Published in final edited form as:

Vet Immunol Immunopathol. 2016 December ; 182: 69–73. doi:10.1016/j.vetimm.2016.10.003.

A Comparison of Serum and Plasma Cytokine Values using a Multiplexed Assay in Cats

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Abstract

Background—Degenerative joint disease (DJD) is highly prevalent in cats, and pain contributes to morbidity. In humans, alterations of cytokine concentrations have been associated with joint deterioration and pain. Similar changes have not been investigated in cats. Cytokine concentrations can be measured using multiplex technology with small samples of serum or plasma, however, serum and plasma are not interchangeable for most bioassays. Correlations for cytokine concentrations between serum and plasma have not been evaluated in cats.

Objective—To evaluate the levels of detection and agreement between serum and plasma samples in cats.

Animals—Paired serum and plasma samples obtained from 38 cats.

Methods—Blood was collected into anti-coagulant free and EDTA Vacutainer® tubes, serum or plasma extracted, and samples frozen at -80°C until testing. Duplicate samples were tested using a 19-plex feline cytokine/chemokine magnetic bead panel.

Results—Agreement between serum and plasma for many analytes was high, however correlation coefficients ranged from -0.01 to 0.97 . Results from $>50\%$ of samples were below the

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lower limit of quantification for both serum and plasma for nine analytes, and for an additional three analytes for plasma only.

Conclusions and clinical importance—While serum and plasma agreement was generally good, detection was improved using serum samples.

Keywords

biomarker; immunoassay; feline

Introduction

Cytokines and chemokines are of great interest as biomarkers, and in veterinary medicine, development and discovery of biomarkers for disease are areas of active research and with a growing literature base. Many studies in human and veterinary medicine have begun to explore and identify cytokines involved in a variety of disease processes, however methodological issues and a lack of standardization have contributed to inconclusive or contradictory findings. The advent of multiplex technologies that can measure a panel of cytokines and chemokines in small volumes of biological samples has increased the ability to screen for potential biomarkers and targets for further research, however concerns have been raised about the ability to compare results from studies using differing assays and media (Elshal and McCoy 2006; de Jager et al. 2009; Burska, Boissinot, and Ponchel 2014; Keustermans et al. 2013).

Cytokines are proteins that are produced by a variety of cells, particularly the T-lymphocytes and macrophages, and are integrally involved in inflammation and cell signaling. The vast majority of cytokines are produced and consumed locally, acting in autocrine, paracrine, and juxtacrine manners. This makes detection of cytokines in systemic circulation challenging, and findings from the serum or plasma might not be reflective of local tissue activity. Despite this, obtaining whole blood is more convenient than most other sampling modalities, particularly in clinical cases, and offers a potential for screening large populations. In fact, concentrations of several cytokines have been found to be altered in the serum and plasma of patients during disease, including IL-6 in humans with rheumatoid arthritis (Burska, Boissinot, and Ponchel 2014) and dogs with immune-mediated polyarthritis (Foster et al. 2014), and stromal cell-derived factor-1 in horses with osteochondral injury (Dymock et al. 2014). These reports pertain to serum or plasma samples, but the two sample types are not necessarily interchangeable.

Several recent studies have investigated the relative difference in multiplex measurements of cytokines between serum and plasma from humans, and have demonstrated a generally high correlation, but have identified problems including matrix impedance and non-specific background binding that is not completely eradicated by the use of manufacturer-provided buffer solutions (Rosenberg-Hasson et al. 2014). In addition, the anti-coagulant used to obtain plasma can affect the concentrations of certain cytokines (Thavasu et al. 1992). Processing and sample handling post-blood draw can further complicate findings as time to separation of serum or plasma, storage, and number of freeze-thaw cycles have all been shown to affect cytokine concentrations (Keustermans et al. 2013; Flower et al. 2000;

Thavasud et al. 1992). Even when these methodological issues are controlled by common treatment of samples, the optimal medium could be cytokine specific (de Jager et al. 2009). When using commercially available multiplex kits with a panel of cytokines, selection of serum or plasma might depend on the relative benefits of one over the other, including number of detectable samples, and the ability to observe a difference in a group with a known disease as compared to a control group. While it has been noted that serum and plasma are not interchangeable for multiplex assays in humans (Chaturvedi et al. 2011), to our knowledge the comparison of multiplex-measured cytokine concentrations in serum versus plasma have not been assessed in cats. Therefore, the objective of this study was to compare the concentrations of 19 cytokines in matched serum and plasma samples from cats using a commercially available multiplex magnetic bead assay and analysis software in order to guide the selection of medium (serum or plasma) for future work.

