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Research paper

Production of seven monoclonal equine immunoglobulins isotyped by multiplex analysis

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ABSTRACT

Horses have 11 immunoglobulin isotypes: IgM, IgD, IgA, IgE, and seven IgG subclasses designated as IgG1–IgG7, each of which are distinguished by separate genes encoding the constant heavy chain regions. Immunoglobulin (Ig) isotypes have different functions during the immune response and pathogen-specific isotypes can be used as indicators for immunity and protection from disease. In addition to existing monoclonal antibodies to various equine Igs, quantification of the individual isotypes requires pure isotype standards. In this report, we describe a fusion between X63-Ag8.653 mouse myeloma cells and horse PBMC to create equine–murine heterohybridomas. Initial screening for Ig production was performed by ELISA. Further testing was performed by a new 5-plex fluorescent bead-based assay able to simultaneously detect equine IgM, IgG1, IgG4/7, IgG5, and IgG6. Production of IgG3 and IgE was tested by separate bead assays. Seven stable heterohybridoma clones producing monoclonal equine IgM, IgG1, IgG3, IgG4/7, IgG5, IgG6 and IgE were created. Purified Ig isotypes were then tested by SDS–PAGE. The pure, monoclonal equine Ig isotypes and the new equine Ig multiplex testing developed here are valuable tools to quantify antibody responses and to accurately determine individual isotypes concentrations in horses.

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1. Introduction

Equine immunoglobulins have been intensively studied in the 1970s (Wagner, 2006). Renewed interest in the fundamental understanding of equine antibodies has led to characterization of the associated genes (Home et al., 1992; Ford et al., 1994; Wagner et al., 2004; Almagro et al., 2006; Sun et al., 2010) and in monoclonal antibody development to various Ig isotypes of the horse (Sheoran et al., 1998; Sugiura et al., 1998; Wagner et al., 2003, 2008; Wilson et al., 2006) in order to better understand and characterize humoral immunity to pathogens or vaccination.

Horses have 11 immunoglobulin isotypes: IgM, IgD, IgA, IgE, and seven IgG subclasses, named IgG1–IgG7 (Wagner

et al., 2004; Lewis et al., 2008). Each isotype is distinguished by the gene encoding the constant heavy chain region (Wagner, 2006). In serum, IgG is the major immunoglobulin of horses (Sheoran et al., 1998). IgG plays a key role in neutralizing immune responses to many horse pathogens including equine herpesvirus type 1 (EHV-1) and EHV-4 (Mizukoshi et al., 2002; Goehring et al., 2010), equine influenza virus (Nelson et al., 1998), *Rhodococcus equi* (Lopez et al., 2002), *Theileria equi* (Mealey et al., 2012) and nematodes (Dowdall et al., 2002). The seven isotypes of IgG have been characterized on the molecular (Wagner et al., 2004) and protein level. The latter was performed by cloning each of the seven IgG heavy chain genes and by expressing the corresponding IgG isotypes in Chinese Hamster Ovary cells (Lewis et al., 2008). Functional studies revealed marked biochemical differences in complement activation, Fc-receptor binding and the bacterial protein binding capacity between the IgG isotypes of

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the horse (Lewis et al., 2008). Experimental infection and disease studies further suggest that IgG4, IgG7 and probably IgG1 protect against various intracellular infections (Nelson et al., 1998; Lopez et al., 2002; Goodman et al., 2006; Soboll-Hussey et al., 2011; Goodman et al., 2012), while extracellular pathogens mainly induce IgG3 and/or IgG5 responses (Dowdall et al., 2002; Mealey et al., 2012). IgM normally precedes IgG antibodies and appears early in acute infection, functioning in the primary antibody-mediated immune response (Wagner et al., 2008). IgE is one of the minor immunoglobulin isotypes in serum (Wagner et al., 2003). IgE is involved in type I hypersensitivity reactions (Larsen et al., 1988; Hellberg et al., 2006; Wagner et al., 2006a) and parasite immunity in horses (Suter and Fey, 1981). IgA is the major immunoglobulin involved in antibody-mediated immune responses at mucosal surfaces (Lewis et al., 2010). Lastly, a functional gene encoding IgD exists in the horse genome. Thus, IgD may be expressed by equine B cells. The serum concentration of IgD is likely low and its function remains unknown in horses (Wagner et al., 2004).

