

## In Vitro Characterization of Inflammatory Biomarkers across Species

Elizabeth A. Kenyon, Sharla M. Peters, Yolanda L. Jones, Christine M. Deaver, Michael J. Myers,  
Haile Yancy\*

U.S. FDA Center for Veterinary Medicine, Laurel, MD 20708

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### Abstract

There are currently no validated animal models or suitable biomarkers with which to ascertain the efficacy of non-steroidal anti-inflammatory drugs (NSAIDs) in equine, bovine or ovine species during conditions of endotoxemia. This has resulted in approval of only one NSAID, flunixin meglumine, for controlling inflammation due to endotoxemia in bovine and equine animals, while none are approved in ovine animals for this claim. This study aims to investigate biomarkers with which to test efficacy of NSAIDs in these species. To this end, the effects of *Escherichia coli* lipopolysaccharide (LPS) induced inflammation on gene expression were investigated. Whole blood from each species was cultured and stimulated with LPS, after which RNA was extracted at various times. RNA was analyzed via quantitative RT-PCR (qRT-PCR) to determine differential expression of biomarkers. Results indicated up-regulation of cluster of differentiation 1 (CD1) gene in bovine and serum amyloid A (SAA) gene in ovine cultures. Down-regulation of cluster of differentiation 4 (CD4) gene and Caspase 1 was seen in bovine, and of CD1 in equine cultures. This work demonstrates that LPS stimulation alters expression of these genes in these species. These genes may be useful biomarkers for inflammation which could serve as markers for NSAID efficacy.

*Keywords:* Inflammation; biomarker; LPS; equine, bovine, ovine

### Introduction

Reliable models of LPS-induced inflammation are lacking for many species important in veterinary medicine. In the absence of models with reliable biomarkers of inflammation, it is difficult to substantiate anti-inflammatory claims for new drugs in these species. Non-steroidal anti-inflammatory drugs (NSAIDs) represent a common class of drugs used to treat inflammation in a variety of species. NSAIDs reduce fever, pain and inflammation in both animals and people by inhibiting prostaglandin synthesis (Lees *et al.*, 2004). Evidence that models for inflammation are lacking can be seen in that only one NSAID, flunixin meglumine (Banamine; Schering-Plough), is currently approved by the FDA to control endotoxin-induced inflammation, pyrexia and the inflammation and pain associated with musculoskeletal disorders. The approval of flunixin meglumine by the FDA was based on claims that thromboxane B2 (TXB2) and prostaglandin E2 (PGE2) synthesis were reduced by the drug in two different models: a sys-

temic endotoxemic inflammation model and a bovine soft tissue model of inflammation (U.S. Food and Drug Administration, 1998). Evidence for the control of inflammation through reduction in eicosanoid production was derived from published literature studies not conducted under the drug review process.

Identifying and verifying biomarkers for inflammation in species would expedite the discovery, evaluation and drug approval process for new NSAIDs. Previous work in this area identified *in vitro* inflammatory biomarkers in swine utilizing a genomic approach (Peters *et al.*, 2011).

Some of the previously identified biomarkers for inflammation in swine might prove to be universal across species as reliable biomarkers for inflammation due to similarities in the physiological responses to inflammation in many veterinary species. This study aims to correlate expression patterns identified in the aforementioned study with those observed in LPS-stimulated bovine, ovine and equine whole blood cultures. The knowledge gained will help to ascertain if there are universal genetic biomarkers. A single set of biomarkers common to multiple species would aid in verifying the efficacy of novel NSAIDs by presenting a uni-

\*Corresponding author: Haile Yancy

Address: U.S. FDA Center for Veterinary Medicine

E-mail address: haile.yancy@fda.hhs.gov

versal test with which to analyze the drugs.

## Materials and methods

### Blood Collection and Treatment:

Bovine, equine and ovine blood were obtained from Bioreclamation, Inc. (Hicksville, NY). Blood was collected from 5 animals of each species to be utilized in qRT-PCR analysis. Ultraculture media (Lonza, Walkersville, MD) was supplemented with L-glutamine (20mM), gentamicin (50µg/ml), HEPES (10mM), sodium bicarbonate (0.075%) and amphotericin B (0.25 µg/ml). The fortified media was used to dilute the blood samples to a 1:1 ratio, starting with approximately 15 ml of blood in each flask (final volume 30 ml per flask; Nalgene-Nunc, Rochester, NY). A concentration of 1 µg/ml lipopolysaccharide E. coli O55:B5 (Sigma-Aldrich, St. Louis, MO) was used to stimulate the whole blood cultures at 24 and 48 hours. Unstimulated 0 h, 24 h and 48 h control cultures were also employed.

