At high concentrations of bFMDV A24, the background signal (OD of day 0 serum) was high. At the lowest dilution of biotinylated virus added, 1:200, the signal to noise ratio was 2.7 and the background O.D was 0.697. Over the range analyzed, as virus was titrated (diluted), the background OD decreased, with a concurrent increase in signal:noise ratio. The virus dilution of 1:6400 gave a signal to noise ratio of 12.5 with a background O.D of 0.109. This bFMDV concentration was chosen for the subsequent FMDV A24 ACA-ELISA assays used.

Initial performance of bFMDV O1 in the ACA-ELISA proved to be inadequate as the background OD was 1.769 at the highest concentration of antigen (1:100 dilution) (Fig. 4A). To remedy this problem, neutravidin-HRP, available from Thermo Fisher Scientific, was used. Neutravidin is a deglycosylated form of avidin with an isoelectric point at near neutral pH (6.3), which has the lowest non-specific binding as compared to all other biotin binding proteins. The removal of carbohydrate moieties and the reduction of the isoelectric point decreases the non-specific binding to both lecithin and negatively-charged complexes. Fig. 4B shows results comparing the FMDV O1 ACA ELISA using Neutravidin-HRP as the detection reagent. As would be expected, the lowest dilution of bFMDV O1 demonstrated the greatest difference in the percentage of non-specific binding between neutravidin (23%) and streptavidin (76%). The dilution with the highest signal:noise ratio, 1:1600, showed the signal:noise ratio increased from 3.5 to 8.3 when replacing streptavidin-HRP with neutravidin-HRP (Fig. 4C). All subsequent ELISA and ELISpot assays were done using neutravidin-HRP.

Serum from animals infected with FMDV A24 were taken daily, and assayed using the bFMDV, biotinylated at a ratio of 1:4 (this biotinylation ratio was used for all subsequent parts of this study), and used at a concentration of 1:6400. As shown in Fig. 5, the signal from the preinfection serum samples (Day 0) remained ~~low, and~~ constant up to

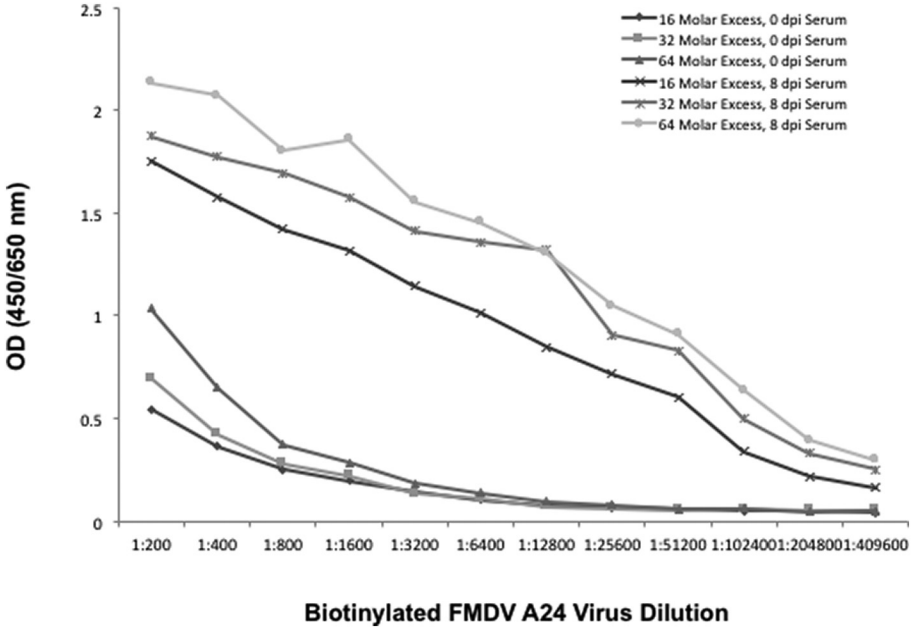

Fig. 3. Effect of biotin excess molar concentration on biotinylated FMDV A24 assay performance in the antibody capturing assay ELISA. Serum samples were obtained from bovine pre-and post FMDV A24 infection. This graph demonstrates changes in signal (8 dpi serum) and noise (0 dpi serum) using 3 different biotin:virus molar excess ratios. A biotin molar ratio of 32:1 gave the best signal:noise ratio at all biotinylated virus dilutions. Results represent a single replicate.

