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Research paper

Development of a bead-based multiplex assay for simultaneous quantification of cytokines in horses

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ABSTRACT

The detection and quantification of equine cytokines has been hampered by the lack of antibodies for many years. With the development of antibody pairs for equine cytokines during the past years, the quantification of these essential regulators of the immune response became possible. After being successfully tested by enzyme-linked immunosorbent assays (ELISA), three of these anti-cytokine reagents were used here to establish the first cytokine multiplex assay for equine IL-4, IL-10 and IFN-α. A fluorescent bead-based system was used as matrix for this assay that allows the simultaneous detection of the cytokines in a single sample by a Luminex analyzer. Equine recombinant cytokine/IgG fusion proteins were validated as standards for quantification of the individual cytokines. The analytical sensitivities of the multiplex assay were found to be 40 pg/ml for IL-4 and 15 pg/ml for IL-10 and IFN-α. The sensitivity of cytokine detection by the multiplex assay was increased by 13- to 150-fold compared to the corresponding ELISA. The specificity of the multiplex assay was validated using cell culture supernatants from equine peripheral blood mononuclear cells (PBMC) stimulated with different mitogens or infected with equine herpesvirus type 1 (EHV-1). As predicted, supernatants from PBMC stimulated with different mitogens contained IL-4 and IL-10, but no IFN-α. EHV-1 infection of PBMC resulted in a dose-dependent secretion of IFN-α. Low concentrations of IL-10 were also measured. IL-4 was not detectable in these samples. The resulting detection pattern found for the multiplex analysis and assays performed with individual standard cytokines indicated that individual bead assays did not interfere or cross-react during simultaneous detection of equine IL-4, IL-10 and IFN-α. The equine cytokine multiplex assay is a valuable and cost-effective tool for quantification of IL-4, IL-10 and IFN-α and can be used for manifold immunological applications. In the future, the assay can also be expanded by adding bead assays for other equine cytokines and chemokines to the existing platform.

1. Introduction

Multiplex assays are a new technology using the principle of simultaneous detection of soluble analytes, such as cytokine, in biological samples. These samples can be serum or other body fluids, or culture supernatants from cells stimulated *in vivo* or *in vitro*. Different multiplex systems have become commercially available during the past few years and are used in a wide variety of protein expression profiling applications, including diagnosis of infectious diseases, cancer, autoimmune and allergic diseases, cardiac diseases, and in neurobiological applications in human patients. In diagnostic and basic immunological research applications, multiplex assays are

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Abbreviations: EDC, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride; MES, 2-[N-morpholino]ethanesulfonic acid.

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frequently used for cytokine and chemokine analysis, isotyping of antibodies or cell signal profiling (Carson and Vignali, 1999; Vignali, 2000; Kellar and Douglass, 2003; Morgan et al., 2004; Prabhakar et al., 2004, 2005; DuPont et al., 2005).

One of the available multiplex systems is Luminex technology which is based on fluorescent beads ('microspheres') that are color-coded into 100 distinct sets (http://www.luminexcorp.com/). Each bead set can be coated with a reagent specific to a particular assay, allowing the capture and detection of a specific analyte from the sample. Within the Luminex analyzer, lasers excite the internal dyes that identify each microsphere particle, and also any reporter dye captured during the assay. Multiple readings are made on each bead set and result in an individual fluorescent signal for each bead assay. This way, the technology allows rapid and accurate analysis of up to 100 unique assays within a single sample (multiplexing).

The advantages of multiplex cytokine assays, compared to conventional tests like ELISA are their enhanced sensitivity and greater dynamic quantification ranges (Kellar and Douglass, 2003; Morgan et al., 2004; Prabhakar et al., 2004). The protein array platform allows the detection of multiple analytes in a single sample simultaneously. Thus, less sample volumes are required and the procedure reduces time and costs of the analysis (Kellar and Douglass, 2003; Morgan et al., 2004). Despite these advantages, multiplex assays are yet rarely used for clinical diagnostic purposes or basic immunological research, such as cytokine and chemokine analysis in domestic species. A major limitation for the development and use of multiplex technology in these species has been the lack of specific antibody pairs to cytokines and chemokines. Only a very few commercial products are currently available for the detection of selected cytokines in cattle or dogs. In addition, the first microsphere-based multiplex assays were recently described for the detection of three cytokines in swine and cattle (Johannisson et al., 2006; Dernfalk et al., 2007).