### RNA Isolation:

The LeukoLock Total RNA Isolation kit (Ambion,

Austin, TX) was utilized to extract RNA from whole blood cultures of the different species according to the manufacturer's protocol with minor modifications. Briefly, a 10 cc syringe was connected directly to a LeukoLock filter and used to flush approximately 15 ml of blood through the filter. The filters were then each flushed with 3 ml of PBS, followed by 3 ml of RNAlater and 2.5 ml of pH-adjusted Lysis/Binding Solution. Twenty-five µl of proteinase K was then used to treat the collected lysate. RNA isolation was performed using RNA binding beads. Samples were incubated and centrifuged, after which the beads were transferred to a clean microcentrifuge tube. Next, a series of wash steps was performed to purify the product. RNA was recovered in 40 µl of elution buffer and was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The RNA samples were normalized to a concentration of 50 ng/µl for qRT-PCR analysis.

### Real-time RT-PCR:

Custom primers (Table 1) were designed for the tested species using Allele ID (PREMIER Biosoft International, Palo Alto, CA) and synthesized by Integrated DNA Technologies (Coralville, IA). Quantitative real-time RT-PCR (qRT-PCR) was

Table 1. Selected genes differentially expressed in leukocytes from whole blood post-LPS treatment

Accession No.	Gene	Gene symbol	Expression	GO biological process
<i>Equine</i>				
XM_001489374	CD1 antigen	CD1	Down-regulated	Immune response
XM_001497051	CD4 molecule	CD4	Down-regulated	Immune response Cellular Adhesion
NM_001081842	Caspase 1	CASP1	Down-regulated	Proteolysis Regulation of apoptosis
AJ251189	Monocyte chemoattractant protein-1	MCP-1	Up-regulated	Inflammatory response
<i>Ovine</i>				
EU366476	Serum amyloid A3.2 precursor protein	SAA3	Up-regulated	Acute-phase response
NM_001009472	Monocyte chemoattractant protein-1	MCP-1	Up-regulated	Inflammatory response
NM_001129902	CD4 molecule	CD4	Up-regulated	Immune response Cell Adhesion
<i>Bovine</i>				
NM_001102024	CD1 antigen	CD1	Up-regulated	Immune response
NM_001103225	CD4 molecule	CD4	Down-regulated	Immune response Cell Adhesion
AB285205	Caspase 1	CASP1	Down-regulated	Proteolysis Regulation of apoptosis

performed with these primers using the Mastercycler® ep Realplex (Eppendorf, Hamburg, Germany). The SuperScript III Platinum Sybr Green One-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA) was utilized for qRT-PCR analysis of samples. The master mix used was comprised of 25 µl of 2X Sybr Green Reaction Mix, 21 µl of DEPC-treated water, 1 µl of Platinum Taq Mix, and 1 µl each of forward and reverse primers at a concentration of 10 µM, with 50 ng of the total RNA sample. The thermocycling parameters were: cDNA synthesis at 50°C for 3 min, 95°C for 5 min, then 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 60°C for 30 sec.

### Statistical Analysis:

The delta-delta Ct method was used to assess relative expression of genes with GAPDH as the normalizing gene. Data are presented as mean ± SEM. Comparison of qRT-PCR data across samples to their respective 0h values was done by performing a Friedman test. Both the 24h and 24h control, and the 48h and 48h control data were compared using the Wilcoxon signed rank test. Statistical significance was considered to be a *P* value < 0.0625 based on small sample size.

## Results

GAPDH specific to each species was utilized as a reference gene for the study to establish the metabolic activity of the cells. No significant change in GAPDH expression was observed due to time or LPS treatment in any of the species (Fig. 1).