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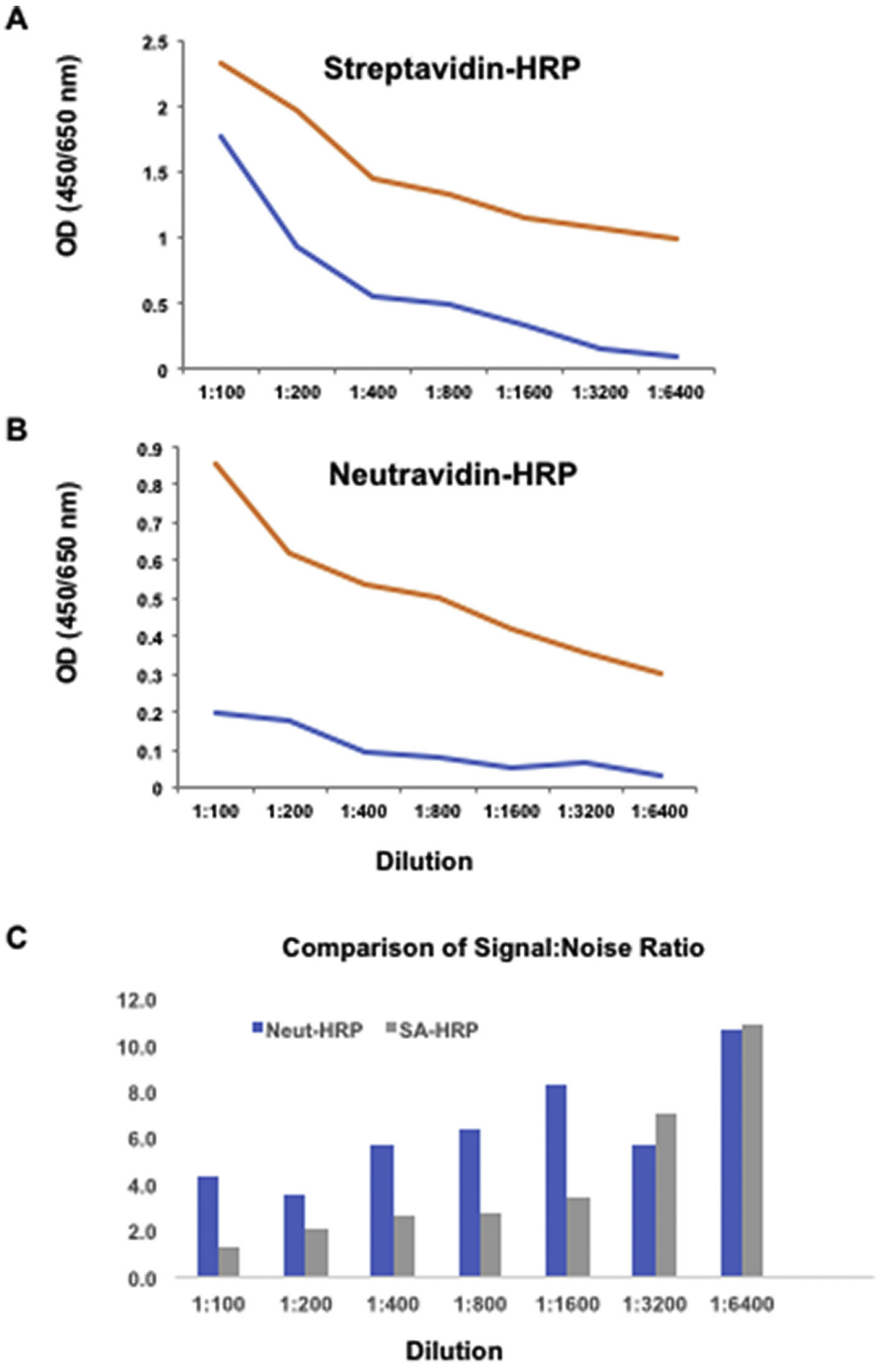