To our knowledge, no multiplex assay has yet been developed for cytokine detection in horses. Here, we describe the development of the first Luminex assay for simultaneous analysis of equine IL-4, IL-10 and IFN-α in biological samples.

2. Materials and methods

2.1. Coupling of monoclonal anti-equine cytokine antibodies to fluorescent beads

The following mAbs were coupled to fluorescent beads (Luminex Corp., http://www.luminexcorp.com): anti-equine IL-4 (Wagner et al., 2006) was coupled to bead 33; anti-equine IL-10 clone 492-2 (Wagner et al., 2008a) was coupled to bead 34; and anti-IFN-α clone 29B (Wagner et al., 2008b) was coupled to bead 35. All mAbs were murine IgG1 isotypes. The coupling was performed according to the recommended protocol from the bead supplier (http://www.luminexcorp.com/uploads/data/Protein%20Protocols%20FAQs/Protein%20Coupling%20Protocol%200407%2010207.pdf).

In brief, the entire procedure was performed at room temperature. All centrifugations were performed at 14,000 × *g* for 4 min. Afterwards, the beads were resuspended by vortexing and sonication for 20 s. For activation, 5×10^6 beads were washed once in H_2O. Beads were resuspended in 80 μl of 100 mM sodium phosphate buffer, pH 6.2. Then, 10 μl Sulfo-NHS (50 mg/ml,) and 10 μl 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC, 50 mg/ml, both from Pierce Biotechnology Inc., Rockford, IL) were added and incubated for 20 min. The beads were washed twice with 50 mM 2-[N-morpholino]ethanesulfonic acid pH 5.0 (MES) and resuspended in MES solution. These activated beads were used for antibody coupling using between 40 and 100 μg of mAb. The coupling of the mAbs was performed for 3 h with rotation. After coupling, the beads were resuspended in blocking buffer (PBS with 1% (w/v) BSA and 0.05% (w/v) sodium azide) and incubated for 30 min. The beads were washed three time in PBS with 0.1% (w/v) BSA, 0.02% (v/v) Tween 20 and 0.05% (w/v) sodium azide (PBS-T), counted and stored in the dark at 2–8 °C.

2.2. Recombinant equine IL-4, IL-10 and IFN-α standards

Supernatants of recombinant equine cytokines/IgG fusion proteins were used as standards in the multiplex assay. The IL-4/IgG1, IL-10/IgG4, and IFN-α/IgG4 are composed of equine cytokines and IgG constant heavy chain regions. The supernatants have been previously shown to contain the biological active cytokines (Wagner et al., 2005, 2008a,b). The concentrations of the cytokine/IgG fusion proteins were determined by ELISAs using purified *r*IL-4, *r*IL-10 and *r*IFN-α proteins for quantification as previously described (Wagner et al., 2006, 2008a,b).

2.3. Luminex assay

The set-up of the multiplex assay developed here is shown in Fig. 1. Beads coupled with anti-IL-4, anti-IL-10 and anti-IFN-α mAbs were sonicated, mixed and diluted in blocking buffer to a final concentration of 1×10^5 beads/ml each. For the assay, 5×10^3 beads/each were used per microtiter well. The cytokine standard containing all three cytokines was prepared in blocking buffer using threefold dilutions of the IL-4/IgG1, IL-10/IgG4 and IFN-α/IgG4 fusion protein supernatants. Millipore Multiscreen HTS plates (Millipore, Danvers, MA) were soaked with PBS-T using a EL × 50 plate washer (Biotek Instruments Inc., Winooski, VT) for 2 min. The solution was aspirated from the plates and 50 μl of each diluted standard concentration or the samples were applied to the plates. Then, 50 μl of bead solution was added to each well and incubated for 30 min on a shaker at room temperature. Then, the plate was washed with PBS-T, 50 μl of the primary detection antibody mixture diluted in blocking buffer was added to each well and incubated for 30 min as above. The primary detection antibody mixture was composed of two biotinylated mAbs to IL-10 (anti-equine IL-10 clone 165-2; Wagner et al., 2008a) and IFN-α (anti-equine IFN-αclone 240–2; Wagner et al., 2008b), and a polyclonal goat anti-equine IL-4 antibody (R&D Systems Inc., Minneapolis, MN).

