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Detection of IgM responses to bovine respiratory syncytial virus by indirect ELISA following experimental infection and reinfection of calves: abolition of false positive and false negative results by pre-treatment of sera with protein-G agarose

D.A. Graham[a,*], J.C. Foster[a], K.A. Mawhinney[a], M. Elvander[b], B.M. Adair[a], M. Merza[c]

[a]Veterinary Sciences Division, Department of Agriculture for Northern Ireland, Stoney Road, Stormont, Belfast BT4 3SD, Northern Ireland, UK

[b]National Veterinary Institute, PO Box 7073, S-750 07 Uppsala, Sweden

[c]National Veterinary Institute, SVANOVA Biotech, Glunten, Uppsala Science Park, S-751 83 Uppsala, Sweden

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Abstract

The IgM responses in three panels of sera generated by infection and reinfection of calves with bovine respiratory syncytial virus (BRSV) were measured by indirect ELISA (I-ELISA). The effect of depleting serum IgG by pre-treatment with protein G agarose (PGA) was evaluated. Following primary infection a weak IgM response was detected in the untreated sera of 3 out of 4 calves with maternally derived antibody (MDA). Both the magnitude and duration of the specific IgM responses in these calves were increased by pre-treatment with PGA. In addition, the fourth infected calf tested gave a single positive IgM result following PGA treatment. Transient or persistent IgM responses which were abolished by pre-treatment of sera with PGA were detected in 4/8 calves following reinfection. These were considered to be false positive results, consistent with the influence of IgM rheumatoid factor (IgM-RF). One of these calves and two additional calves showed transient increases in IgM which were resistant to PGA treatment. These were considered to represent specific IgM responses to reinfection. The results indicate the ability of PGA treatment to eliminate both false positive and false negative results and emphasise the necessity for controlling

* Corresponding author. Tel.: +44 1232 525749; fax: +44 1232 525787
E-mail address: david.graham@dani.gov.uk (D.A. Graham)

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the influence of IgM-RF in IgM-specific indirect ELISAs. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Bovine respiratory syncytial virus -specific IgM; ELISA; Protein G agarose; IgM rheumatoid factor

Abbreviations: ACC-ELISA, antibody class capture ELISA; BRSV, bovine respiratory syncytial virus; BRSV-IgG, BRSV-specific immunoglobulin G; BRSV-IgM, BRSV-specific IgM; Dpi, days post infection; Dpr, days post reinfection; COD, corrected optical density; FBL, foetal bovine lung; I-ELISA, indirect ELISA; IgM-RF, IgM-isotype rheumatoid factor; MDA, maternally derived antibody; PGA, protein G agarose; PNT, positive negative threshold; S/P%: sample/positive percentage

1. Introduction

Serodiagnosis of viral infections using IgM-restricted assays offers the possibility of obtaining a more rapid result compared to testing for seroconversion, since a diagnosis can be made without waiting to collect a convalescent serum sample (Meurman, 1983). In addition, such assays have been shown to be more sensitive than testing for seroconversion in the face of maternally derived antibodies (MDA) (Kimman et al., 1987a; Westenbrink and Kimman, 1987; Graham et al., 1998c). Immunoassay protocols for IgM detection typically use either indirect or antibody class capture ELISAs (ACC-ELISA). In indirect ELISAs (I-ELISAs), specific IgM must compete with specific IgA and/or IgG to bind with antigen on the solid phase (inter-isotypic competition). In ACC-ELISAs, total serum IgM, a proportion of which is antigen-specific, is captured by polyclonal or monoclonal anti-IgM antibodies on the solid phase. Such assays are, therefore, subject to intra-isotypic competition. Both, inter- and intra-isotypic competition may reduce the sensitivity of such assays (Meurman, 1983). In addition, rheumatoid factor of IgM isotype (IgM-RF) may generate false positive IgM signals, especially when an I-ELISA protocol is followed. This requires the presence of both antigen-specific IgG and IgM-RF, and is well documented in relation to human pathogens (Salonen et al., 1980; Yolken and Leister, 1981; Tuokko, 1984).