Fig. 4. Comparison of FMDV O1 Antibody Capture Assay ELISA performance using streptavidin-HRP vs. Neutravidin-HRP. The two top graphs represent ACA ELISA experiments performed with two different forms of avidin-HRP: Streptavidin-HRP (A), and avidin, Neutravidin-HRP (B). Serial two-fold dilutions were tested starting at a low dilution of 1:100 (x-axis). The raw OD values (450 nm/570 nm) are shown on the y-axis. Avidin, Neutravidin greatly reduces non-specific binding, especially at lower dilutions of the avidin forms. Panel C shows the same data expressed as a signal to noise ratio obtained with varying concentrations of both avidin:HRP types. The background (noise) was determined by the OD (450 nm/570 nm) generated when using a 1:100 dilution of naïve bovine serum (0 dpi) at each dilution. Signal was determined by the OD (450 nm/570 nm) generated when using a 1:100 dilution of O1 Manisa infected bovine serum (7 dpi). The highest signal:noise ratio was seen at the highest dilution of both avidin forms.

and including 4 dpi. At 5 dpi, the signal increased significantly above the background, and the O.D proceeded to increase on the subsequent days. The serum was assayed up to and including 8 dpi.

3.2. Detection of B cells secreting FMDV specific antibody by ELISpot

We tested whether biotinylation of FMDV could be useful in assays for B cells secreting anti-virus antibody, specifically an ELISpot for ASCs. For this assay we used the mouse hybridoma cell lines secreting antibody specific for FMDV, serotype A, named 6HC4 (Saiz et al., 1989) and FMDV, serotype O, named 12FE9 (Stave et al., 1988). bFMDV A24 bound by antibody 6HC4 showed clear, strong, and discrete spots in the ELISpot assay. There was no background in the negative control, the 6HC4 antibody probed with bFMDV O1 (Fig. 6). The inverse was observed when bFMDV O1 was used in an ELISpot assay with the mouse monoclonal anti-FMDV serotype O antibody, 12FE9, as the

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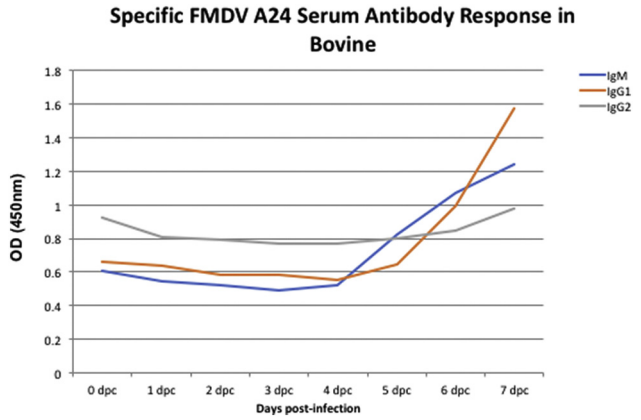

Fig. 5. Isotype-specific antibody response to FMDV A24. This graph depicts the OD (450 nm/570 nm) values obtained when using biotinylated FMDV A24 to determine relative antibody responses in pre- and post-FMDV A24 infected bovine serum using IgM, IgG1 and IgG2 isotype-specific monoclonal antibodies in the Antibody Capture Assay ELISA. While IgG2 levels do not rise during the timepoints tested, IgM and IgG1 levels increase after 4 dpi and 5 dpi, respectively.

coating antibody (Stave et al., 1988). bFMDV A24 gave no signal in this assay while bFMDV O1 gave many discrete spots (Fig. 6). Different spot numbers were observed for 6HC4 and 12FE9 due to differing numbers of hybridoma cells seeded. These assays were done in quadruplicate, and variation in numbers between identical wells in a row was low.

We optimized the procedures by conducting a checkerboard dilution series of bFMDV in one dimension, and neutravidin-HRP in the other, yielding optimal reagent concentrations and comparing multiple plate types. For the ELISpot assays, the Multiscreen HA Filter Plate (Millipore, Billerica, MA, Cat# MSHAS4510) plate was chosen to give the best spot morphology and intensity out of the 3 tested (data not shown). We have now demonstrated this assay is just as clear using B cells from cattle peripheral blood of vaccinated and/or infected animals (Kenney et al., manuscript in preparation).