Materials and Methods

Subjects

Matched serum and plasma samples were used from 38 cats that had been screened for one of three clinical studies at the North Carolina State University College of Veterinary Medicine (NCSU-CVM). All cats were examined under approval from the Institutional Animal Care and Use Committee at NCSU.

Serum and plasma were collected from each cat during a single visit to the NCSU-CVM. For serum samples, whole blood was collected into a 3mL anti-coagulant free plastic tube (red top) and allowed to clot at room temperature for at least 30 but no more than 60 minutes. Clotted samples were centrifuged at $1163 \times g$ for 10 minutes, and serum was removed, aliquoted, and stored in cryovials at -80°C until use. For plasma samples, whole blood was collected into a 2.7 mL plastic tube (lavender top) containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. The volume of blood added was according to manufacturer recommendation to achieve a concentration of EDTA of approximately 1.8 mg/mL of blood. Within 30–60 minutes of collection, samples were centrifuged at $1163 \times g$ for 10 minutes and plasma was similarly processed and stored. All samples had a maximum of one freeze-thaw cycle prior to use; while multiple freeze-thaw cycles can affect cytokine concentrations and should be avoided, one freeze-thaw has been shown to have minimal effects on measured cytokine concentrations in previous studies (Flower et al. 2000; Thavasud et al. 1992).

Feline-specific cytokine kits (19-plex)¹ were purchased and used according to the manufacturer's recommendations. Quality control samples were run on each plate, in duplicate, and sets of serum and plasma (in duplicate) were run on the same plate (i.e. no pairs of serum or plasma for an individual cat were run on different plates). Plates were analyzed using a dedicated plate reader and software^{2,3}.

¹FCYTMAG-20K-PMX Feline Cytokine/Chemokine Magnetic Bead Panel Premixed 19-Plex; EMD Millipore, Billerica, MA USA

²MAGPIX; Luminex Corporation, Austin TX USA

³xPONENT v.4.2; Luminex Corporation, Austin TX, USA

Results were analyzed using dedicated software^{3,4}; bead count, standard curves, quality control samples, and coefficients of variation (CVs) for each analyte were assessed. Quality controls were compared with the range provided by the kit. As per manufacturer's recommendation and industry-accepted standards for multiplex assays, CVs less than or equal to 20% were considered acceptable. In cases where CVs were >20%, no value for that sample was recorded, and the number of samples for that analyte was therefore reduced. Reported concentrations for each analyte and each sample (cat) represent the mean of the duplicate samples.

For each sample type (serum and plasma), detection was reported as the number of results that were in range, those below or above the limit of quantification (LOQ), and the number of analytes that had >25% and >50% of the results below the lower LOQ (LLOQ). Correlations between the serum and plasma results for each analyte were generated using Pearson correlation coefficients for any analyte with 9 serum/plasma pairs in range (25% of total pairs). To evaluate overall differences, a repeated measures model (ANOVA) was used with fixed effects for analyte, sample type (serum or plasma) and plate (1 or 2), and a random subject effect. To evaluate the effect of sample age, a repeated-measures model was used with random effects for subject and plate, fixed effects of the analyte and age of sample (in days), and a response variable of the ratio of serum:plasma. To evaluate the consistency of results across cats, the ratio of serum:plasma results for each cat and each analyte was tabulated. Where appropriate, a p-value of 0.05 was considered significant, and statistical analysis performed using a standard statistical software program⁵.