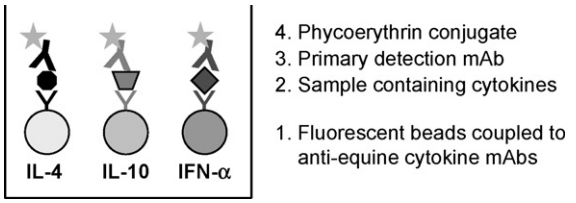

Fig. 1. Components and individual steps of the multiplex cytokine assay to detect equine IL-4, IL-10 and IFN-α. The reaction shown in the figure is performed in a single microtiter plate well. (1) Individual color-coded fluorescent bead sets have been coupled with mAbs to IL-4, IL-10 or IFN-α. Anti-cytokine mAb coupled beads are mixed and applied to microtiter plates. (2) In this step, the sample is added. Additional plate wells are used for a serial dilution of the standard cytokines. (3) The cytokines bound to each bead are detected by biotinylated mAbs specific for IL-4, IL-10 or IFN-α. (4) During the last step, the plate is incubated with streptavidin–phycoerythin. Alternatively, a secondary, phycoerythrin-conjugated antibody can be used if the detection antibody is not biotinylated. Additional details of the coupling procedure, the reagents and the assay steps are given in Section 2.

After washing, 50 μl of a mixture containing streptavidin–phycoerythrin (Invitrogen, Carlsbad, CA) and a phycoerythrin-conjugated donkey anti-goat antibody (Jackson Immunoresearch Laboratories, West Grove, PA) was added. Plates were incubated for 30 min as above and washed. The beads were resuspended in 100 μl of blocking buffer and the plate was placed on the shaker for 15 min. The assay was analyzed in a Luminex IS 100 instrument (Luminex Corp., http://www.luminexcorp.com). The data were reported as median fluorescent intensities. For standard curve fitting and subsequent calculation of the cytokine concentrations in samples the logistic 5*p* formula ($y = a + b/(1 + (x/c)\hat{}d)\hat{}f$) was used (Luminex 100 Integrated System 2.3).

2.4. Cell culture supernatants from stimulated or infected PBMC

Heparinized blood samples from four horses were collected for PBMC isolation. The procedure was approved by the Cornell University Institutional Animal Care and Use Committee. Equine PBMC were isolated by density gradient centrifugation and stimulated with mitogens (PWM, ConA, and PHA) or LPS for 4 days as previously described (Wagner et al., 2006, 2008a). For IFN-α secretion, PBMCs were infected for 24 h with different dilutions of purified equine herpesvirus type 1 (EHV-1) virions obtained from the EHV-1 wild-type strain RacL11 (300 μg/ml; kindly provided by Dr. Klaus Osterrieder). The virion purification was performed as previously described (Von Einem et al., 2004). Untreated or uninfected cultures of PBMC were used as controls. The cell culture supernatants from all PBMC cultures were tested in the multiplex assay.

2.5. Analytical sensitivity and specificity of the assay

The analytical sensitivity was determined for each cytokine and corresponded to the lowest detectable cytokine concentration in the linear range of the individual cytokine standard curve in the multiplex assay. The specificity of the multiplex assay was determined by two approaches. First, assays containing each individual cytokine/IgG fusion protein (instead of a mixed standard with all three of them) were performed on the otherwise complete three-bead multiplex assay. This approach tested for potential cross-reactivity of each recombinant cytokines with the remaining two cytokine assays. Second, cell culture supernatants from PBMC were used as samples in the multiplex assay. The supernatants were expected to contain native IL-4 and IL-10 (mitogen stimulated PBMC; Wagner et al., 2006, 2008a), IL-10 (LPS stimulated PBMC; Wagner et al., 2008a) or IFN-α (virus infected PBMC; Wagner et al., 2008b). These samples were tested for potential interference of the native cytokines or other components in the cell culture supernatants with the performance of the assay.

3. Results

3.1. Equine IL-4/IgG1, IL-10/IgG4 and IFN-α/IgG4 fusion proteins as standards for cytokine detection

To investigate the performance of cytokine/IgG fusion proteins as standards for cytokine quantification, they were compared to purified equine *r*IL-4, *r*IL-10 and *r*IFN-α of known concentrations by ELISA (Fig. 2). Both, the purified recombinant cytokine and the respective IgG fusion protein resulted in parallel curves in the linear quantification ranges for equine IL-4, IL-10 and IFN-α. This suggested that the purified recombinant cytokines and the cytokine/IgG fusion proteins performed identical in the assays. The cytokine concentration in the fusion protein preparations were determined after repeated measurements by ELISA. The comparison of the purified recombinant cytokines and the cytokine/IgG fusion proteins was repeated in the multiplex assay. Although fewer comparisons were used on the bead assay, they resulted again in parallel curves and in identical performance of the purified recombinant cytokines and the cytokine/IgG fusion proteins.