3.3. Flow cytometric analysis of virus specific B cells

The use of bFMDV as a probe in analysis of B cell responses by flow cytometry was very attractive as many fluorophores coupled to streptavidin are available from multiple commercial sources, simplifying staining schemes to identify B cell subsets. To test our strategy, we analyzed mouse B cell hybridomas secreting anti-FMDV monoclonal antibody. Our initial observations noted that all controls, including no primary antibody controls, gave no non-specific labelling. The data presented have not been adjusted for compensation. Since the fluorophores were spread across 3 excitation lasers, minimal to no compensation was necessary.

The gating strategy used to analyze the results is given in Fig. 7. Hybridoma cells which were not viable stained intensely with the LIVE/DEAD® stain (designated Qdot 565 on plot), due to large levels of free amine staining. The viable cells stained significantly less with the LIVE/DEAD® stain, and thus these cells were selected with this "viability gate". Next, in order to include only single cell events, a gate was drawn around cells to exclude doublet, triplets, etc., cells (cell clumps) by adjusting the axes to Forward scatter (FSC) area (FCS-A) and height (FSC-H) and gating on the singlets as shown in Fig. 7.

Using the hybridoma IL-A21 (mouse anti-bovine class II MHC) as a negative control, and 6HC4, specific for serotype A FMDV, as a positive sample, live, single cells were then plotted on a scatter plot with Alexa Fluor® 647 (designated as APC) on the Y axis (labelling murine IgG2a expressing cells, IL-A21) and PE-Cy7 (labelling IgG2b expressing cells, 6HC4). bFMDV A24 was incubated with the different hybridomas and probed with streptavidin coupled with the fluorophore BV-421. As seen in Fig. 8A, there were both IgG2b and IgG2a single positive cells, and also a significant portion of double negative cells. A quadrant gate was applied, such that single positive cells were colored and displayed on a daughter scatterplot, with SSC on the Y axis and BV421 staining indicating virus labelling on the X axis. All cells which stained for IgG2a expression were negative for BV421 signal (and thus virus binding) while cells stained for IgG2b expression were all positive for BV-421, binding bFMDV A24. There was clear separation of the 2 populations