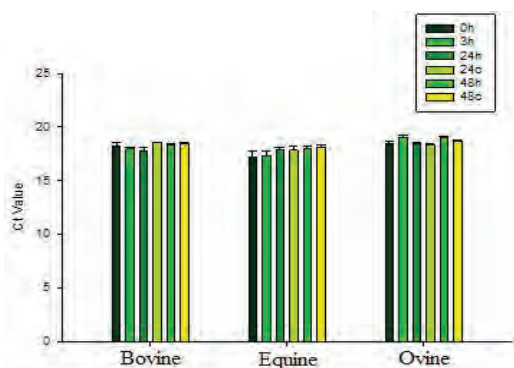


Fig. 1. qRT-PCR analysis of reference (housekeeping) gene GAPDH in individual animals, graphed by species (n=5 for all species). Data are expressed as mean ± SEM. No significant differences were observed between any of the time points ( $P < 0.05$ ).

cultures demonstrated significant up-regulation as compared to the 0 hour time point for monocyte chemoattractant protein-1 (MCP-1). Significance

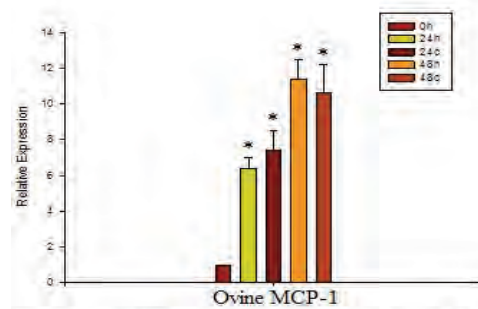


Fig. 2. qRT-PCR analysis of MCP-1 in individual sheep (n=5). Data are expressed as mean ± SEM. \*Statistically different based on comparison to the 0 h control for each gene ( $P < 0.05$ ).

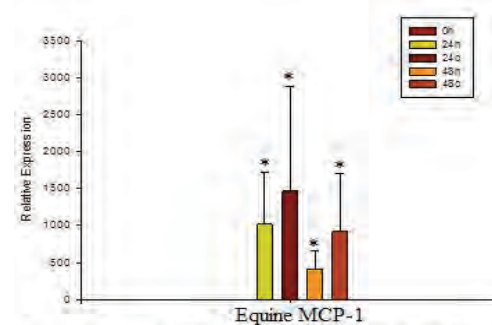


Fig. 3. qRT-PCR analysis of MCP-1 in individual horses (n=5). Data are expressed as mean ± SEM. \*Statistically different based on comparison to the 0 h control for each gene ( $P < 0.05$ ).

between LPS-stimulated and control samples at each time point was not obtained in either species due to extreme variability in expression of the gene.

The cluster of differentiation 1 (CD1) gene was evaluated in two of the three species. CD1 was not analyzed in ovine samples due to the lack of ovine-specific sequence information for the gene. Expression of CD1 differed between equine and bovine cultures. CD1 was up-regulated with statistical significance at all time points through 48 hours in the bovine blood. The 24h LPS-stimulated culture was significantly less up-regulated than the 24c culture (Fig. 4). Conversely, marked down-regulation of the gene is evident in equine blood over time. The stimulated 48h culture was significantly more down-regulated than the 48c culture (Fig. 5).

Expression of the cluster of differentiation 4 (CD4) gene shows down-regulation at all time points in bovine and equine cultures (Fig. 6). Ovine expression of CD4 showed the opposite trend with up-regulation through the 48 hour time point (Fig. 7). Bovine samples for 24h and 24c were also statistically different showing less down-regulation in the presence of LPS.

Ovine (Fig. 2) and equine (Fig. 3) whole blood



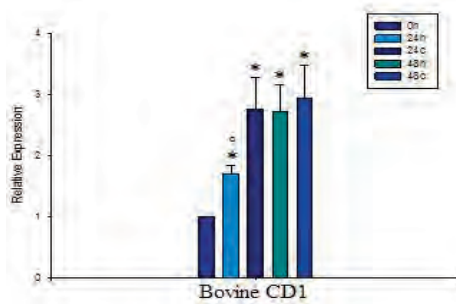


Fig. 4. qRT-PCR analysis of CD1 in individual cows (n=5). Data are expressed as mean ± SEM. \*Statistically different based on comparison to the 0 h control for each gene (P<0.05); °Statistically different from respective control (P<0.0625).

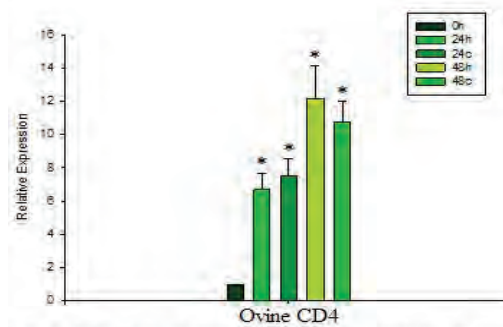


Fig. 7. qRT-PCR analysis of CD4 in individual sheep (n=5). Data are expressed as mean ± SEM. \*Statistically different based on comparison to the 0 h control for each gene (P<0.05).