Here, we describe the development of seven equine-murine heterohybridomas producing seven distinct Ig isotypes of the horse including equine IgE, which to our knowledge, has not been described before. These equine Igs produced by heterohybridomas were purified and can be used for quantification of isotypes in diagnostic testing and immunological research. Furthermore, we developed a multiplex assay for the simultaneous detection of equine Ig isotypes to characterize equine antibodies produced by the heterohybridomas. The equine Ig multiplex assay provides a valuable research tool to further analyze antibody responses in equine health and disease.

2. Materials and Methods

2.1. Myeloma cell line

X63-Ag8.653 mouse myeloma cells were used for cell fusion. Cells were maintained in Hybridoma-SFM culture medium (Gibco, Grand Island, NY), supplemented with 10% fetal calf serum (Thermo Scientific, Logan, UT). At the time of fusion, cells were in the logarithmic phase of growth.

2.2. Equine peripheral mononuclear cells

Heparinized blood was obtained from the jugular vein of a healthy 7-year-old thoroughbred mare. This mare previously donated cells to obtain the first genome sequence of the horse (<http://www.ncbi.nlm.nih.gov/genome?term=equus%20caballus>). PBMC were obtained by Ficoll-Paque isolation (GE Healthcare, Piscataway, NJ) as described previously (Wagner et al., 2003). All animal procedures were approved by the Cornell University Institutional Animal Care and Use Committee and were in accordance with the guidelines established by the NIH.

2.3. Cell fusion

Equine PBMC were carefully mixed with murine X63-Ag8.653 in a 1:10 ratio and centrifuged to remove

supernatant. Pre-warmed PEG 1500 (Roche Diagnostics, Indianapolis, IN) was slowly added to the cell pellet (1.5 mL PEG per 3×10^8 cells) and incubated for 1 min at 37 °C. Hybridoma-SFM was carefully added to the cells over a period of 3 min (1 mL in first minute; 3 mL in second minute; 16 mL in third minute). Cells were then centrifuged and decanted. HAT Fusion medium (Hybridoma-SFM containing, 10% (v/v) fetal calf serum, 100 μ M hypoxanthine, 0.4 μ M aminopterin, 16 μ M thymidine, 1% (v/v) Pen/Strep, 0.1% (v/v) Gentamicin, 1.5 ng/mL of rhum IL-6) was added to yield 5×10^5 cells/mL and cells were carefully resuspended and plated in 24 well plates (Corning Incorporated, Corning, NY) with 2 mL per well. Fusion plates were incubated in a humidified CO₂ incubator at 37 °C. After three weeks of incubation, supernatants were tested for Ig production by ELISA. Individual clones were isolated from Ig positive wells with a pipetter and transferred to fresh 24-well plates with 2 mL HAT medium.

After four weeks in HAT medium, clones were weaned into HT medium (Hybridoma-SFM containing 10% (v/v) FCS, 100 μ M hypoxanthine, 16 μ M thymidine, 1% (v/v) Pen-Strep, 0.1% (v/v) Gentamicin), then into Hybridoma-SFM supplemented with 10% FCS two weeks later and finally into plain Hybridoma-SFM medium. Limiting dilutions were performed if Ig testing indicated that more than one clone existed in a well.