IgM-RFs are autoantibodies with a specificity for the $c\gamma_2$–$c\gamma_3$ interface of homologous and heterologous IgG (Waron et al., 1987; Sasso et al., 1988). The presence of IgM-RF in bovine sera has been demonstrated (Ungar-Waron et al., 1991; Graham et al., 1998b). By re-testing positive sera following pre-treatment with hyperimmune antiserum to bovine IgG(Fc), Ungar-Waron and Abraham (1991) demonstrated a false positive rate of 16.0% when field sera were tested by I-ELISA for bovine herpes virus-1-specific IgM. Subsequently, using a novel method of depleting IgG from bovine sera based on treatment with protein G bound to a cross-linked agarose matrix (PGA), Graham et al. (1998c) detected a false positive rate of 17.7% in field sera tested by I-ELISA for bovine respiratory syncytial virus-specific IgM (BRSV-IgM). The generation of such false positive results continues to be an obstacle to the wider use of IgM-restricted assays in veterinary serodiagnosis.

The BRSV-IgM results obtained when this I-ELISA was used to test sera generated by experimental infection of seronegative calves have been described previously (Graham

et al., 1998a). Following on from this, the aims of the work described in the present paper were as follows:

1. to determine the ability of this I-ELISA to detect BRSV-IgM responses following experimental infection of calves in the presence of MDA and following experimental reinfection, and to compare with seroconversion results;
2. to determine whether or not the development of false positive IgM signals could be observed in one or more calves after infection or reinfection;
3. to examine the effect of PGA treatment of sera on the magnitude and duration of IgM responses; and
4. to compare results with those described previously when similar sets of sera were tested by ACC-ELISA (Kimman et al., 1987a, b).

2. Materials and methods

2.1. Sera

Three panels of sera were generated by experimental infection or reinfection of calves. Six conventionally reared, colostrum supplemented Friesian X calves were bought at ≤ 1 week of age and infected/mock-infected seven weeks later. These were housed in isolation facilities throughout the experimental period (calves 1–6, Panel 1). Testing of blood samples collected serially between purchase and infection indicated declining levels of BRSV-specific IgG (BRSV-IgG), consistent with this antibody being maternally derived. The two calves with the lowest levels of BRSV-IgG served as controls and were housed separately prior to inoculation with mock-infected foetal bovine lung cells (FBL) which had been frozen and thawed once. Each calf received 10 ml intra-tracheally and 5 ml in each nostril. In the same way, the remaining four calves were inoculated with a crude lysate of FBL cells infected with a Swedish BRSV isolate SVA 274/92 which were frozen and thawed once, when >80% viral cytopathic effect was observed. A clotted blood sample was collected from each calf 0, 2, 5, 7, 9, 12, 14, 16, 20, 24, 28, and 34 days post infection (dpi). Fifty-four days after initial infection, the six calves were reinoculated as above, and bled 0, 2, 4, 7, 9, 11, 14, 16, 21, 24 and 28 days later (Panel 2 sera). The viral titres of the inocula used for infection and reinfection were $10^{4.1}$ and $10^{4.3}$ $TCID_{50}$/ml, respectively. Panel 3 sera were generated by reinfection of a further four calves (68, 69, 70 and 73) with Swedish BRSV isolate SVA 187/92 ($10^{4.3}$ $TCID_{50}$/ml) at 5-6 months of age, following the same protocol. Serial serum samples were collected 0, 2, 5, 7, 9, 12, 16 and 20 days post reinfection (dpr). These calves had initially been infected with the same isolate 37 days earlier, at which time they were all seronegative (Elvander et al., 1998). All sera were held at $-20^{\circ}C$ or below prior to testing.