Results and Discussion

Subjects

Matched serum and plasma were available for 38 cats that had been screened at the NCSU-CVM. Cats were screened for participation in studies of normal cats as well as treatment trials for degenerative joint disease. All samples were obtained during the period from 2007–2014, and were taken prior to any treatments being given. Cats were between 1 and 19 years of age (mean 11.5 years) and 2.08–8.46 kg (mean 4.9 kg).

Serum and plasma comparisons

All quality control results were within range with acceptable CVs. Results were removed for low bead counts for 72 individual results (5.0%) across all the samples and cytokines measured, however these were generally clustered within a sample, so these 72 wells represented results from five cats. Overall, CVs and bead counts were generally within acceptable range for all of the analytes measured, although 6.8% of the plasma samples and 6.1% of the serum samples were removed from the analysis due to either low bead counts or high CVs. The majority of CVs were below 15%.

The number of results that were in range (above the LLOQ) for each analyte and each matrix are shown in Tables 1 and 2. No results were above the upper LOQ. As shown in Table 1 and

⁴MILLIPLEX ANALYST v.5.1, EMD Millipore, Billerica, MA USA

⁵SAS v.9.4; Cary, NC USA

described further in Table 2, many results were below the LLOQ for either serum, plasma, or both. In general, there were more results above the LLOQ for serum than plasma, but this was not true for all analytes, with 6/19 analytes having results above the LLOQ for plasma but not serum. Correlations between serum and plasma were calculated for 11 analytes with 9 or more pairs of results, and are shown in Table 3. Correlation coefficients were generally positive; six of 11 analytes (55%) had correlation coefficients >0.9 , however correlation coefficients were below 0.9 for five of the 11 analytes (45%). The ratio of serum:plasma results for each analyte and each cat are shown in Figure 1. The ratios show the tendency for serum to be higher than plasma, but this varied by analyte and by cat within an analyte.

Results of the repeated measures model evaluating sample type showed no significant difference between plasma and serum ($p=0.620$). There was no significant sample type (plasma or serum) by analyte interaction; serum values were generally higher than plasma, regardless of the analyte being considered (Figure 1). There was also no significant effect of sample age on the ratio of serum:plasma ($p=0.510$).

The objective of this pilot study was to compare serum and plasma cytokine concentrations in matched samples from cats using a feline specific multiplex assay. Results indicated that the correlation between serum and plasma was generally positive, and was high for over half of the analytes with a reasonable number of pairs for comparison (set at 25% of total sample pairs). However, correlations for 45% of the analytes considered were below 0.9, and ranged from -0.01 (negligible) to 0.89. Both detection and overall values were higher in the serum samples than in the plasma samples, though the effect was not significant overall or for analyte by sample interaction. In the serum samples, results were undetectable (below the lower limit of quantification) in 75% of the samples for five analytes, while this was true for eight analytes in the plasma samples. Discussion with the manufacturer of the plates revealed that median values obtained during our study were comparable to those obtained during development of the assay (for serum; Terry Whitehead [application scientist], personal communication), and is similar to findings in humans in the absence of disease (de Jager et al. 2009; Chaturvedi et al. 2011). Higher detection and higher concentrations of cytokines in serum over plasma have also been shown in other studies in humans (de Jager et al. 2009; Rosenberg-Hasson et al. 2014; Tvedt et al. 2015), though occasionally the opposite has been reported (Wong et al. 2008). Plasma is the fluid fraction of the blood and contains clotting factors and fibrinogen; these components are absent from serum, as they are consumed during clot formation. In the process of clot formation, however, leukocytes and platelets can be activated, leading to cytokine release, and potentially falsely elevating concentrations of some cytokines in serum over plasma. This has been shown for certain cytokines, including IL-1 and IL-8, but can be minimized by standardized and rapid processing of samples following clot formation (Skogstrand et al. 2008). Prompt processing is critical for accurate measurement in plasma samples, too, as important effects of time to processing and storage have been demonstrated with both increased and decreased concentrations of several cytokines as samples age (Jackman et al. 2011).