2.3.1. Detection of Ig isotypes from equine-murine heterohybridomas by enzyme linked immunosorbent assay (ELISA)

The initial screening of the heterohybridoma supernatants for equine Ig production was performed by ELISA. Nunc brand polystyrene plates (Thermo Scientific, Rochester, NY) were incubated overnight at 4 °C with 4 μ g/mL of antibody (Table 1) in carbonate coating buffer (1 M NaHCO₃, 1 M Na₂CO₃, pH 9.6). Plates were washed four times with phosphate buffered saline, 0.05% TWEEN (Sigma–Aldrich, St. Louis, MO) and then incubated with cell culture supernatants for 1 h at room temperature. Plates were again washed with PBST, as described. Plates used to screen for IgG1, IgG4/7, and IgG6 were incubated at room temperature for 30 min with peroxidase-conjugated goat anti-horse IgG(H+L) antibody (Jackson ImmunoResearch, West Grove, PA), diluted 1:10,000. Plates were washed and incubated 15 min in the dark with substrate buffer (33.3 mmol citric acid, 66.7 mmol NaH₂PO₄, pH 5.0), combined with 130 μ g/mL 3,3',5,5' Tetramethylbenzidine (Sigma–Aldrich) and 0.012% hydrogen peroxide. The reaction was stopped using 1 N H₂SO₄. Plates were assessed using an automatic plate reader (Biotek, Winooski, VT) at 450 nm absorbance.

For detecting IgE producing heterohybridomas, plates were coated with anti-IgE 176, incubated with culture supernatant and detection was accomplished by incubating the plates for 1 h with biotinylated anti-IgE 134 diluted 1:2000, followed by peroxidase-conjugated streptavidin (Sav-Px) diluted 1:5000 for half an hour (Jackson ImmunoResearch, West Grove, PA). All other steps were the same as those described above. Likewise, to screen for IgM clones, plates were coated with anti-IgM-1-22 and detection was by biotinylated anti-IgM-2B63 at 1:2000

Table 1

Monoclonal antibodies used for characterization of heterohybridomas secreting different equine Ig isotypes.

Monoclonal antibody	Equine Ig isotype recognized	Used for		Reference
		ELISA	Bead-based assay	
Anti-IgM-1-22	IgM	Coating	Bead coupling	Wagner et al. (2008)
Anti-IgM-2B63		Detection		
CVS 39		Coating		
CVS 45	IgG4 and IgG7 (IgGb)	Coating	Bead coupling	Sheoran et al. (1998)
Anti-IgG6-267	IgG1 (IgGa)			
Anti-IgG5-416	IgG6 (IgGc)			
Anti-IgG5-522-9	IgG5	Coating	Bead coupling	Lewis (2010)
Anti-IgG1/3-159	IgG5			
Anti-IgG3/5 586	IgG1 and IgG3			
Anti-IgE-176	IgG3 and IgG5 (IgG(T))	Detection (IgG3)	Detection (IgG3)	Wagner et al. (unpublished)
Anti-IgE-134	IgE	Coating	Bead coupling	
Anti-IgA-135	IgE	Detection	Detection	Wagner et al. (unpublished)
	IgA	Coating	Not used	

and Sav-Px. IgG3 clones were determined by coating with anti-IgG3-159 and detecting with biotinylated anti-IgG5-586 diluted 1:2000 and Sav-Px. IgG5 clones were determined using anti-IgG5-416 as the coating antibody and biotinylated anti-IgG5-522 at 1:2000 as the detecting antibody along with Sav-Px. Lastly, IgA clones were determined by coating with anti-IgA-135 and then detected using peroxidase-conjugated goat-anti-horse IgA (Serotec, Raleigh, NC) diluted 1:4000.