3.2. Quantification and analytical sensitivities of equine IL-4, IL-10 and IFN-α detection by multiplex cytokine analysis.

The analytical sensitivities and the linear quantification ranges for the detection of equine IL-4, IL-10 and IFN-α were determined in the multiplex assay using serial dilutions of the IL-4/IgG1, IL-10/IgG4 and IFN-α/IgG4 fusion proteins for quantification (Fig. 3). Compared to the corresponding ELISA, the multiplex assay increased the analytical sensitivity of the cytokine detection between 13- and 150-fold (Table 1). In addition, the linear quantification ranges of the standard curves were wider for the multiplex assay than for the corresponding ELISA. The multiplex assay allowed the quantification of IL-4 in a sample in the range between 40 and 80 ng/ml. The linear quantification ranges for IL-10 and IFN-α were found to be 15 pg/ml to around 30 ng/ml. Thus, cytokine concentrations approximately 2000-fold higher than the analytical sensitivity could still be detected in the same sample dilution with the multiplex assay. In contrast, the respective ELISAs detected concentrations of around

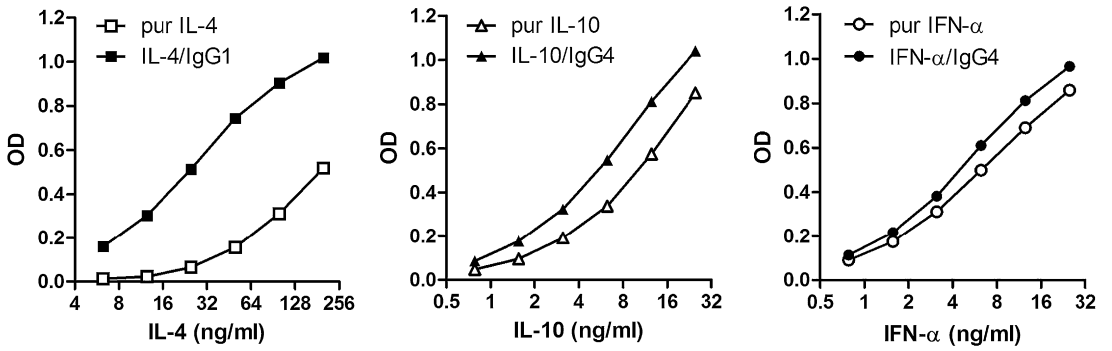

Fig. 2. Detection of purified recombinant equine IL-4, IL-10 and IFN-α and the corresponding cytokine/IgG fusion proteins by ELISA. The concentrations (ng/ml) on the *x*-axes correspond to the purified cytokines. The cytokine/IgG fusion proteins were of unknown concentration and were diluted within the linear ranges of the curves to determine their cytokine concentrations. The graphs show means of 5 (IFN-α) or 12 (IL-4 and IL-10) repeated ELISAs. OD = optical density.

35- to 125-fold higher than the analytical sensitivity of the assays.

3.3. Specificity of cytokines detection in the multiplex assay

The specificity of the multiplex assay was tested by using the individual standards for IL-4, IL-10 or IFN-α on the multiplex array. The three cytokines were only detected by their corresponding bead-array resulting in standard curves identical to those in Fig. 3. No cross-reactivity was detected with the remaining two bead-arrays (Table 2). The data also indicated that the IgG

Table 1
Comparison of analytical sensitivities and linear quantification ranges for IL-4, IL-10 and IFN-α detection by ELISA and by the Luminex multiplex assay.

	Analytical sensitivity (pg/ml)			Linear quantification range (pg/ml)	
	ELISA[a]	Multiplex assay	Increase	ELISA[a]	Multiplex assay
IL-4	6000	40	150-fold	6,000–200,000	40–81,000
IL-10	200	15	13-fold	200–25,000	15–33,000
IFN-α	700	15	47-fold	700–25,000	15–28,000

[a] The analytical sensitivities and linear quantification ranges for detecting IL-4 (Wagner et al., 2006), IL-10 (Wagner et al., 2008a) and IFN-α (Wagner et al., 2008b) by ELISA were determined previously.

Table 2
Cross-reactivity of cytokine/IgG fusion proteins and detection antibodies in the bead-based multiplex assay for equine cytokines.