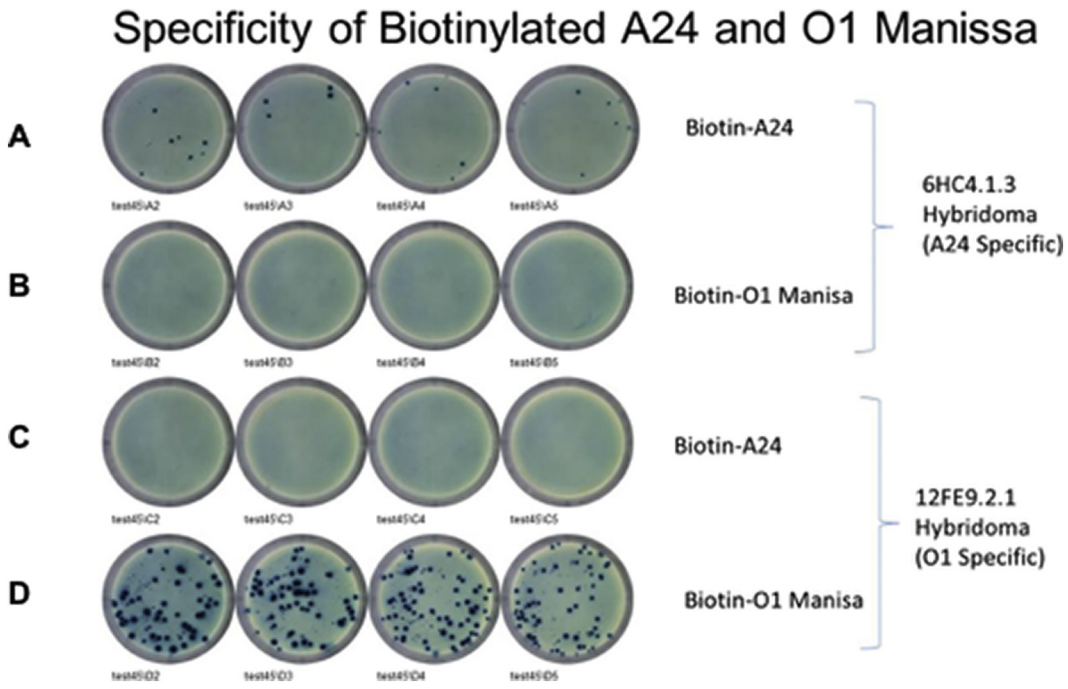

Fig. 6. Biotinylated FMDV A24 and FMDV O1 are serotype specific. The two top rows of this ELISpot plate show results obtained when antibodies secreted from FMDV A24-specific hybridoma cells, cultured overnight, were probed with biotinylated FMDV A24 (A) and biotinylated FMDV O1 (B). The two bottom rows of this ELISpot plate show results obtained when antibodies secreted from FMDV O1-specific hybridoma cells, cultured overnight, were probed with biotinylated FMDV A24 (C) and biotinylated O1 (D). No spots were visible in wells containing a mismatch in virus and antibody serotype. Four biological replicates were tested for each FMDV virus (serotype A24 and O1) and serotype-specific antibody combination.

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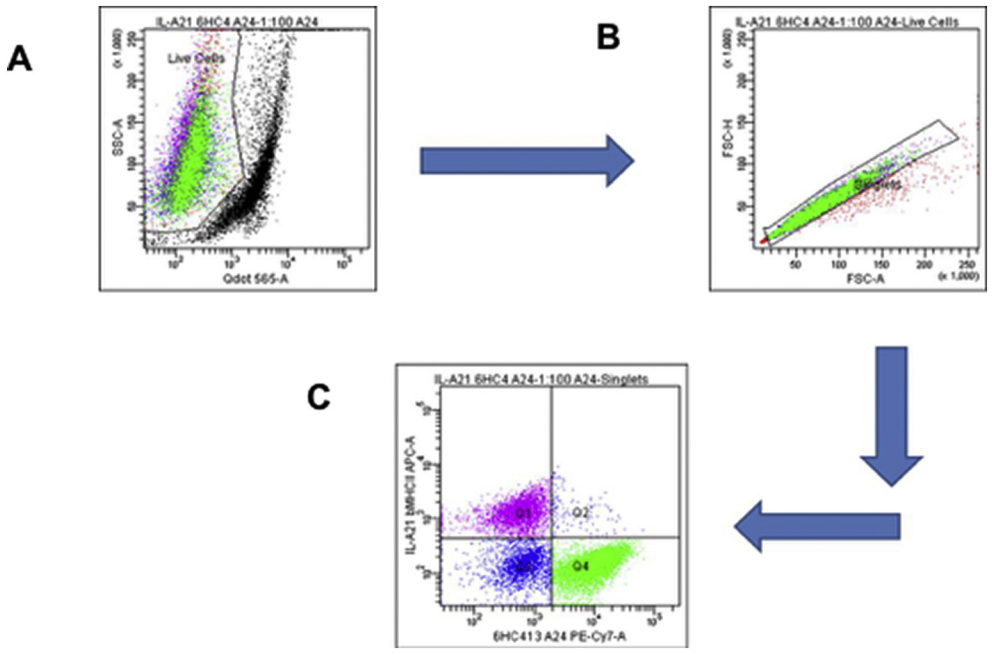

Fig. 7. Gating strategy used for flow cytometric analysis of hybridoma cells. The first gate selects only the live cells (A). The second gate selects only single cells (B). The quadrant gate in the third panel C divided up the cells into IgG2a + IgG2b- (purple, Q1), and IgG2a-IgG2b + (green, Q4). Cell populations were mutually exclusive using this gating strategy making it possible to identify biotinylated FMDV A24 specific binding in additional graphs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of hybridomas by bFMDV A24 labelling, allowing clear demarcation of positive and negative cells.