2.3.2. Ig Isotype determination by multiplex analysis

The continuous testing of the supernatants was performed using a 5-plex assay for detecting equine IgM, IgG1, IgG4/7, IgG5 and IgG6. Separate bead-based assays were performed for IgG3 and IgE detection. The multiplex assay uses fluorescent beads as matrix for the assay and allows for the simultaneous detection of multiple analytes in a single sample or, as in this case, the differentiation of five different equine Ig isotypes using a single assay. Monoclonal antibodies specific for equine isotypes were coupled to fluorescent beads (Luminex Corp., Austin, TX) and the assay was performed exactly as described previously for other analytes (Wagner and Freer, 2009). Here, a total of 100 µg anti-equine Ig isotype mAb was used for coupling of 5×10^6 beads. All mAbs were murine IgG1 isotypes and are listed in Table 1. For the 5-plex Ig isotype assay, anti-IgM clone 1-22 was coupled to bead 34, anti-IgG1 clone CVS45 to bead 38, anti-IgG4/7 clone CVS39 to bead 35, anti-IgG5 clone 416-2 to bead 33, and anti-IgG6 clone 267 to bead 37. Cell culture supernatants from heterohybridomas were used as samples. Equine serum served as positive control and sample buffer as negative control for the assay. For detection, biotinylated goat anti-horse IgG(H+L) (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:10,000 was used followed by an incubation with streptavidin-phycoerythrin (Sav-PE; Invitrogen, Carlsbad, CA) at 1:100. The IgG3 and IgE assays were run as single-plex assays. IgG3 was detected in the supernatants using anti-IgG1/3 clone 159 coupled to bead 36 and by using biotinylated anti-IgG3/5 clone 586 at 1:1000 for detection. IgE was measured by an assay based on bead 35 coupled with anti-IgE clone 176 and detection using biotinylated anti-IgE clone 134 at 1:250. Both assays used a secondary detection step with Sav-PE as described above. The incubation time for

each step was 30 min and plates were washed between incubation steps as described (Wagner and Freer, 2009). Then, 100 µL of blocking buffer was added per well and each plate was placed on the shaker for 15 min to resuspend the beads after the assay. The assay was analyzed in a Luminex 200 instrument (Luminex Corp., Austin, TX). The data were reported as median fluorescent intensities (MFI).

2.3.3. Purification of mAbs

Purification of equine IgG isotypes from serum-free cell culture supernatants was performed using a Protein G column (GE Healthcare, Piscataway, NJ). IgE was purified using an anti-IgE affinity column. IgG and IgE purification was done on a FPLC instrument (GE Healthcare, Piscataway, NJ). Both purification methods were previously described in detail (Wagner et al., 2003, 2006a). Supernatant from the IgM heterohybridoma was 40-fold concentrated using a 4 mL, 30 kDa concentrator (Millipore, Billerica, MA) and was not further purified for SDS-PAGE.

2.3.4. SDS-PAGE

SDS-PAGE was performed as described previously (Wagner et al., 2003). The heavy and light chains of the antibodies were separated in 15% polyacrylamide gels with 2-beta mercaptoethanol (ME). Afterwards, gels were stained with Coomassie Brilliant Blue.

3. Results

The single fusion of peripheral blood mononuclear cells from a healthy horse and X63-Ag8.653 cells resulted in 170 wells containing heterohybridoma clones. Supernatants from more than 60% of the wells (107 wells) contained equine Ig. Initial isotype testing by ELISA showed 31 wells positive for IgG1; 27 wells positive for IgG6; 13 wells positive for IgM, 8 wells positive for IgG3; 5 wells positive for IgG4/7; 5 wells positive for IgG5; and 1 well positive for IgE. IgA positive wells were not detected. Seventeen wells were positive for Ig, but the isotypes were not determined suggesting that these clones contained either isotypes we could not test for (IgD, IgG2) or light chains only. From positive wells, 53 cell lines were maintained and further tested using a multiplex 5-plex assay for isotyping. Of these,