The higher levels of detection in serum over plasma, and relative greater ease in obtaining serum suggest that serum would be the most suitable medium for future, large scale screening studies in cats. However, a recent study by Rosenberg-Hasson et al. (Rosenberg-

Hasson et al. 2014) found that while detection was improved in serum over plasma, the sensitivity of plasma to changes in measured cytokine concentrations in multiple myeloma patients compared to non-affected patients was superior to serum. They suggest that greater background non-specific binding in serum samples masks small changes in cytokine concentrations that are better detected using plasma, and that 'higher' is not necessarily better. In contrast, Dymock et al. (Dymock et al. 2014) concluded that stromal cell-derived factor 1 measured in serum was more sensitive than plasma or synovial fluid in distinguishing horses with osteochondral injury from uninjured horses. In the current study, the objective was to compare the values obtained from serum and plasma in cats, however future work should evaluate the relative sensitivity of serum and plasma to detect changes in cytokine concentrations in disease in a larger sample of cats.

In this study, evaluation of the ratio of serum to plasma for individual cats and analytes showed that the relationship between the values for serum and plasma were not consistent across analytes or across individual cats, suggesting that a simple correction factor would not be appropriate for comparing serum and plasma values for cytokines. For example, for RANTES, the one analyte where the ratios were in the same direction for all cats (serum:plasma ratio greater than 1), the ratios ranged from close to one to over six.

It is clear that sample handling and storage can affect measured concentrations of cytokines, and this has been rigorously evaluated in studies using spiked samples and various blood collection tubes and anticoagulants, and differing freeze-thaw cycles (see Zhou (Zhou et al. 2010) and Keustermans (Keustermans et al. 2013) for reviews). Other handling concerns include timing of blood draw, as several cytokines show circadian patterns with predictable peaks (Petrovsky, McNair, and Harrison 1998; Altara et al. 2015). This is generally related to the concentration of plasma cortisol, which follows a circadian pattern in dogs (Palazzolo and Quadri 1987) and humans (Petrovsky, McNair, and Harrison 1998). While this is a potential concern, in cats it does not appear that cortisol follows the same circadian pattern, showing instead an episodic release, making effects on measured cytokine concentrations difficult to predict (Leyva, Addiego, and Stabenfeldt 1984; Kemppainen and Peterson 1996). For this study, samples were generally obtained in the morning, and the serum and plasma obtained during the same blood draw. However, effects of the timing of sampling should be evaluated in future work with cats. Other factors shown to affect measured cytokine concentrations, including feeding (Payette et al. 2009), were not controlled for in this study, but are unlikely to differentially affect serum versus plasma. A further limitation of the current study is the different ages of the samples used. Storage over time, even at -80°C has been shown to decrease measured concentrations of cytokines (de Jager et al. 2009), but it is unknown if the level of degradation is different for serum versus plasma. In this study, the differences between serum and plasma results were not different for samples of varying ages, but it is not known what the concentrations were at the time the samples were taken.

In summary, this study found that while results from matched serum and plasma samples in cats were positively correlated, both detection (readings above the LLOQ) and measured values were higher in the serum samples. The ratio of serum:plasma results was not consistent across analytes or cats, thus a correction factor could not be applied to make serum and plasma samples comparable, which supports the recommendation to select one or

the other medium when running multiplex assays. While these findings are not necessarily unexpected, they do represent the first report of a comparison of serum and plasma concentrations of cytokines in samples from cats. Future work should evaluate the effects of storage on relative values for serum and plasma compared to values obtained when running fresh samples, as well as evaluate whether relative changes in cytokine concentrations are more sensitively detected in serum or plasma. This study focused on samples obtained through clinical practice, but future work to verify these findings using pooled samples of serum and plasma, and known (spiked) concentrations of cytokines.

Acknowledgments

The authors thank Alex Taylor for technical support with cytokine assays. Margaret E. Gruen received funding support from the National Institutes of Health Ruth L. Kirschstein National Research Service Award [T32OD01130].