	Bead-specificity[a]		
	Anti-IL-4	Anti-IL-10	Anti-IFN-α
Cytokine standard added[b]			
IL-4/IgG1	5498/11	2/3	4/2
IL-10/IgG4	28/46	10225/85	20/30
IFN-α/IgG4	19/36	57/55	10726/86
Background[c]	−1.4	−115	−142

[a] The median fluorescence intensity (MFI) values are given for each cytokine standard and bead set.
[b] MFI-values for the highest/and the lowest concentration of each cytokine standard are shown in the table (IL-4/IgG1 = 81 ng/ml and 40 pg/ml; IL-10/IgG4 = 33 ng/ml and 15 pg/ml; IFN-α/IgG4 = 28 ng/ml and 15 pg/ml).
[c] The background values are mean MFIs of 32 measurements.

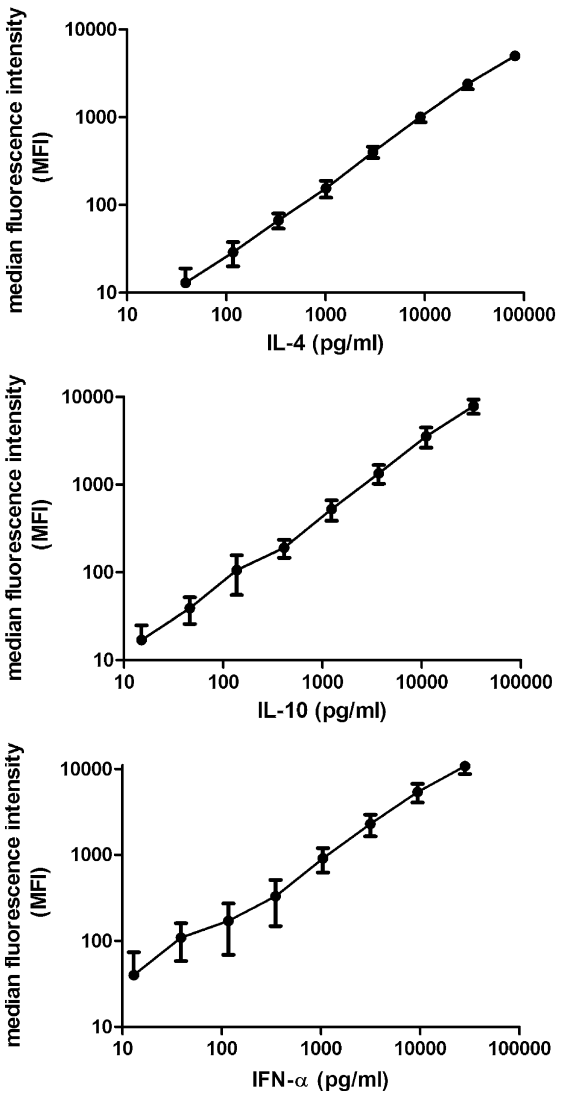

Fig. 3. Standard curves of equine IL-4, IL-10 and IFN-α obtained by the multiplex assay. Serial dilution of cytokine/IgG fusion proteins were used as standard proteins. Each standard curve shows means and standard deviations obtained from 14 assays.

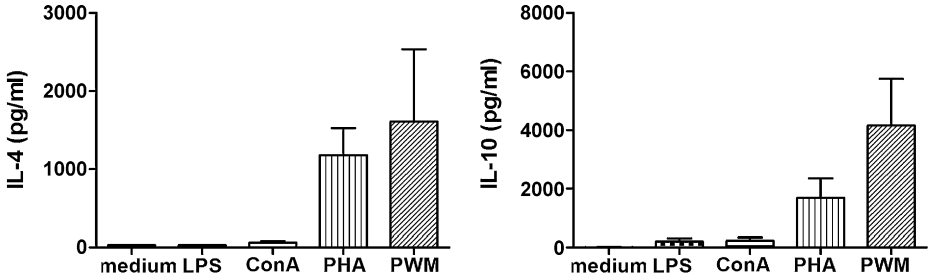

Fig. 4. IL-4 and IL-10 in supernatants of mitogen or LPS-stimulated PBMC detected by the multiplex assay. IFN-α was not detectable in these supernatants. PBMC were stimulated for 4 days. The bars represent means and standard errors from four horses with eight repeated multiplex measurements for each supernatant. The relatively high standard errors observed for IL-4 and IL-10 production after stimulation with PHA and PWM were a result of different responses of PBMC from individual horses to these mitogens. The repeated measurements of the supernatants resulted in similar values for each sample.

portion of the cytokine/IgG fusion proteins did not interfere with the multiplex assay format. If this would have been the case, additional reactions would have been observed by using the individual cytokine standard.