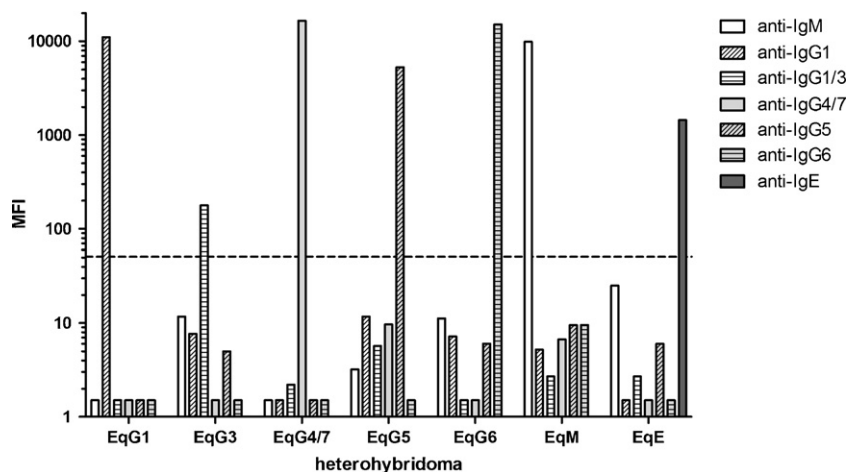


Fig. 1. Multiplex characterization of equine–murine heterohybridomas. Cell culture supernatants were analyzed using fluorescent bead-based multiplex assays to determine the isotype of each monoclonal antibody. IgM, IgG1, IgG4/7, IgG5 and IgG6 were detected simultaneously in a 5-plex assay. Individual assays were performed for IgG3 and IgE. The dotted line represents the lowest doubled blank value of the assay and was used as a cut-off value. Mean fluorescence intensity (MFI) values above that cut-off were considered positive. A value of 1.5 MFI was assigned to all non-detectable samples in the multiplex assay. Clone numbers were as follows: EqG1 11, EqG3 39, EqG4/7 120, EqG5 18-1, EqG6 5-1, EqM 49, EqE 37-1.

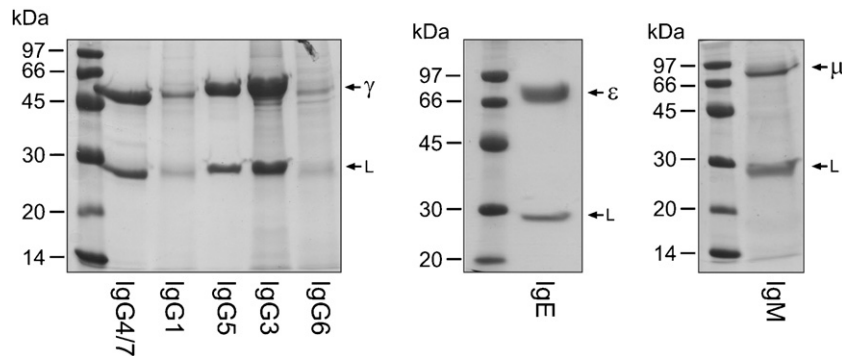


Fig. 2. SDS–PAGE analysis of purified equine Ig isotypes produced by equine–murine heterohybridomas. Igs were purified from clones EqG1 11, EqG3 39, EqG4/7 120, EqG5 18-1, EqG6 5-1, EqM 49, EqE 37-1. Two micrograms of each protein were loaded onto the gels. Gels were run under reducing conditions and stained with Coomassie blue. L = light chain; γ , ϵ , μ = Ig heavy chains of IgG, IgE and IgM, respectively.

23 heterohybridomas maintained equine Ig production for longer than 4 months.

Seven stable heterohybridoma clones, one of each isotype, designated as EqG4/7 120, EqG1 11, EqG5-43, EqG3 147-1, EqG6 5-1, EqM 49 and EqE 37-1, were further characterized from culture supernatants using equine Ig multiplex analysis (Fig. 1). Each of the heterohybridomas produced only one equine Ig isotype. Each of the seven Ig isotypes were subsequently purified from supernatants and analyzed by SDS-PAGE using reducing conditions. As expected, IgG isotypes contained gamma-chains of approximately 52–58 kDa and Ig light chains of 27–29 kDa. An ϵ -chain of around 70–78 kDa was detected for the IgE producing heterohybridoma and a μ -chain for approximately 78–82 kDa was found for the IgM producing heterohybridoma (Fig. 2).

