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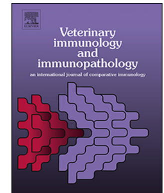

Survey of cytokines in normal canine tears by multiplex analysis: A pilot study

Paoul S. Martinez*, Eric S. Storey, Cherie M. Pucheu-Haston

Department of Veterinary Clinical Sciences, School of Veterinary Medicine Louisiana State University, Skip Bertman Drive, Baton Rouge, LA, 70803, United States

A B S T R A C T

Objectives: The purpose of this pilot study was to use a multiplexed assay to measure cytokines in normal stimulated canine tears.

Methods: 25 healthy dogs were included in the study. Stimulated tears were collected in capillary tubes from the right (OD) and left (OS) eyes and stored at −80 °C until batch sample analysis was performed. The samples were analyzed utilizing Luminex® canine-validated multiplex beads on a Bio-Rad multiplex analyzer for IL-2, IL-6, IL-7, IL-8, IL-10, TNF-α, and IFN-γ. Based upon previous human studies, tears were initially evaluated at a 1:10 dilution. Eight random samples were later re-analyzed without dilution.

Results: Diluting the samples 1:10 rendered all analytes undetectable except IL-8. A repeat analysis of eight randomly selected undiluted samples still demonstrated very low cytokine levels except for IL-8 (16/16 eyes; 2254 ± 1677 pg/ml OD, 1095 ± 786.8 pg/ml OS); and IFN-γ (15/16 eyes; 13.37 ± 13.08 pg/ml OD,16.08 ± 19.4 pg/ml OS).

Conclusion: This pilot study is the first to analyze cytokines in canine tears. This study demonstrated that IL-8 is consistently detected in both diluted and undiluted samples, but undiluted samples may be superior to 1:10 diluted samples for evaluation of other cytokines in canine tears.

1. Introduction

The precorneal tear film (PTF) is a multi-layered structure that provides lubrication, nutrients, antimicrobial properties, a clear refractive media, and removal of debris. The aqueous layer of the precorneal tear film is composed of 98.2% water and 1.8% solids, mainly proteins. (Gelatt, 2013). Some examples of these proteins include globulins, albumin, lysozomes, epidermal growth factor, lacritins, and cytokines. Low levels of cytokines and chemokines have been demonstrated to be present in healthy human tears (Lam et al., 2009; Carreno et al., 2010; Enriquez-de-Salamanca et al., 2008; Enriquez-de-Salamanca et al., 2010; Nakamura et al., 1998; Tan et al., 2014) compared to the elevated levels that have been found in patients suffering from ocular surface diseases such as dry eye syndrome, allergic conjunctivitis, vernal keratoconjunctivitis and atopic keratoconjunctivitis (Lam et al., 2009; Uchio et al., 2000; Huang et al., 2012; Lee et al., 2013; Posa et al., 2013; Nagineni et al., 2016). Patients with inflammatory diseases (dry eye, atopic keratitis, vernal keratitis) have demonstrated an increase in cytokines present in their tears such as interleukin (IL) 1, IL-6, IL-8, tumor necrosis factor- alpha (TNF- α), and interferon- gamma (INF- γ) (Lam et al., 2009; Huang et al., 2012; Ambroziak et al., 2016; Barabino et al., 2012).

The collection of sufficient sample volume is a critical factor in any analysis of tear samples. Acquisition of a sufficient volume of tears for analysis can be performed by pooling samples from different patients into a single sample, pooling a single patient's tear samples over a week's duration, or by collecting stimulated (or "reflex") tears from a single patient. Stimulated tear production can be achieved through the use of noxious substances or a Schirmer tear strip placed in the ventral fornix. Once the method to produce an appropriate volume of tears has been decided, the next choice is how those samples may be acquired for analysis. Several methods have been proposed for the acquisition of tear samples. (Nakamura et al., 1998; Posa et al., 2013; VanDerMeid et al., 2011; Dionne et al., 2016; Agrawal et al., 2016). These include the capillary tube collection method and the collection (and subsequent extraction) of tear fluid from Schirmer tear strips or absorbent sponges. Each method of tear fluid collection has benefits and pitfalls. Tear collection using Schirmer tear test strips or sponges is safe and painless, but the extraction process may result in loss of sample or analyte. In contrast, sample loss is expected to be minimal when samples are collected using capillary tubes, but this technique may be associated with a greater risk of accidental trauma to the globe or lid.

* Corresponding author.
E-mail address: pmartinez@lsu.edu (P.S. Martinez).

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In human medical literature, there has been extensive research to determine the cytokine profiles of patients' tears utilizing a variety of techniques. Analyses of human tear samples have been performed using multiplex analyzers, enzyme-linked immunosorbent assays (ELISA), and quantitative protein microarrays (Dionne et al., 2016). Some of these techniques (such as ELISA) are limited to single-analyte analysis, while other techniques (such as multiplex assays) assess for multiple analytes in a single sample. Multiplex bead-based assays can measure multiple analytes in relatively small sample volumes. This technique benefits researchers investigating small volumes of tears.

Information regarding cytokine levels in the canine tears is extremely limited. To the authors' knowledge, there is currently no peer-reviewed literature investigating the cytokine profiles in healthy canine tears. Our hypothesis was that there are detectable levels of cytokines and chemokines in stimulated canine lacrimal tear fluid, and that these substances can be quantified using a canine-specific multiplex analysis system.