Abbreviations

CV	Coefficient of variation
DJD	Degenerative Joint Disease
EDTA	Ethylene-diamine-tetra-acetic acid
LOQ	Limit of Quantification
NCSU-CVM	North Carolina State University College of Veterinary Medicine

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	FAS	FLT3	GM-CSF	IFN- γ	IL-1 β	IL-2	PDGF-BB	IL-12	IL-13	IL-4	IL-6	IL-8	CXCL-1	SDF-1	RANTES	SCF	MCP-1	TNF- α	IL-18
Aramis		1.00						0.93							1.08				0.82
Biscuit		0.92		0.94				0.89	1.11					30.45	1.32	1.56	0.91		1.19
Bruce	0.82	0.88	0.70			0.83		0.85	0.87	0.70					0.84	2.59	0.79	0.74	0.84
Brulser		1.40		1.84	1.47	1.24		1.47	1.18	1.43	1.53	1.41	14.73	1.27	1.52	1.47	1.32	1.58	1.44
Bunker		1.25		1.84				1.24		1.30					0.73	2.04	1.33	0.02	0.99
Caol Ila		1.68			2.29			1.96	2.04										0.77
Chester		1.05			1.23			1.08		1.50	1.06				1.45			1.71	0.94
Chloe		1.16						1.04	1.02	1.13			1.19	92.28	1.64	1.13	0.01		1.05
Cookie		1.50		1.19		1.00		1.31							1.39	1.70			0.83
Cosmo		1.20		1.52	1.37			1.26	1.12	1.03	1.24	1.65		1.20	2.22	1.27	1.19	1.48	
Diablo		1.43						1.63	1.32	1.83		1.93		1.28	1.68		1.19		1.06
Ellie	1.03	1.06				1.04		0.97	1.14	1.14	1.00	1.02		1.05	1.24	1.06	1.00	1.11	0.92
Elvis		0.58						0.51			1.01				1.01		0.82		0.95
Jasper		1.15						1.19							1.38				
KC		1.61				0.95		1.53						1.63		1.64			
Lemur	2.90													3.57					
Lily		3.67						3.27								4.38			
Lucky																			
Magic		0.92						0.99	0.97	0.86				0.77	0.65	0.84	0.79		1.14
Malachi									1.37					0.11	0.05				
Mister		2.34		1.88				2.56	1.25	2.22				0.49	2.43	2.59	2.35		0.02
Monterey Jack		1.62						1.55							2.16	169.48			1.91
Moose		1.48						1.57						1.61	2.24	2.07			
Muffin		1.95						1.74	0.80	1.83					5.42	1.90	0.78		0.85
Nother		1.25		1.33				0.01	1.06	1.14		3.84		1.31	2.94	1.15	1.25	2.19	0.92
Otis		8.83						6.67											2.16
Pee Dee		1.44						1.29		1.50					1.45		1.40		1.65
Rat		1.64						1.37							3.25	1.78	0.75		1.13
Sade		1.41						1.30	1.14	1.32	1.19				3.35	1.45	1.05		0.91
Scout		2.08						2.64	2.85						6.51	3.46	0.01		1.66
Sophie	1.89	1.60	1.65	2.58		1.42		1.51	1.39	0.60	2.82			1.82	280.67	1.79	2.17		0.02
Stanley		1.44						1.44			1.55			1.47	2.25	1.51	1.13		
Tabi		1.37						1.20						1.23		1.31			
Tasha		1.77						1.50				2.40			2.44	1.48			1.03
Teddy		1.44						1.71	1.33					1.62	1.44	1.34	1.13		0.80
Tessa		1.02		1.22	1.12			0.91	1.21	1.11	1.08	1.23		1.00	1.08	1.25	1.07	1.19	1.02
Thunder		1.60						1.48									0.81		0.96
Trinity		1.23						1.42						0.22	450.25	1.49	1.21		1.21

Figure 1.

Serum: plasma ratios for each cytokine analyte and each cat. Ratios below 1.0 are in yellow, between 1.0 and 2.0 are in green, and above 2.0 are in pink. This figure shows the variability in ratios between analytes and between individuals for any single analyte.

Table 1

Number of results above (in range) and below the lower limit of quantification (LLOQ) for each of the 19 cytokines measured. Results for plasma and serum are presented separately, and show that perfect concordance, with both serum and plasma in range or below the LLOQ, was not found. A varying number of results were above the LLOQ for serum or for plasma, but not for both for most of the analytes.