For further validation of the specificity of the multiplex assay in biological samples, cell culture supernatants from mitogen or LPS stimulated equine PBMC were used. Mitogen stimulation of equine PBMC has been shown previously to induce the secretion of high concentrations of IL-4 and IL-10. LPS was found to induce low concentrations of IL-10 in previous experiments (Wagner et al., 2006, 2008a). Mitogens were not expected to induce the secretion of IFN-α. Here, the results obtained by multiplex analysis were similar to previous observations. PHA and PWM induced high concentrations of IL-4 and IL-10 (Fig. 4). ConA stimulation induced detectable IL-4 and IL-10 concentrations and low IL-10 concentrations were also found after LPS stimulation. IFN-α was not detectable in any of the supernatants.

In addition, PBMC were infected with EHV-1. Highest concentrations of IFN-α were detected in the supernatants from infected cells at the highest virus concentration. The IFN-α production decreased dose-dependent with reduction of the viral dose (Fig. 5). Low concentrations of IL-10 were also detectable in the supernatants. The kinetics of IL-10 secretion differed from that for IFN-α after EHV-1 infection. The IL-10 peak was detected at an intermediate viral dose, whereas lower IL-10 concentrations were found with higher or lower EHV-1 doses. IL-4 was not detectable in supernatants from EHV-1 infected PBMC.

The combined results for stimulated and virally infected PBMC demonstrated that the individual bead assays for IL-4, IL-10 and IFN-α specifically detected the respective cytokines in biological the samples.

4. Discussion

Multiplex assays for cytokine and chemokines are available for basic immunology research applications in humans and rodents. They combine a sensitive technology with the advantages of protein arrays that analyze multiple analytes in a single sample and at the same time (Kellar and Douglass, 2003). Despite these advantages cytokine multiplex assays are yet rarely used in veterinary species. Only a few studies describe the simultaneous detection of three cytokines each in swine and cattle (Johannisson et al., 2006; Dernfalk et al., 2007). A major drawback for veterinary species was the lack of specific anti-cytokine antibody pairs that are suitable for detecting soluble cytokines and chemokines. However, several reagents have been generated for veterinary species during the past few years and many initiatives to develop more reagents are still ongoing. These tools are now becoming available for the development of assays using the advantages of multiplex technology. The new tools and technologies are likely to improve the analysis of cellular

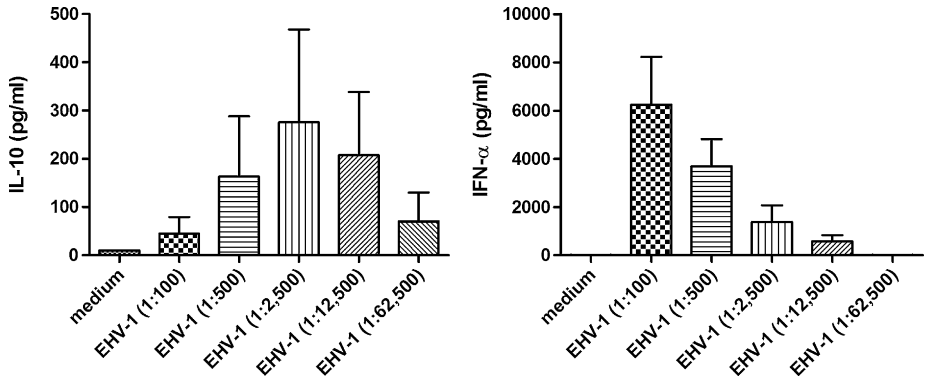

Fig. 5. IFN-α and IL-10 concentrations in supernatants of PBMC infected with EHV-1 were determined in the multiplex assay. Supernatants were obtained 24 h after infection. IL-4 was not detectable in these samples. The bars represent means and standard errors from six horses.

immune responses in veterinary species tremendously within the next few years.