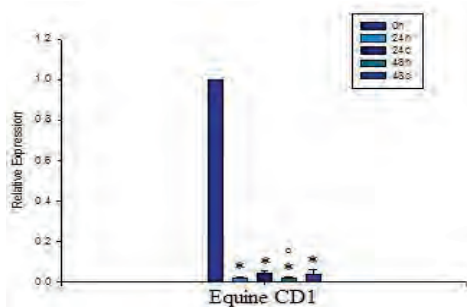


Fig. 5. qRT-PCR analysis of CD1 in individual horses (n=5). Data are expressed as mean ± SEM. \*Statistically different based on comparison to the 0 h control for each gene (P<0.05); °Statistically different from respective control (P<0.0625).

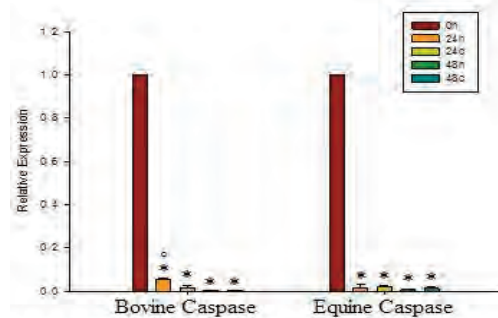


Fig. 8. qRT-PCR analysis of Caspase 1 in individual cows (n=5) and horses (n=5). Data are expressed as mean ± SEM. \*Statistically different based on comparison to the 0 h control for each gene (P<0.05); °Statistically different from respective control (P<0.0625).

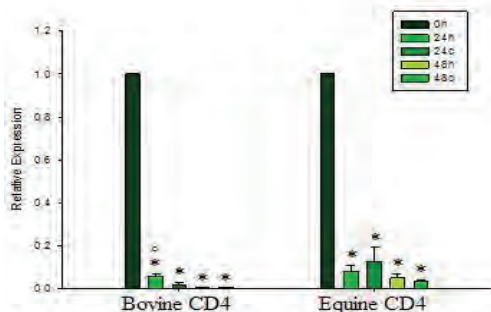


Fig. 6. qRT-PCR analysis of CD4 in individual cows (n=5) and horses (n=5). Data are expressed as mean ± SEM. \*Statistically different based on comparison to the 0 h control for each gene (P<0.05); °Statistically different from respective control (P<0.0625).

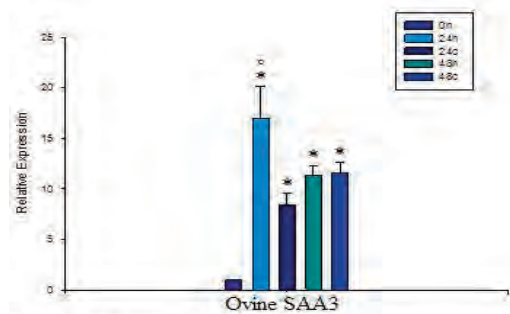


Fig. 9. qRT-PCR analysis of SAA3 in individual sheep (n=5). Data are expressed as mean ± SEM. \*Statistically different based on comparison to the 0 h control for each gene (P<0.05); °Statistically different from respective control (P<0.0625).

Equine and bovine blood cultures demonstrated significant down-regulation of Caspase 1 at all time points relative to the 0 hour time point (Fig. 8). Bovine Caspase 1 indicated a statistically different response between the 24h and 24c time points with less down-regulation in the presence of LPS.

Analysis of serum amyloid A 3 (SAA3) gene expression in the ovine blood cultures showed significant up-regulation of the gene through 48 hours (Fig. 9). A statistically significant difference was also noted with the 24h time point showing more elevated expression than the 24c time point.

## Discussion

The candidate biomarkers analyzed in this study have all been previously identified as components of inflammatory or immune responses (Table 1). MCP-1 is the primary chemoattractant for recruitment of monocytes during the inflammatory response (Maus *et al.*, 2002). The lack of significance between the control and LPS-stimulated samples indicates that there is no consistent effect of LPS treatment in culture that might not also be seen solely due to time in culture. Use of MCP-1 as a biomarker would not be advisable based on the un-

reliability of results caused by this variation.