2.2. BRSV-IgM ELISA

BRSV-IgM was detected by I-ELISA, as previously described (Graham et al., 1998c). All sera were tested with, and without, prior depletion of IgG using a 40-mg/ml suspension of PGA in 0.01 M phosphate buffered saline, pH 7.4, containing 0.05% Tween

20 (Graham et al., 1998c). Results for each serum were expressed as a percentage of the positive reference serum included on each plate (S/P%). Sera with an S/P% value ≥22.0% were considered to have given a positive BRSV-IgM result (Graham et al., 1998c).

2.3. Measurement of BRSV-IgG

Changes in BRSV-IgG levels, following infection/reinfection, were detected using a commercially available I-ELISA (RSV-Ab, SVANOVA Biotech, Uppsala). Sera were tested at a dilution of 1/25, according to the manufacturers' recommendations, being deemed positive if the corrected optical density (COD) ≥0.2. In addition, sera collected 0 and 20 dpi (Panel 1), 0 and 21 dpr (Panel 2) and 0 and 20 dpr (Panel 3) were tested for evidence of seroconversion by single-dilution ELISA, as previously described (Graham et al., 1998d). Sera were tested at a dilution of 1/100 with an increase in S/P% ≥40% between acute and convalescent samples, indicating a fourfold or greater increase in titre.

3. RESULTS

3.1. Panel 1

When sera were tested for BRSV-IgM without pre-treatment with PGA, calves 1, 2 and 4 showed a transient response (Fig. 1), with peak S/P% values of 103.5%, 37.4% and

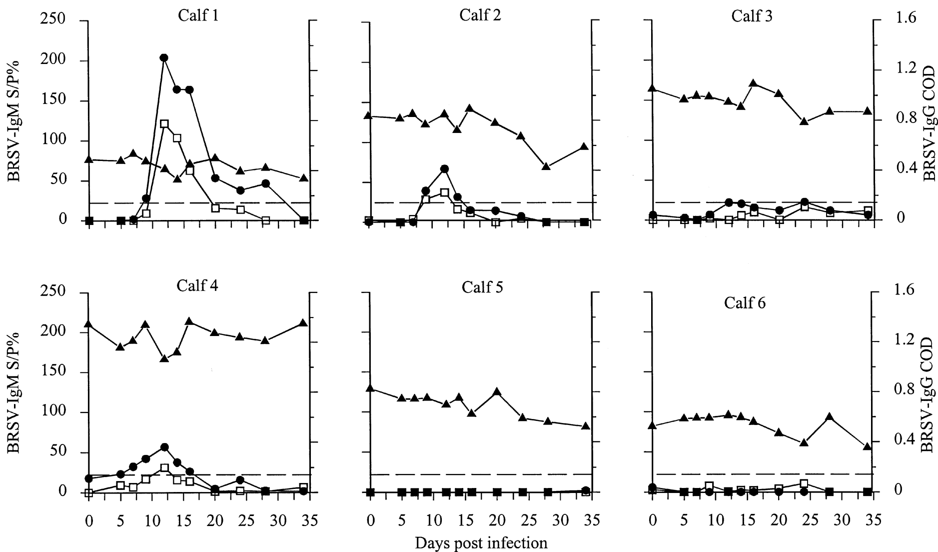

Fig. 1. BRSV-specific IgM (S/P%) and IgG (▲) (COD) responses in Panel 1 sera following primary inoculation of calves with either BRSV strain SVA/274/92 (calves 1–4) or with a control inoculum (calves 5 and 6). BRSV-IgM responses were measured both with (●), and without (□), pre-treatment with PGA. BRSV-IgM PNT = 22% (– –).

Table 1
Summary of BRSV-IgM and -IgG responses for virus-inoculated calves from panels 1, 2 and 3. Results in parentheses were obtained following pre-treatment of sera with PGA.

