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Research paper

Quantification of equine immunoglobulin A in serum and secretions by a fluorescent bead-based assay

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ABSTRACT

Only few quantitative reports exist about the concentrations and induction of immunoglobulin A (IgA) in mucosal secretions of horses. Despite this, it is widely assumed that IgA is the predominant immunoglobulin on mucosal surfaces in the horse.

Here, two new monoclonal antibodies (mAbs) against equine IgA, clones 84-1 and 161-1, were developed and characterized in detail. Both IgA mAbs specifically bound monomeric and dimeric equine IgA in different applications, such as Western blots and fluorescent bead-based assays. Cross-reactivity with other equine immunoglobulin isotypes was not observed. The new IgA mAb 84-1 was used in combination with the previously characterized anti-equine IgA mAb BVS2 for the development and validation of a fluorescent bead-based assay to quantify total IgA in equine serum and various secretions. The IgA assay's linear detection ranged from 64 pg/ml to 1000 ng/ml. For the quantification of IgA in serum or in secretions an IgA standard was purified from serum or nasal wash fluid (secretory IgA), respectively. The different standards were needed for accurate IgA quantification in the respective samples taking the different signal intensities of monomeric and dimeric IgA on the florescent bead-based assay into account.

IgA was quantified by the bead-based assay established here in different equine samples of healthy adult individuals. In serum the median total IgA was 0.45 mg/ml for Thoroughbred horses (TB, $n = 10$) and 1.16 mg/ml in Icelandic horses (ICH, $n = 12$). In nasopharyngeal secretions of TB ($n = 7$) 0.13 mg/ml median total IgA was measured, and 0.25 mg/ml for ICH ($n = 12$). Saliva of ICH ($n = 6$) contained a median of 0.15 mg/ml, colostrum of Warmbloods ($n = 8$) a median of 1.89 mg/ml IgA. Compared to IgG1 and IgG4/7 quantified in the same samples, IgA appeared as the major immunoglobulin isotype in nasopharyngeal secretions and saliva while it is a minor isotype in serum and colostrum.

The newly developed monoclonal antibodies against equine IgA and the resulting bead-based assay for quantification of total IgA can notably improve the evaluation of mucosal immunity in horses.

1. Introduction

Quantification of total immunoglobulin A (IgA) concentrations in horses has mostly been performed in serum. In the available reports, serum IgA was quantified by immunodiffusion techniques with polyclonal antisera (McGuire and Crawford, 1972; Mair et al., 1987; Kohn et al., 1989; McFarlane et al., 2001; de Camargo et al., 2009), a commercial ELISA based on polyclonal antibodies (Holznagel et al., 2003; Jenvey et al., 2012), or an inhibition ELISA using the anti-IgA mAb BVS2 (Sheoran et al., 2000). Quantification was performed by comparison to standard sera or purified serum IgA (Sheoran et al., 2000). The previous studies roughly agreed in their reported mean total IgA serum concentration within a range of 0.1–3.5 mg/ml. Nevertheless, assay validation or assay detection ranges were hardly reported.

As in other mammals, IgA has been considered the predominant Ig on mucosal surfaces and in secretions of horses (McGuire and Crawford, 1972; Mair et al., 1987; Sheoran et al., 2000; Wagner et al., 2003a). However, total IgA quantification in mucosal secretions of horses is rare. Some recent studies quantified equine IgA in colostrum and milk (Sheoran et al., 2000; Bondo and Jensen, 2011; Jenvey et al., 2012). These studies reported varying mean total IgA concentrations between 0.96 and 9 mg/ml and 0.3 and 0.7 mg/ml for colostrum or milk, respectively. In other secretions, quantification of equine IgA was reported sporadically. The existing reports used different samples and sampling techniques and thus cannot be directly compared. They investigated nasopharyngeal mucus (Galan and Timoney, 1985) and uterine secretions (Troedsson et al., 1993) by radial immunodiffusion

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(RID), or nasal wash samples by an inhibition ELISA (Sheoran et al., 2000). Mair et al. (1987) quantified IgA in respiratory tract lavage fluids by immunoelectrophoresis and reported ratios of IgA to albumin to be highest in nasal lavages. Nevertheless, the three reports on upper respiratory secretions agreed in their findings of higher IgA than IgG contents in those samples. Assay validations for the quantification methods were again not described. In other studies (Breathnach et al., 2001; Crouch et al., 2005), standardization of pathogen-specific IgA to total IgA in nasal wash samples was performed. These reports stated an induction of virus-specific IgA (relative to total IgA) on the nasal mucosa by challenge infection. Nonetheless, normal values of the total IgA for the standardization or assay validation for its measurement in nasal wash samples were not reported.

In summary, the quantification of total IgA in mucosal secretion samples of horses is fragmentary. Total IgA quantification would benefit from further investigation and improved reagent tools, such as a well-defined IgA standard and a pair of IgA-specific mAbs. In addition, the analysis of IgA in local mucosal immunity is of interest for equine respiratory pathogens. For this purpose a reliable quantification of antigen-specific IgA is needed and it must accordingly be validated for secretory samples. This report describes the quantification of equine IgA in serum and various equine mucosal secretions by the generation of monoclonal antibodies against equine IgA and the establishment of a fluorescent bead-based assay for total equine IgA.

2. Materials and methods

2.1. Samples for assay evaluation and establishment of normal ranges

All samples were collected from healthy horses in the research herds of Cornell University (Table 1). All animal procedures were approved by the Cornell University Institutional Animal Care and Use Committee under the protocol number 2011-0011.