The low level of auto-fluorescence of cells in the violet channel, compared to other channels, was also noted in preliminary experiments (data not shown) and was the reason for using a UV excited dye such as BV421 for the virus labelling. Fig. 8B shows the results for analysis of staining the hybridoma, 12FE9 (mouse anti-FMDV, IgG1 isotype). This hybridoma is specific for FMDV serotype O and was probed with the bFMDV O1 strain. Here we used PE labelled anti-murine-IgG1 to identify the 12FE9 hybridoma cells. Labelling of 12FE9 hybridomas with bFMDV-O 1 showed an even greater separation of positive to negative cells. These comparisons show that the technique of probing

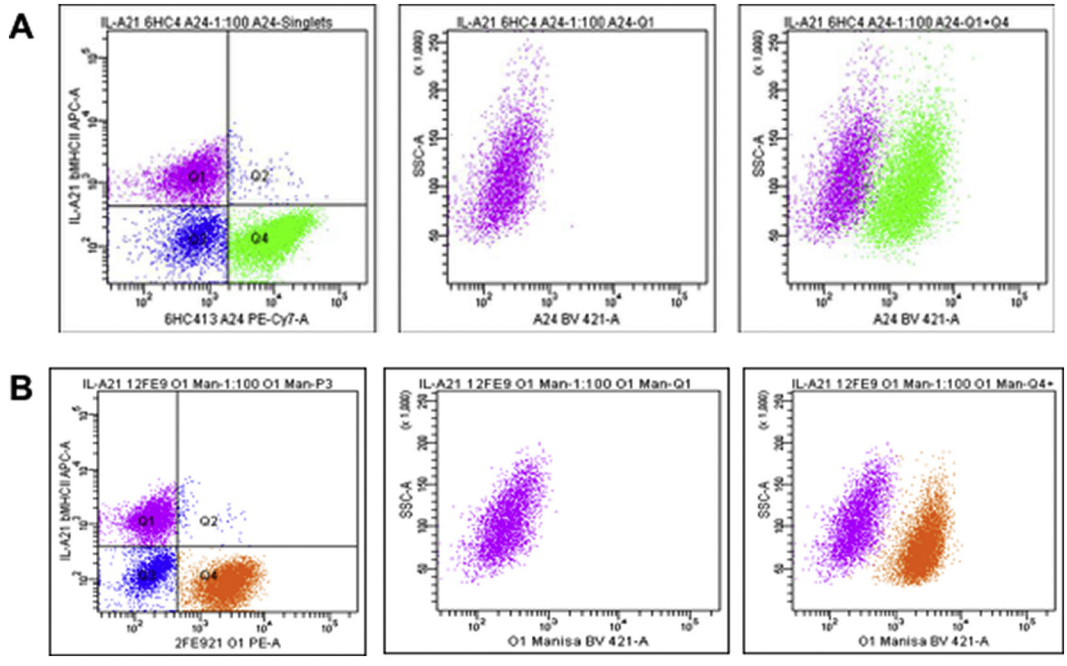

Fig. 8. Flow cytometry analysis of biotinylated FMDV A24 (A) and FMDV O1 (B) binding to FMDV serotype-specific hybridoma cells. Using the gating strategy depicted in Fig. 7, FMDV-specific hybridoma cells were combined with hybridoma cells specific to bovine MHC II (negative control) and probed with biotinylated FMDV A24 (A) and FMDV O1 (B). The center plot in both panels, depicting only the negative control MHC II specific hybridoma cells, represent specific biotinylated FMDV. The right plot in both panels represents biotinylated FMDV A24-specific binding to FMDV A24-specific hybridoma cells (A) and biotinylated FMDV O1-specific binding to FMDV O1-specific hybridoma cells (B) when combined with MHC II hybridoma cells. These results show two distinct populations, FMDV-positive and FMDV-negative, when the relevant biotinylated FMDV serotypes is used to probe a sample containing both the FMDV-specific hybridoma cells and MHC II-specific hybridoma cells.

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FMDV specific B cells with biotinylated virus works with different serotypes of FMDV and different isotypes of antibody.