Calf number	Panel 1				Panel 2				Panel 3			
	1	2	3	4	1	2	3	4	68	69	70	73
IgM Response[a]	+ (+)	+ (+)	− (+)	+ (+)	+ (+)	+ (−)	− (+)	+ (−)	− (+)	− (+)	− (+)	+ (+)
First IgM[b]	12 (9)	9 (9)	na[i] (24)	12 (5)	7 (9)	11 (na)	na (14)	9 (na)	na (0)	na (0)	na (0)	0 (0)
Last IgM[c]	16 (28)	12 (14)	na (24)	12 (16)	28 (7)	21 (na)	na (14)	28 (na)	na (16)	na (7)	na (12)	20 (2)
Duration IgM[d]	4 (19)	3 (5)	na (na)	na (11)	21[h] (2)	10 (na)	na (na)	19 (na)	na (16)	na (7)	na (12)	20 (2)
Peak IgM (day)[e]	12 (12)	12 (12)	na (24)	12 (12)	28 (9)	16 (na)	na (14)	28 (na)	na (0)	na (5)	na (0)	20 (0)
Peak IgM (S/P%)[f]	103.5 (203.6)	37.4 (66.8)	na (22.5)	31.2 (57.0)	39.8 (33.1)	38.4 (na)	na (23.6)	39.6 (na)	na (40.3)	na (37.2)	na (63.2)	57.8 (28.3)
Seroconversion[g]	−	−	−	−	+	+	−	+	−	−	−	−

[a] For each calf, the presence or absence of an IgM response.
[b] The first day on which a BRSV-IgM response was detected.
[c] The last days on which the BRSV-IgM response was detected.
[d] The duration of the BRSV-IgM response.
[e] The day of peak response.
[f] Size (S/P%) of the peak response.
[g] The results of testing acute and convalescent samples, collected approximately three weeks apart, for seroconversion (IgG response) are also given.
[h] BRSV-IgM negative 14 and 16 dpr.
[i] Not applicable.

31.2%, respectively, (Table 1). BRSV-IgM was detectable in calf 1 from 12–16 dpi, and in calf 2 from 9–12 dpi. BRSV-IgM was detectable in calf 4 only on Day 12 post infection. No response was detected in the two control calves.

When sera were tested after PGA treatment, the day on which peak BRSV-IgM response was observed in these three calves was unchanged, but an increase in both the magnitude and duration of the BRSV-IgM response was observed (Fig. 1). Peak S/P% values increased to 203.6%, 66.8% and 57.0% for calves 1, 2 and 4, respectively (Table 1). BRSV-IgM was detectable in calf 1 from 9–28 dpi, in calf 2 from 9–14 dpi and in calf 4 from 9–14 dpi. In addition, calf 3 gave a single positive BRSV-IgM result of 22.5% on Day 24 post infection when sera were pre-treated with PGA. None of the six calves showed a BRSV-IgG response to inoculation sufficient to indicate seroconversion, with levels remaining relatively stable or showing an overall decline over the sampling period.

3.2. Panel 2

No response to reinoculation was detected in control calves 5 and 6, either with or without prior treatment with PGA (Fig. 2). The remaining four calves were all BRSV-IgM negative at reinfection, irrespective of prior treatment with PGA. In the absence of PGA treatment, the plots for calves 1 and 4 were similar and consisted of three components. Firstly, S/P% values increased and became positive, then decreased and, finally, increased sharply. However, the responses generated for these two calves differed