CD1 is involved in antigen recognition by T cells during the immune response (Porcelli *et al.*, 1989). Bovine RNA indicated that CD1 was up-regulated with statistical significance at all time points through 48 hours. The significance found between the 24h LPS-stimulated culture and the 24c culture demonstrates that this change would not have happened simply due to time in culture. Conversely, marked down-regulation of the gene is evident in equine blood over time. The stimulated 48h culture was significantly more down-regulated than the 48c culture which also demonstrates a change not solely due to time in culture. The differences in expression of CD1 between equine and bovine cultures were not unexpected as previously there have been conflicting reports about the expression of CD1.

Previous studies indicate that there is a localized up-regulation of CD1 subsets that, it has been proposed, may suggest that there is a pro-inflammatory positive feedback loop involving dendritic cells that express CD1 and CD1 restricted T cells (Vincent *et al.*, 2002). CD1 has also been reported to be down-regulated in a number of studies following *in vitro* exposure to Mycobacteria (Gagliardi *et al.*, 2004, 2007; Mariotti *et al.*, 2004; Stenger *et al.*, 1999). Peters *et al.* (2011) also found that CD1 was down-regulated in swine whole blood cultures as a result of LPS exposure, leading them to conjecture that the differences in CD1 expression may result from different signal transduction pathways initiated by the different pathogens, or pathogen components, being studied. Our findings also might suggest that there is a difference in CD1 expression based on species differences. The down-regulation of CD1 seen in the equine blood in our study has also been observed in equine peripheral blood adherent cells (PBAC) which suggests that *Rhodococcus equi* infection results in down-regulation of CD1b (Pargass *et al.*, 2009). This information could allow for the contention that it may be the species of animal rather than, or in addition to, the pathogen that accounts for the difference in CD1 gene expression. There are unaccounted for differences in CD1 regulation between the equine and bovine which indicate that the response of CD1 to inflammation may not be universal across species. However, it may serve as a useful biomarker if differences between each species are taken into account.

Expression patterns for CD4, which is involved in the development and activation of helper T cells for the immune response (Zamoyska, 1998), were measured in all three species. The gene was down-regulated at all time points in bovine and equine cultures showing that the gene does change over time. Ovine expression of CD4 also showed a change with up-regulation through the 48 hour time point. Bovine samples for 24h and 24c were the only ones demonstrating a statistical difference showing less down-regulation in the presence of LPS, and indicating that the change was not a result of time in culture. This leads us to believe that CD4 may be an effective biomarker for inflammation in bovine through the 24 hour time point.

Caspase 1 is involved in regulation of apoptosis (Earnshaw *et al.*, 1999) and initiation of a pathway that results in expression and secretion of interleukin-1beta (IL-1 $\beta$ ) and IL-18 during inflammation (Tschopp and Schroder, 2010). The statistical difference in bovine RNA between the 24h and 24c time points suggests that the gene may be an effective bovine biomarker for inflammation when analyzing to the 24h time point.

SAA proteins are involved in the acute-phase response to inflammation both in up- and down-regulating genes and processes involved in the inflammatory response (Malle *et al.*, 1993). The statistically significant difference noted at the 24h time point in ovine cultures demonstrates that LPS treatment has an effect on the expression of SAA3 and is not being caused solely by time in culture. This resembles the finding that SAA2 is up-regulated at all time points in swine with significance by the 24h time point and a statistically significant difference between the LPS-stimulated and control samples at that point in time (Peters *et al.*, 2011). This pattern indicates that SAA may be a biomarker for inflammation in swine and ovine.

In summary, we have demonstrated that a subset of the *in vitro* biomarkers identified in swine is also indicative of LPS-stimulated inflammation in bovine, ovine and equine whole blood culture *in vitro*. Specifically, these are CD1, CD4 and Caspase 1 in bovine; SAA3 in ovine and CD1 in equine cultures. The similarities in expression of these genes between species makes them potential candidates for use in testing anti-inflammatory drugs in a species to assess the effects of the drugs against the expression of these candidate biomarkers in response to inflammation. Further study of

the response of these genes in the presence of NSAIDs as well as in vivo will be necessary to firmly establish this relationship. This work may assist in the development of a model to study inflammation and test anti-inflammatory drugs more efficiently in vivo. Our findings indicate that it may not be possible for a single genetic marker to serve as a universal biomarker for all species. However, some of the genes may prove to be useful biomarkers for inflammation within a given species.

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