2. Materials and methods

2.1. Participant selection

All procedures were approved by the Institutional Animal Care and Use committee of the Louisiana State University. Twenty-five healthy dogs were recruited from students, staff and faculty. Each participant had a complete medical and surgical history taken, and physical and ophthalmic examinations performed. Participants were included in the study if they satisfied the following criteria: 1. no history of major medical or surgical illness, 2. not on any medications outside of preventative heartworm and flea medications, 3. normal physical examination as determined by one of the authors (PSM), 4. normal ophthalmic examination as determined by a board certified ophthalmologist (ESS), 5. good temperament that would allow for tear collection. Ophthalmic examination included cranial nerve assessment, pupillary light reflexes, menace response, palpebral reflex, dazzle reflex, Schirmer tear test I (Intervet, Inc. Summit, NJ), intraocular pressure measurement following instillation of topical analgesia (Proparacaine, Alcon Laboratories, Inc. Fort Worth, Texas) and applanation tonometry (Medtronic Tono-Pen XL), fluorescein staining (Contracare Ophthalmics and Diagnostics, Gujarat, India), mydriasis (Tropicamide, Akorn, Inc. Lake Forest, IL), biomicroscopy slit lamp for anterior segment evaluation, and indirect ophthalmoscopy for fundic evaluation.

2.2. Sample collection/storage

A minimum of 48 h were allowed to elapse after the initial ophthalmologic examination before sample acquisition, to allow for recovery from manipulation associated with the initial examination. This withdrawal period was chosen based upon prior professional experience suggesting that clinical signs of inflammation (if any) following a routine, uncomplicated ophthalmologic examination would be expected to have resolved well prior to this point. Each participant was minimally restrained and had a dye-free Schirmer's tear strip placed into the ventral cul-de-sac to stimulate lacrimal tear production. A plain non-heparinized glass microcapillary tube was placed near the lateral canthus, taking extreme care not to touch the conjunctival surface. Once the capillary tube was greater than 75% filled, the tube was sealed with clay. This procedure was then repeated on the other eye. A minimum of three tubes per eye was obtained in 23/25 patients. The tubes were immediately transported to a −80 °C freezer for storage until batch analysis was performed.

2.3. Multiplex cytokine immunoassay

Each sample was transferred from the capillary tubes to microcentrifuge tubes and held on ice at the time of analysis. The samples were initially analyzed utilizing a custom Milliplex® Map canine Multiplex assay (EMD Millipore, Billerica, MA, USA) at a 1:10 dilution. The samples were prepared and analyzed as per the manufacturer's protocol. Briefly, 7.5 μL of each sample was diluted in 67.5 μL of assay buffer (L-AB, EMD Millipore) to make 75 μL of analyte. All reagents were allowed to come to room temperature. Two hundred microliters of assay buffer was added to each well of a 96-well microtiter plate. The plate was placed on a plate shaker for 10 min at 500 rpm, after which the wells were emptied by inverting the plate and gently tapping the contents out onto an absorbent pad. Diluted samples, standards and control solutions (25 μL each) were added in duplicate to appropriate wells, followed by 25 μL of assay buffer. Twenty-five microliters of a mixture of antibody-coated magnetic fluorescent beads were then added to each well. The plates were incubated on an orbital plate shaker (500 rpm) overnight in the dark at 4 °C. The next day, the plate was placed on a magnetic separation block, and the supernatant was decanted. The plate was washed twice by adding 200 ul of wash buffer to all wells, agitating for 30 seconds on the shaker, then replacing on the magnetic plate and decanting the buffer. Then 25 μL of detection antibodies were added to each well (LCC, EMD Millipore). The plate was placed on an orbital plate shaker for 1 h at room temperature, after which 25 μL of Streptavidin/phycoerythrin (LSAPE3, EMD Millipore) was added to each well and the plate was replaced on the shaker for 30 min. The plates then were washed twice as previously described. Sheath fluid (Bio-Plex Sheath Fluid, Bio-Rad Laboratories, Hercules, CA, USA; 150 μL) was added to all wells, and the plates were agitated for 5 min at room temperature. The plates were then analyzed on a Bio-Plex® 200 multiplex analyzer (Bio-Rad Laboratories, Hercules, CA, USA), using a five-parameter regression formula relative to the standard curve. Subsequent to the initial analysis, eight random samples were re-evaluated without dilution as described above. The estimated minimum detectable concentration for each parameter as provided by the manufacturer was as follows: IL-2, 3.5 pg/ml; IL-6, 3.7 pg/ml; IL-7, 7.5 pg/ml; IL-8, 21.7 pg/ml, IL-10, 8.5 pg/ml; TNF-α, 6.1 pg/ml; and IFN-γ 18 pg/ml.

2.4. Statistical analysis

Sample mean and median values and standard deviations for each cytokine were calculated using Prism 4 for Windows (Graph Pad Software, La Jolla, CA, USA). Determination of sample normality was performed using a Kolmogorov-Smirnov test. Because the samples were not normally distributed, comparisons between eyes was performed using a Mann-Whitney test. Comparison of cytokine concentrations between analyses was performed using a paired Wilcoxin signed rank test. The significance level for all analyses was defined as $p < 0.05$.

3. Result and discussion

3.1. Study participant analysis

Forty-nine dogs were screened for participation in the study. Twenty-four participants were excluded from the study because of abnormal physical and ophthalmic examinations. Twenty-five healthy dogs were identified for inclusion in the study. There were 10 males (three intact and seven neutered) and 15 females (one intact and fourteen spayed) with a mean age of 4.08 years ± 2.25 years. One patient admitted to the study developed later neurologic signs (seizure activity) and was later euthanized due to poor response to medications. On necropsy an intracranial mass was observed.

3.2. Cytokine analysis

The tear samples were initially analyzed at a 1:10 dilution, based on reports from human literature (Lam et al., 2009; Carreno et al., 2010;

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