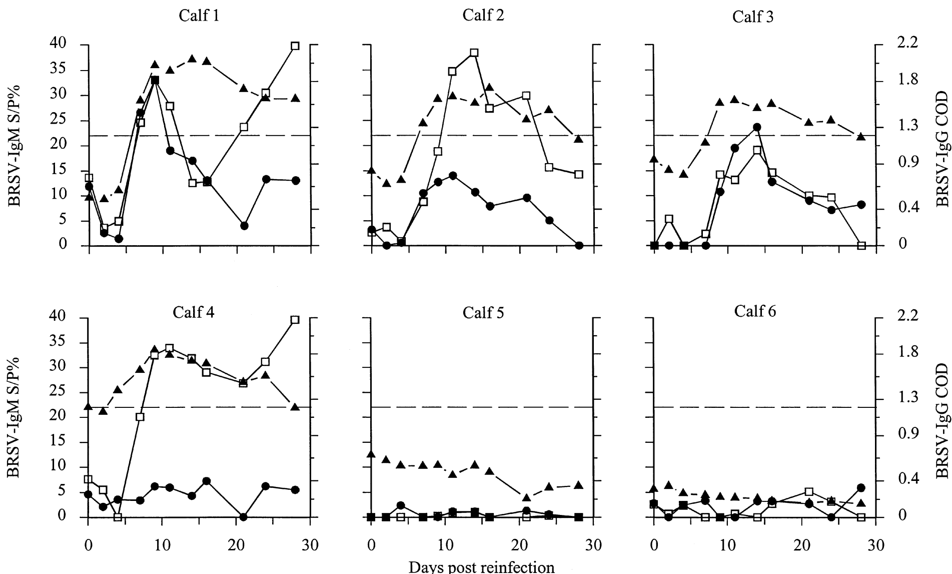

Fig. 2. BRSV- IgM (S/P%) and -IgG (▲) (COD) responses in Panel 2 sera following reinoculation of calves with either BRSV strain SVA/274/92 (calves 1–4) or with a control inoculum (calves 5 and 6). BRSV-IgM responses were measured both with (●), and without (□), pre-treatment with PGA. BRSV-IgM PNT = 22% (– –).

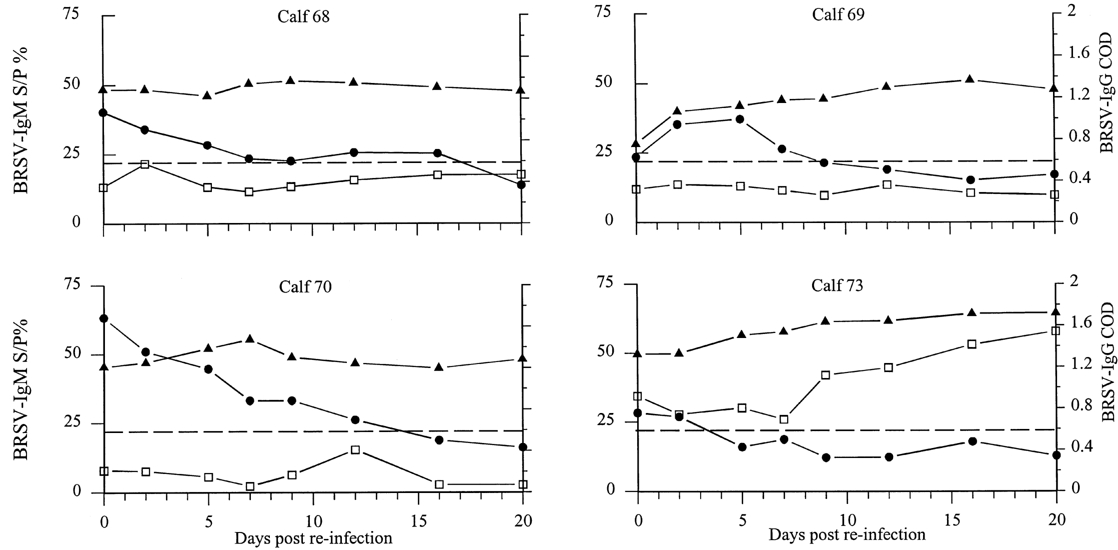

Fig. 3. BRSV-IgM (S/P%) and -IgG (▲) (COD) responses in Panel 3 sera following reinoculation of calves with BRSV strain SVA/187/92. BRSV-IgM responses were measured both with (●), and without (□), pre-treatment with PGA. BRSV-IgM PNT = 22% (– –).