	Plasma	Serum	Count		Plasma	Serum	Count
FAS	In range	In range	4	IL-6	In range	In range	9
	↓LOQ	In range	1		↓LOQ	In range	3
	In range	↓LOQ	0		In range	↓LOQ	0
FLT-3L	↓LOQ	↓LOQ	31	IL-8	↓LOQ	↓LOQ	22
	In range	In range	35		In range	In range	7
	↓LOQ	In range	0		↓LOQ	In range	18
GM-CSF	In range	↓LOQ	0	CXCL1	In range	↓LOQ	0
	↓LOQ	↓LOQ	0		↓LOQ	↓LOQ	11
	In range	In range	2		In range	In range	3
IFN- γ	↓LOQ	In range	0	MCP-1	↓LOQ	In range	11
	In range	↓LOQ	0		In range	↓LOQ	0
	↓LOQ	↓LOQ	34		↓LOQ	↓LOQ	20
IL-12 p40	In range	In range	9	PDGF-BB	In range	In range	22
	↓LOQ	In range	4		↓LOQ	In range	6
	In range	↓LOQ	0		In range	↓LOQ	2
IL-13	↓LOQ	↓LOQ	20	RANTES	↓LOQ	↓LOQ	5
	In range	In range	35		In range	In range	0
	↓LOQ	In range	0		↓LOQ	↓LOQ	35
IL-18	In range	In range	17	SCF	In range	In range	30
	↓LOQ	In range	3		↓LOQ	In range	5
	In range	↓LOQ	1		In range	↓LOQ	0
	↓LOQ	↓LOQ	15		↓LOQ	↓LOQ	0
	In range	In range	28		In range	In range	26

		Plasma	Serum	Count		Plasma	Serum	Count
IL-1 β		↓LOQ	In range	1	SDF-1	↓LOQ	In range	5
		In range	↓LOQ	1		In range	↓LOQ	0
		↓LOQ	↓LOQ	4		↓LOQ	↓LOQ	3
		In range	In range	5		In range	In range	21
IL-2		↓LOQ	In range	2	TNF- α	↓LOQ	In range	4
		In range	↓LOQ	0		In range	↓LOQ	2
		↓LOQ	↓LOQ	27		↓LOQ	↓LOQ	10
		In range	In range	6		In range	In range	6
IL-4		↓LOQ	In range	0		↓LOQ	In range	3
		In range	↓LOQ	0		In range	↓LOQ	0
		↓LOQ	↓LOQ	29		↓LOQ	↓LOQ	27
		In range	In range	17				
IL-4		↓LOQ	In range	4		↓LOQ	In range	4
		In range	↓LOQ	1		In range	↓LOQ	1
		↓LOQ	↓LOQ	13		↓LOQ	↓LOQ	13
		In range	In range	13				

Table 2

Analytes with less than 50% and less than 25% of samples within range (above the lower LOQ) for serum and plasma.

<25% Serum (<9)	<25% Plasma	<50% Serum (<19)	<50% Plasma
FAS	FAS	FAS	FAS
GM-CSF	GM-CSF	GM-CSF	GM-CSF
IL-1 β	IL-1 β	IFN- γ	IFN- γ
IL-2	IL-2	IL-1 β	IL-13
PDGF-BB	IL-8	IL-2	IL-1 β
	CXCL-1	IL-6	IL-2
	PDGF-BB	CXCL-1	IL-4
	TNF- α	PDGF-BB	IL-6
		TNF- α	IL-8
			CXCL-1
			PDGF-BB
			TNF- α

Table 3

Correlations between serum and plasma results for each analyte with 9 or more pairs of results.

Analyte	N	Pearson's Correlation	P-value
FLT 3L	35	0.945	<0.001
IFN- γ	9	0.893	0.001
IL-12p40	35	0.724	<0.001
IL-13	17	0.990	<0.001
IL-18	28	0.561	0.002
IL-4	17	0.931	<0.001
IL-6	9	0.948	<0.001
MCP-1	22	0.416	0.054
RANTES	30	0.973	<0.001
SCF	26	0.954	<0.001
SDF-1	21	-0.010	0.966