In the horse, monoclonal antibodies to equine IL-4, IL-10 and IFN-γ were previously developed and tested in ELISA (Wagner et al., 2006, 2008a,b). Previous studies in other species showed high correlations between cytokine detection by ELISAs and by bead-based multiplex assays (Kellar et al., 2001; Kellar and Douglass, 2003; DuPont et al., 2005). Here, we have used these reagents to establish and validate the first multiplex assay for equine cytokines. A comparison of the ELISA with the multiplex assay using the same antibodies showed that the multiplex assay improved the analytical sensitivity for the three cytokines between 13- and 150-fold. A similar increase in sensitivity was previously reported for multiplex assays simultaneously detecting rodent and human cytokines and respective ELISAs. The multiplex assays for human cytokine detection was found to detect cytokines in the range of a few pg/ml to 10 ng/ml (Kellar and Douglass, 2003; Prabhakar et al., 2004), which is similar to the assay sensitivities and linear quantification ranges described here for equine cytokines. However, multiplex assays developed for three proinflammatory cytokines in swine or cattle were found to have analytical sensitivities ranging from 2000 to 6500 pg/ml for bovine (Dernfalk et al., 2007) and 180–1600 pg/ml for porcine cytokines (Johannisson et al., 2006). One factor that might have influenced the differences in the analytical sensitivity of cytokine detection could be the antibodies that were used for the design of the assays. For the bovine assay polyclonal antibody were coupled to the fluorescent beads and the porcine assay used different polyclonal antibodies for cytokine detection. In contrast, the human multiplex assays were based on pairs of mAbs (Kellar and Douglass, 2003; Prabhakar et al., 2004; DuPont et al., 2005). Our assay for equine cytokines used pairs of mAbs for IL-10 and IFN-α detection, both of which resulted in an analytical sensitivity of 15 ng/ml in the multiplex format. In the absence of a second mAb to equine IL-4, we used a polyclonal antibody for IL-4 detection in the multiplex assay together with an anti-equine IL-4 mAb coupled to the beads resulting in an analytical sensitivity of 40 ng/ml for IL-4 in the multiplex assay. In our experience, the use of a polyclonal anti-IL-4 antibody in the multiplex assay increased the background reactivity and thereby negatively influenced the analytical sensitivity of IL-10 and IFN-α detection compared to the corresponding singleplex assays for these cytokines (data not shown). It should also be noted that the multiplexing of cytokine assays has been found to reduce the sensitivity of detection compared to singleplex assays by 7- to 32.5-fold if various polyclonal reagents were used (Dernfalk et al., 2007). This suggests that pairs of mAbs are the preferred tools for multiplex assay development and detection of cytokines in ranges that are expected to be physiologically relevant.

In our approach, testing of individual cytokine standards, supernatants from equine PBMC stimulated with mitogens or PBMC infected with EHV-1 indicated that the bead-based assays for the individual cytokines did not interfere with each other in the multiplex approach. The same observation has been reported previously for multiplex cytokine assays of other species (Kellar and Douglass, 2003; Johannisson et al., 2006; Dernfalk et al., 2007). The higher specificity and sensitivity together with the enlarged linear quantification range of the multiplex assay compared to the ELISA suggested that this technology could become a valuable approach for the detection of cytokines in horses and other veterinary species in the near future. Similar to already existing multiplex assays for humans and rodents, the existing equine assay could be expanded to detect up to 20 or 30 cytokines and chemokines.

Commercial assays for rodent or human multiplex cytokine detection are usually performed with purified recombinant cytokines as standards for quantification. Here, we tried a different approach and tested equine IL-4/IgG1, IL-10/IgG4 and IFN-α/IgG4 fusion proteins for quantification in the multiplex assay. This had several advantages compared to the use of purified recombinant cytokines. Most important was the stability of the cytokine/IgG fusion proteins. As indicated by commercial vendors, most purified recombinant cytokines have a short half-life and are only stable for a few months. In contrast, the equine IL-4, IL-10 and IFN-α/IgG fusion proteins did not show any notable loss of detectable cytokine concentration for at least 2 years after storage at −20 °C and for at least 6 months after storage at 4 °C. In addition, cytokine/IgG fusion proteins are relatively easy to produce and inexpensive compared to purified recombinant cytokines. The multiplex assay has a much wider linear quantification range than an ELISA. To cover the entire linear range of the standards, the multiplex assay often uses higher amounts of the standard proteins than the ELISA. This can be costly for purified recombinant proteins. Thus, cytokine/IgG fusion proteins provide a stable and affordable alternative to purified recombinant cytokines for protein detection assays.

5. Conclusion

Multiplex cytokine assays are a valuable tool to analyze soluble indicators of the cellular immune response in veterinary species. Applications for this technology range from the analysis of the host immune response for research, diagnostic and clinical questions to a wide variety of application to test efficacy of vaccines targeting cellular immunity. Currently, reagent availability is still inhibiting the rapid progress in developing multiplex cytokine and chemokines assays for all veterinary species. However, efforts of international reagent initiatives, such as the BBSRC Immunological Toolbox (http://www.immunologicaltoolbox.co.uk), the Cytokine Center at the University of Ütrecht (http://www.cytocen.com) and the US Veterinary Immune Reagent Network (http://www.vetimm.com) in generating a variety of recombinant cytokines and mAbs to cytokines for various veterinary species are ongoing. These reagents are likely to become available within the next few years, and will provide valuable tools to improve multiplex assay development, immunological research and vaccine development to existing and emerging diseases in veterinary species.