after PGA treatment. Calf 1 again demonstrated a BRSV-IgM response to reinfection, peaking nine days after reinfection at 33.1%. Thereafter, S/P% values fell below the positive/negative threshold (PNT) and remained there. In contrast, the BRSV-IgM response in calf 4 was completely abolished. Calf 2 also appeared to give a BRSV-IgM response on reinfection, with S/P% values of untreated sera becoming positive, rising to a peak, and then decreasing to negative values again. However, the plot for this calf following PGA treatment of the sera was similar in shape but reduced in magnitude, such that all sera became negative. The two plots for calf 3 were similar in shape and magnitude, with a rise to a peak value 14 days after reinfection, followed by a steady decrease. All S/P% values, with the exception of PGA-treated sera collected 14 days after reinfection (23.6%), were negative. Following reinfection, BRSV-IgG levels in calves 1–4 rose sharply to an initial plateau, before declining toward the end of the sampling period (Fig. 2). Seroconversion was detected in calves 1, 2 and 4. The BRSV-IgG levels in calves 5 and 6 continued to decline after mock reinfection, consistent with these antibodies being maternally derived.

3.3. Panel 3

When sera were tested without prior treatment with PGA, calves 68, 69 and 70 were negative for BRSV-IgM at the time of reinfection and throughout the following period (Fig. 3). Following PGA treatment however, all three of these calves tested positive at the time of reinfection and for varying periods thereafter. The BRSV-IgM S/P% values of calves 68 and 70 showed a downward trend, respectively becoming negative 20 and 16 days after reinfection. In contrast, calf 69 showed a transient low level IgM response to reinfection, with S/P% values rising from 23.6% at reinfection to 37.2% five days later and becoming negative after a further four days (Fig. 3, Table 1). Calf 73 showed a different pattern, testing positive at reinfection with a subsequent upward trend in BRSV-IgM S/P% values when testing was performed without prior treatment of sera with PGA, giving a maximum value of 57.8% at the end of the sampling period (20 dpr). However, PGA treatment resulted in an opposite downward trend, becoming negative five days after reinfection. BRSV-IgG levels were stable or rose slowly following reinfection, with none of the calves seroconverting.

4. Discussion

The suppressive effect of MDA on the humoral response to BRSV infection is well recognised, with the IgM-specific response having been shown to be more resistant to suppression by MDA than the IgG response (Kimman et al., 1987a, b). This differential suppression is evident in the IgM- and IgG-specific responses to primary BRSV infection shown by the four infected Panel 1 calves. Generally, an inverse relationship was evident between BRSV-IgG levels at the time of infection and the subsequent IgM responses, consistent with the findings of Kimman et al. (1987a). None of these four calves seroconverted following infection, as defined by a fourfold or greater increase in specific IgG levels, and the IgM profiles of two of the three calves, which showed a response

when sera were tested without pre-treatment with PGA, were of short duration and low intensity (Table 1, Fig. 1). These findings are in contrast to previously described results obtained with this I-ELISA in the absence of MDA. When the four calves used to generate Panel 3 sera were initially infected with BRSV when seronegative, all four seroconverted and tested positive for IgM, with IgM responses being detectable for 16–30 days and peak S/P% values ranging from 165.3% to 279.8% (Elvander et al., 1998; Graham et al., 1998a).

Protein G is an immunoglobulin-binding bacterial cell wall protein which, unlike protein A, binds bovine IgG_1 and IgG_2 (Björck and Kronvall, 1984; Åkerström et al., 1985). Pre-treatment of bovine sera with PGA prior to testing for BRSV-IgM by I-ELISA is a novel procedure which has previously been shown to be effective in depleting serum IgG (Graham et al., 1998c). Removal of competition between these two isotypes for binding sites on the solid phase increased the magnitude of the BRSV-IgM responses in sera which contain both BRSV-IgG and -IgM (Graham et al., 1998c). The results reported here for Panel 1 sera confirm and extend these previous results. The overall shape of the IgM profiles for calves 1, 2 and 4 were similar with, and without, PGA treatment of sera. However, a marked increase in the magnitude of the IgM S/P% value of some sera was apparent following treatment. In a proportion of sera this resulted in a change in status from negative to positive, with a concomitant increase in the period for which each calf tested positive. In addition, calf 3, which was BRSV-IgM negative throughout the sampling period when sera were tested without PGA treatment, gave a single low positive IgM result (24 dpi) following PGA treatment. A similar effect was evident at the start of the sampling period for Panel 3 sera. Calves 68, 69 and 70 were BRSV-IgM negative when tested without PGA treatment, but tested positive, with values ranging from 23.6% to 63.2%, when treated with PGA. This IgM is considered to represent the tail end of the response to initial infection 37 days previously. This is supported by the steady decline of the signal to less than the PNT value in calves 68 and 70.