4. Discussion

The equine antibody response to antigenic agents is frequently used in diagnostic assays to evaluate immunity to

pathogens and efficacy of vaccines, or to determine seroprevalences of emerging and re-emerging diseases. Here we have described the production of seven heterohybridomas secreting single equine Ig isotypes, including an IgE producing clone, which, to our knowledge, has previously been unavailable. The generation of equi–murine heterohybridomas has been described previously (Appleton et al., 1989; Richards et al., 1992; Wagner et al., 1995). The goal for some of these attempts was to generate equine mAbs against equine Influenza virus and in one study [(horse $\times$ mouse) $\times$ horse] fusions were performed to obtain these mAbs (Appleton et al., 1989; Richards et al., 1992). Previous equi–murine heterohybridoma production had to be performed in the absence of isotype-specific reagents for horses and resulted in few equine IgG (IgG1, IgG3, IgG4) and one IgM producing cell lines (Wagner et al., 1998). In this study, we had detection tools for most equine isotypes available. The generation of monoclonal equine Ig isotypes using these tools was a very efficient procedure and resulted in multiple heterohybridoma clones of seven different isotypes from a single fusion.

In horse serum, IgG is the predominant immunoglobulin (Sheoran et al., 2000). Of the IgG isotypes, IgGb (IgG4/7) is prevalent at the highest concentration, followed by IgG(T) (IgG3 and IgG5); IgGa (IgG1); and last, IgGc (IgG6) (Lewis et al., 2008). IgM and IgA serum concentrations are believed to be greater than that of IgG6 (Wagner, 2006). Out of the currently detectable isotypes, IgE is present at the lowest concentration in serum (Wagner et al., 2003). The serum concentrations of IgG2 or IgD are still unknown and are assumed to be very low. Based on the observed serum concentrations our expectation was to see a similar proportion of clones after fusing peripheral blood cells, such that IgG4/7 > IgG3 = IgG5 > IgG1 > IgM > IgA > IgG6 > IgE. However, we did observe a majority of IgG1 secreting clones followed by IgG6 > IgM > IgG3 > IgG4/7 = IgG5 > IgE. The observed discrepancy between serum concentrations of immunoglobulins versus obtained clones, especially for IgG6, may simply reflect differences in the affinity and avidity of anti-isotypes antibodies used previously to determine serum Ig concentrations (Sheoran et al., 2000) and some of the new mAbs to equine IgG isotypes used here. Revisiting normal serum Ig concentrations using these additional mAbs to equine isotypes and pure equine standard Igs, such as the equine heterohybridoma antibodies, may result in a more accurate quantification of normal isotype concentrations at least for some isotypes. The observed discrepancy is also likely to reflect a difference between serum Ig isotype concentrations and the number of corresponding Ig isotype positive B-cells in equine PBMC. Serum isotypes and circulating peripheral B-cell populations are unlikely to match because the majority of serum antibodies is produced by plasma cells located in secondary lymphatic tissues and not by the circulating, mostly naïve B-cells. For example, IgG4 and IgG7 represent dominating serum isotypes. However, IgG4 or IgG7 positive peripheral B-cells are rarely found in clinically healthy horses. Equine peripheral B-cells are mostly IgM⁺ or IgG1⁺ cells (Wagner et al., 2012). IgA, IgG2 and IgD clones were not isolated, as would be expected. Their presence in serum is limited, and present testing methods do not allow for elucidation of the IgD or IgG2 isotypes.