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References

Carson, R.T., Vignali, D.A., 1999. Simultaneous quantification of 15 cytokines using a multiplexed flow cytometric assay. J. Immunol. Methods 227, 41–52.

Dernfalk, J., Waller, K.P., Johannisson, A., 2007. The xMAP™ technique can be used for detection of the inflammatory cytokines IL-1β, IL-6 and TNF-α in bovine samples. Vet. Immunol. Immunopathol. 118, 40–49.

DuPont, N., Wang, K., Wadhwa, P., Culhane, J., Nelson, E., 2005. Validation and comparison of luminex multiplex cytokine analysis kits with ELISA: determinations of a panel of nine cytokines in clinical sample culture supernatants. J. Reprod. Immunol. 2, 175–191.

Johannisson, A., Jonasson, R., Dernfalk, J., Jensen-Waern, M., 2006. Simultaneous detection of porcine proinflammatory cytokines using multiplex flow cytometry by the xMAP™ technology. Cytometry A 69A, 391–395.

Kellar, K.L., Douglass, J.P., 2003. Multiplexed microsphere-based flow cytometric immunoassays for human cytokines. J. Immunol. Methods 279, 277–285.

Kellar, K.L., Kalwar, R.R., Dubois, K.A., Crouse, D., Chafin, W.D., Kane, B.E., 2001. Multiplexed fluorescent bead-based immunoassays for quantification of human cytokines in serum and culture supernatants. Cytometry 45, 27–36.

Morgan, E., Varro, R., Sepulveda, H., Ember, J.A., Apgar, J., Wilson, J., Lowe, L., Chen, R., Shivraj, L., Agadir, A., Campos, R., Ernst, D., Gaur, A., 2004. Cytometric bead array: a multiplexed assay platform with applications in various areas of biology. Clin. Immunol. 110, 252–266.

Prabhakar, U., Eirikis, E., Reddy, M., Silvestro, E., Spitz, S., Pendley, C., Davis, H.M., Miller, B.E., 2004. Validation and comparative analysis of a multiplexed assay for the simultaneous quantitative measurement of Th1/Th2 cytokines in human serum and human peripheral blood mononuclear cell culture supernatants. J. Immunol. Methods 291, 27–38.

Prabhakar, U., Eirikis, E., Miller, B.E., Davis, H.M., 2005. Multiplexed cytokine sandwich immunoassays: clinical applications. Methods Mol. Med. 114, 223–232.

Vignali, D.A., 2000. Multiplexed particle-based flow cytometric assays. J. Immunol. Methods 243, 243–255.

Von Einem, J., Wellington, J., Whalley, J.M., Osterrieder, K., O'Callaghan, D.J., Osterrieder, N., 2004. The truncated form of glycoprotein gp2 of equine herpesvirus 1 (EHV-1) vaccine strain KyA is not functionally equivalent to full-length gp2 encoded by EHV-1 wild-type strain RacL11. J. Virol. 78, 3003–3013.

Wagner, B., Robeson, J., McCracken, M., Wattrang, E., Antczak, D.F., 2005. Horse cytokine/IgG1 fusion proteins—mammalian expression of biologically active cytokines and a system to verify antibody specificity to equine cytokines. Vet. Immunol. Immunopathol. 105, 1–14.

Wagner, B., Hillegas, J.M., Antczak, D.F., 2006. A monoclonal antibody to equine interleukin 4. Vet. Immunol. Immunopathol. 110, 363–367.

Wagner, B., Hillegas, J.M., Brinker, D.R., Horohov, D.W., Antczak, D.F., 2008a. Characterization of monoclonal antibodies to equine interleukin-10 and detection of T regulatory 1 cells in horses. Vet. Immunol. Immunopathol. 122, 57–64.

Wagner, B., Hillegas, J.M., Flaminio, M.J.B.F., Wattrang, E., 2008b. Monoclonal antibodies to equine interferon-α (IFN-α): new tools to neutralize IFN activity and to detect secreted IFN-α. Vet. Immunol. Immunopathol. 125, 315–325.