IgM-RF-induced false positive signals arise in I-ELISAs because the anti-IgM label reacts with IgM-RF bound to specific IgG attached to solid phase antigen, and have been shown to be more dependent on levels of specific IgG than IgM-RF levels (Champsaur et al., 1988). Therefore, depletion of serum IgG is one practical method of eliminating such false positive IgM results. Although the level of false positive IgM signals is greatly reduced in ACC-ELISA, they may still occur when a control antigen is not included (Kryger et al., 1981; Yolken and Leister, 1981).

The finding that following reinfection, some sera from calves 1, 2 and 4 (Panel 2) and 73 (Panel 3) which tested BRSV-IgM positive without PGA treatment became negative following depletion of serum IgG is consistent with these being false positive results due to the interaction of IgM-RF and specific IgG in these sera (Champsaur et al., 1988; Ungar-Waron and Abraham, 1991). In the case of calf 2, the false positive IgM signal was transient, whereas calves 1, 4 and 73 showed a steady increase in signal strength toward the end of the sampling period (Figs. 2 and 3). The PNT value of 22% was determined previously and represents the mean plus two standard deviations of the S/P% values recorded for 59 healthy calves with no previous history of respiratory disease (Graham et al., 1998c). The observation that following PGA treatment calf 2 (Panel 2) showed a transient IgM response which failed to reach a value of 22% may indicate that the PNT

value is too high. Between 0 and 16 dpr, the IgM values for calf 1 were similar before, and after, PGA treatment, with a transient rise to peak values in excess of the PNT value, indicating that the development of a false positive IgM response in this calf was preceded by an initial specific IgM signal. This is the first time that the sequential development of false positive IgM signals in an I-ELISA system has been demonstrated in this way. These results suggest that false positive signals are associated with reinfection rather than with primary infection, possibly due to the higher levels of specific IgG associated with the latter. The generation of false positive results attributable to IgM-RF may help explain previous reports of prolonged IgM responses to BRSV and bovine coronavirus which have been detected by I- ELISA (Stewart and Gershwin, 1990; Heckert et al., 1991).

If BRSV-IgM results, generated following PGA treatment, are considered specific, three out of eight calves (panels 2 and 3 combined) showed weak, transient increases in BRSV-IgM levels following reinfection, while three out of eight calves seroconverted. By comparison, Kimman et al. (1987a) detected no IgM responses to reinfection by ACC-ELISA.This may indicate that the sensitivity of the I- ELISA exceeds that of the ACC-ELISA, possibly reflecting differences in the relative significance of inter-isotypic competition in the former and intra-isotypic competition in the latter. Whilst intra-isotypic competition may produce false negative results (Van Zaane and Ijzerman, 1984), depletion of serum IgG with PGA, as demonstrated in this study, markedly reduces the impact of inter-isotypic competition.

In conclusion, the results obtained with this I-ELISA compare favourably with those previously reported for ACC-ELISA (Kimman et al., 1987a, b), and agree with a previous study which found it to be superior to testing for seroconversion for the serodiagnosis of BRSV infection in field sera (Graham et al., 1998c). The results demonstrate the necessity of controlling non-specific IgM responses and the benefits of amplifying specific responses by removal of competing IgG. The effectiveness of this novel method of pre-treating sera is indicated in both these regards. It is recommended that a protocol to deplete serum IgG should be routinely incorporated into the protocols for I-ELISAs measuring specific IgM in order to eliminate the dual possibilities of false positive results due to IgM-RF and false negative results due to inter-isotypic competition.

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