Given that IgE is minimally present in serum and circulating IgE⁺ cells mainly represent cells that have captured IgE via their Fc-receptors and not IgE producing B-cells (Wagner et al., 2003, 2012), it was exciting to yield an IgE clone in this fusion. IgE is involved in pathological reactions such as IgE-mediated hypersensitivities in horses (Larsen et al., 1988; Hellberg et al., 2006; Wagner et al., 2006a) and is believed to protect against parasite infection (Suter and Fey, 1981). Through cross-linking with Fcε-receptors on basophils or mast cells, IgE stimulates cellular degranulation, resulting in clinical signs of allergy in some horses (Wagner et al., 2006a). In young foals, IgE of maternal origin is transferred through the consumption of colostrum (Wagner et al., 2006b). Interestingly, and despite the presence of intestinal parasites and environmental pressures, endogenous IgE is not detectable in foals before colostrum uptake and at least for the first 3–4 months of life. This suggests that serum IgE present in foals is of maternal origin (Wagner et al., 2006b). While there is uncertainty

what role maternal IgE plays in early immune system development, IgE produced by our heterohybridoma can serve as a reference molecule to quantify IgE in future studies on immune development, parasite immunity and allergy.

The present heterohybridoma Igs provide pure preparations of equine Ig isotypes, which are not contaminated with other isotypes. Prior to this point, IgE has been obtained by purification from serum samples (Suter and Fey, 1981; Wagner et al., 2003; Wilson et al., 2006). While this method has its merits, it is unreliable in producing a pure IgE and some IgG contamination is usually observed. Because of the glycosylation of IgE, there are also issues with utilizing recombinant expression systems. Previous attempts to develop recombinant equine IgE were hampered by either poor antigenic similarity to native IgE after expression in *E. coli* (Marti et al., 1997; Watson et al., 1997) or low protein yields of fully functional IgE after expression in mammalian cells (Wagner et al., 2002). Pure equine IgE derived from a heterohybridoma cell line is both identical to native IgE and is produced in high quantities around 1–10 µg/mL supernatant. This is similar to the concentration obtained from regular mouse monoclonal antibodies and can overcome some of the previously encountered issues in IgE quantification and functional studies on IgE in horses. A heterohybridoma secreting equine IgA was not derived from our approach despite a mAb to equine IgA being available. A likely reason for the absence of an IgA producing cell line is a low frequency of IgA producing B-cells in the peripheral blood. IgA secreting plasma cells are mainly located in the mucosa and mucosa-associated lymphoid tissues (Lewis et al., 2010).

Purified equine monoclonal Ig isotypes provide reliable standards for isotype quantification. They allow the comparison of antibody responses in healthy horses and those with various diseases. Current procedures only allow the quantification of the major Ig classes such as IgM and IgG in horses and IgG standards usually represent a mixture of various isotypes (Wagner, 2006). With known isotype concentrations, more detailed, quantitative comparisons can be made with samples acquired from various natural or experimental diseases and conditions. For example, different isotype responses have been described following vaccination versus natural infection with Equine herpesvirus-1 (Goodman et al., 2006, 2012; Goehring et al., 2010).

Aside from the development of equine-murine heterohybridomas, we have also described the development of a new multiplex assay for detection of equine isotypes using a panel of equine monoclonal anti-isotype antibodies (Sheoran et al., 1998; Wagner et al., 2003; Goehring et al., 2010; Wagner et al. unpublished). This bead-based technology provides a tool for further characterization of the equine Ig isotype response. While most isotyping has been done via an ELISA, this technology is more sensitive and allows for simultaneous characterization of IgM, IgG1, IgG4/7, IgG5 and IgG6 from a single sample. Thus, we can greatly accelerate the detection and measurement of the major equine Ig isotypes present in a sample. IgG3 and IgE however, remain as single assays mainly because of the

much lower concentrations of these two isotypes in equine serum, requiring a higher serum concentration for analysis.

In conclusion, the seven equine–murine heterohybridomas described here are useful reference molecules for quantification and future studies on equine Ig isotype responses. The challenge of obtaining pure immunoglobulins, particularly IgE can be overcome via purification of antibodies from each particular heterohybridoma. Furthermore, the quantification of immunoglobulin isotypes can be efficiently accomplished using the new Ig isotype multiplex assay for horses. In total, these new tools can contribute to further explore antibody responses after infection or vaccination and to design new diagnostic testing strategies for horses.

Conflict of interest statement

None.

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