Not surprisingly, chemically inactivating the virus in the same manner used to produce the killed virus vaccine, does not change the signals detected in these assays. Purified FMDV capsids inactivated with binary-ethyleneimine (BEI) by standard protocols (Aarthi et al., 2004) was subsequently biotinylated and tested in ELISpot and flow cytometry with comparable results shown here, for the live virus. An example is shown in the Supplemental Fig. 1. The difference in the curves shown is likely due to the dilution of the virus preparation during the BEI treatment and subsequent inactivation of the BEI. Still, these data show that whether live virus or inactivated, killed virus are the requirement of the assay, using biotinylated virus will work in either case.

4. Discussion

In order to achieve better sensitivity in assays analyzing the antibody response to FMDV, we assessed the biotinylation of FMDV capsid as a way to enhance these assays. The approach was attractive as the biotin/streptavidin binding interaction is one of the strongest routinely used in detection assays and as such, commercial availability of derivatives of streptavidin are extensive. These provide a range of products that can be compared for relative compatibility with the assay system. Further, most labels used in biological assays, including enzymes, fluorophores, etc., are widely available coupled to streptavidin. These qualities greatly facilitate assay development and refinement.

Data presented here demonstrate we can chemically biotinylate capsids of FMDV, both serotype A and serotype O. Antigenic reactivity of the virus with antibodies is preserved, as evidenced by results from the ACA ELISA, ELISpot and analysis by flow cytometry. This is likely a result of the biotin / virus ratio being kept constant and that the biotinylation reagent includes a spacer arm between the biotin and the reactive site ultimately coupled to the capsid. Further, we were able to biotinylate capsids to a level compatible with these assays that did not alter infectivity of the virus. Again, this indicates that the level of biotinylation described here does not alter capsid structure or function. In addition, we show that neutravidin gives better signal to noise ratios than the standard streptavidin for one of the virus serotypes tested, demonstrating another versatile aspect of this approach. Finally, we also tested whether chemical inactivation of virus alters results in the same assay and detected no difference between when probing with live, biotinylated FMDV and biotinylation of chemically inactivated virus.

The excellent signal:noise achieved in the ACA ELISA using this reagent opens up a potential new method of serum assay for anti-FMDV antibodies. As no polyclonal serum is required, only monoclonal antibodies and biotinylated virus, an ELISA based on this technology would negate the need for laboratory animals to be used for reagent production. Only serum from the test animals under study are part of the assay.

In the ELISpot assays, the fact that there was no detectable cross reactivity of bFMDV A24 with anti-serotype O monoclonal antibodies, and similarly none when assaying bFMDV O1 with anti-serotype A monoclonal antibodies, further demonstrates that biotinylation of the virus had no observable effect on specificity. The negative controls were essentially blank. These results show enhanced sensitivity compared to previous reports (Pega et al., 2013) and indicate using biotinylated FMDV capsid will lead to more informative ELISpot analysis of B cell responses to this virus.

Importantly, the ability of the bFMDV to detect cell surface antibody specific for virus, with a strong separation, will allow for more sensitive analysis of B cell activation and differentiation during the response to FMDV infection or vaccination. Juleff and colleagues (Juleff et al., 2009) have reported the response to FMDV infection in cattle is T cell independent. Use of biotin labelled FMDV capsids to “stain” B cells expressing anti-FMDV antibody in flow cytometric studies will potentially allow for determining if the T independent B cell phenotype (B1 B cells) is the only phenotypes expressed by FMDV specific B cells or if other phenotypes are also stimulated. Further, this provides the potential to identify whether there are virus specific memory B cells generated in response to FMDV infection and vaccination. Since many cells can be isolated from blood and lymphoid tissues of cattle, large numbers of cells (10^8) can be analyzed to detect very minor populations of cells. The many fluorophores commercially available, coupled to streptavidin and other avidins, makes developing such assays more feasible.

5. Conclusions

These methods should improve assays for anti-FMDV antibody responses in all formats. Further, they will allow investigators to focus on only FMDV specific B cells as they measure the development of the B cell responses to FMDV. In particular, the ELISpot assay and analysis of B cells by flow cytometry should provide a much more accurate assessment of the kinetics of the B cell response to virus infection. Analyzing vaccine performance using these enhanced methods for detection of B cell responses to FMDV should lead to improved vaccine design and testing.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jim.2017.07.001.

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