Serum samples were collected by jugular venipuncture by a vacutainer system into serum tubes (BD Franklin Lakes, NJ, USA), allowed to clot and separated by centrifugation.

Nasopharyngeal secretions (NS) were acquired by using two polyester tipped applicators (Puritan Medical Products Company, Gullford, ME, USA) swabbed on the nasopharyngeal mucosa of one nostril for 5–10 s. The swabs were immediately put into 1 ml of PBS (VWR, Solon, OH, USA) and transferred to the laboratory. This resulted in an average dilution of the original secretion of 1:9 (range 1:6.5–1:11.7, determined by volume measurement on $n = 6$). Leaving the swabs in the tube, they were spun down at 1000 × *g* at 4 °C for 5 min. The NS diluted in PBS surrounding the swabs was collected into a 1.5 ml centrifuge tube. The NS was centrifuged again at 100 × *g* at 4 °C for 5 min and the cleared fluid supernatant was used as NS sample.

Nasal wash fluid (NW) was obtained by installation of 50 ml saline into one nostril of the horse by use of rubber catheter tubing. The fluid running out of the nostril was collected and transported to the laboratory. Subsequently, the fluid was filtered through a 100 µm cell strainer (Corning, Durham, NC, USA) to remove debris and the filtrate was spun at 100 × *g* at 4 °C for 5 min. The supernatant of the resulting pellet was used as NW sample.

Saliva was collected as follows: Cotton pieces were placed into spiral bits (1.5 cm diameter) keeping the cotton in place but allowing free contact with the saliva in the horse's mouth for approximately 30 min. In the following, the saliva-saturated cotton pieces were suspended on a pipet tip in a centrifuge tube, transported to the laboratory and centrifuged at 1000 × *g* at 4 °C for 5 min. After removal of the drained cotton and the tip, the undiluted saliva was collected from the bottom.

Colostrum samples were collected pre-suckling of the neonatal foals, within 60 min post-partum on average. The samples were spun down at 2200 × *g* at room temperature for 5 min to separate the fat. The lower hydrophilic phase was collected and used as sample.

All samples were stored in aliquots at −20 °C prior to the measurement of IgA.

Table 1
Horses used to obtain samples.

Breed	Samples	*n*	Female	Male	Age in years Median (range)
TB[a]	Serum	14	11	3	10.5 (5–17)
TB	NS[d]	11	9	2	10 (5–17)
ICH[b]	Serum and NS	12	6	6	5 (5–12)
ICH	Saliva	6	6	0	11 (8–13)
WB[c]	Colostrum	8	8	0	15 (11–19)

Horses used for control samples of body fluids by breed and sample type.
[a] TB, Thoroughbred.
[b] ICH, Icelandic horses.
[c] WB, Warmblood.
[d] NS, nasopharyngeal secretions.

2.2. Control samples for cross-reactivity in serological assays

Bronchoalveolar lavage fluid (BALF) was collected in the course of a diagnostic procedure from an 11-year-old Icelandic mare with recurrent airway obstruction. After sedation of the horse and administration of 10 ml 2% Lidocainehydrochloride into the bronchi, 250 ml sterile saline were injected into one bronchus by sterile BAL tubing and re-aspirated into 50 ml syringes. The yielded liquid was transported to the laboratory, clarified by centrifugation and used as a positive control sample containing natural equine IgA.

Recombinant equine monomeric and dimeric IgA were produced as described by Lewis et al. (2010). These were a kind gift from Dr. Lewis. Heterohybridoma supernatants containing single equine Ig isotypes were produced as described earlier (Keggan et al., 2013). These were used as controls for cross-reactivity in serological assays.

2.3. Affinity purification of IgA

The anti-IgA mAb BVS2 was generated and characterized by Lunn et al. (1995, 1998) and was previously used for the detection of equine IgA (Lunn et al., 1998; Breathnach et al., 2001). Purified anti-IgA mAb BVS2 (10 mg) was coupled to a HiTrap NHS-activated HP 1 ml column (GE Healthcare, Fairfield, CT, USA) according to the manufacturer's instructions. Affinity purification of equine serum IgA was performed on an FPLC instrument (ÄKTA FPLC, GE Healthcare). One ml of horse serum diluted in 50 ml sterile PBS or 50 ml of NW were run over the anti-IgA column in separate purification runs. The bound IgA was eluted with glycine buffer (0.1 M glycine, pH of 2.3) and immediately neutralized in 1 M Tris buffer (pH 8.0). After dialysis against PBS, the eluted IgA was concentrated using a concentrator column (Vivaspin Protein Concentrator Spin Columns, GE Healthcare) with a molecular weight cut-off of 30 kDa. The protein concentration was measured in a BCA assay (Pierce, Thermofisher, Waltham, MA, USA).

IgA was purified from serum of eight healthy adult individuals (four Icelandic horses, one Quarter horse, one Warmblood, one Thouroughbred, one mixed-breed pony) and from nasal wash fluid of three individual healthy adult Icelandic horses (ICH). Purity of the IgA was confirmed by SDS–PAGE (as described below) with Coomassie blue staining and by analysis in an equine Ig isotype fluorescent bead-based multiplex assay (Keggan et al., 2013). In addition, purified IgA from a Coomassie stained SDS gel was submitted for mass spectrometric protein identification via N-terminal sequencing to the Institute of Biotechnology at Cornell University.

Only batches of purified IgA without contaminations of other isotypes (< 0.4 ng/ml IgG1, < 0.06 ng/ml IgG4/7, no detectable IgG5, IgG6, and IgM in a 100 ng/ml total protein solution) were used as controls or standards in serological assays and as an immunogen for