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Authors: J. Filipe, G. Curone, V. Bronzo, G. Pisoni, P. Cremonesi, C. Pollera, L. Turin, D. Vigo, P. Roccabianca, M. Caniatti, P. Moroni, F. Riva

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Pentraxin 3 is up-regulated in epithelial mammary cells during *Staphylococcus aureus* intra-mammary infection in goat

Filipe J. [a], Curone G. [a], Bronzo V. [a], Pisoni G.[b], Cremonesi P.[c], Pollera C.[a], Turin L. [a], Vigo D. [a], Roccabianca P. [a], Caniatti M. [a], Moroni P. [a,d], Riva F. [a]*

[a] Università degli Studi di Milano, Dipartimento di Medicina Veterinaria, via Celoria 10, 20133 Milan, Italy.

[b] Veterinary Practitioner.

[c] Istituto di Biologia e Biotecnologia Agraria, Consiglio Nazionale delle Ricerche, 26900 Lodi, Italy.

[d] Cornell University, Animal Health Diagnostic Center, Quality Milk Production Services, Ithaca, NY 14853, USA.

*Corresponding Author: Federica Riva

Dipartimento di Medicina Veterinaria, Università degli Studi di Milano

Via Celoria 10, 20133, Milano, Italy

federica.riva@unimi.it

tel. +39-02-50318086

fax +39-02-50318089

Authors' emails: Joel Fernando Soares Filipe (joel.soares@unimi.it), Giulio Curone (giulio.curone@unimi.it) , Valerio Bronzo (valerio.bronzo@unimi.it) , Giuliano Pisoni (giulianopisoni@libero.it), Paola Cremonesi (cremonesi@ibba.cnr.it), Claudia Pollera (claudia.pollera@unimi.it) , Lauretta Turin (lauretta.turin@unimi.it), Daniele Vigo (daniele.vigo@unimi.it), Paola Roccabianca (paola.roccabianca@unimi.it) , Mario Caniatti (mario.caniatti@unimi.it) , Paolo Moroni (paolo.moroni@unimi.it).

HIGHLIGHTS

- PTX3 nucleotide sequence is highly conserved among the species
- PTX3 is mainly expressed in goat bone marrow mammary gland aorta rectum pancreas skin
- The infected udder influences the inflammation in the contralateral healthy one
- *Staphylococcus aureus* intra-mammary infection causes systemic and local PTX3 modulation

Abstract

Pentraxin 3 is the prototypic long pentraxin and is produced by different cell populations (dendritic cells, monocytes/macrophages, endothelial cells, and fibroblasts) after pro-inflammatory stimulation. Different studies demonstrated the up-regulation of PTX3 during mastitis in ruminants, but its role is still unknown. We first investigated the conservation of PTX3 sequence among different species and its pattern of expression in a wide panel of organs from healthy goats. We studied the ~~role~~ modulation of PTX3 during natural and experimental mammary infection, comparing its expression in blood, milk and mammary tissues from healthy and *Staphylococcus aureus* infected animals. We confirmed the high conservation of the molecule among ~~the~~ different species. Goat PTX3 was expressed at high levels in bone marrow, mammary gland, aorta, rectum,

pancreas, skin and lungs. PTX3 was up-regulated in epithelial mammary cells and in milk cells after *S. aureus* infection, suggesting that it represents a first line of defense in goat udder.

Abbreviations: Pentraxin 3 (PTX3); Mammary epithelial cells (MEC); Polymorphonuclear leukocytes (PMNs); Toll like receptors (TLRs); Somatic cell count (SCC); Pattern recognition molecules (PRMs); Lipopolysaccharide (LPS); Dendritic cells (DCs); C-reactive protein (CRP); Serum amyloid P (SAP); Antigen presenting cells (APCs); Smooth muscle cells (SMCs); High density lipoprotein (HDLP); Milk fat globules (MFG)

Keywords: Pentraxin 3; *Staphylococcus aureus*; intra-mammary infection; goat; innate immunity.

1. Introduction

Mastitis is one of the most economically important diseases in dairy industry and it is defined as an inflammation of the mammary gland which makes it among the most important concerns for the livestock sector [1]. *Staphylococcus aureus* ~~are~~ is one of the main etiological agents of small ruminants intra-mammary infections (IMI)–establishing clinical or sub-clinical mastitis, usually characterized by the increase of somatic cell count (SCC) in milk from the affected half udder [2]. The invasion of *S. aureus* induces the innate immune response~~,~~ mainly through the activation of ~~.~~ neutrophils and macrophages, which are the first cells to be recruited at the site of inflammation [3]. Early innate immune responses also occur in mammary epithelial cells (MEC) and these responses reinforce subsequent neutrophil infiltration into mammary tissue, activation of these innate immune cells and increased SCC in milk [4, 5]. Recent studies indicate that MEC respond strongly and rapidly to challenge by either low levels of bacteria or bacterial cell wall components~~,~~ with marked changes in expression of pro-inflammatory cytokines, chemokines and other molecules [4, 5, 6, 7].

In different studies on mammary gland response to *S. aureus* infection , Pentraxin 3 (PTX3) showed a large change in its expression levels both in milk and blood, and is consequently a candidate for further studies on immune response associated with mastitis [8-11].

Pentraxins are a family of evolutionarily conserved pattern recognition molecules (PRMs) characterized by a cyclic-multimeric structure. On the basis of structure, pentraxins have been divided into short and long families [12]. C-reactive protein (CRP) and serum amyloid P (SAP) components are prototypes of the short pentraxin family, while PTX3 is a prototype of the long pentraxins [13-15]. PTX3 is a soluble pattern recognition receptor involved in the initiation of protective responses against pathogens. It is produced by somatic and immune cells in response to pro-inflammatory stimuli and Toll Like Receptors (TLRs) engagement, and it interacts with several ligands and uses multifunctional properties. This molecule is a multimeric glycoprotein comprising 381 amino acids, including a 17-residue signal peptide [16]. The C-terminal half containing the pentraxin signature is highly homologous to CRP and SAP, with up to 57% similarity to these molecules; in contrast, the N-terminal domain of PTX3 is not related to other known proteins [13, 16]. The presence of a highly conserved motif of primary amino acid sequence is a unique feature of the members of the pentraxin family (e.g., 92% of amino acid residues in human and murine PTX3 are conserved), suggesting a strong evolutionary pressure to maintain its structure/function relationships [13]. The protein is rapidly and locally produced by different cells, including cells of the myeloid lineage, such as monocytes, dendritic cells (DCs), and macrophages, vascular and lymphatic endothelial cells, smooth muscle cells (SMCs), epithelial cells, and fibroblasts [17]. Primary pro-inflammatory cytokines (specifically IL-1 and TNFα), intact microorganisms or TLR agonists (e.g., LPS or bacterial outer membrane lipoproteins), and high-density lipoproteins (HDLs) induce PTX3 [13, 18]. IL-10 is a mild inducer of PTX3 in monocytes and DCs and can amplify PTX3 production induced by LPS [19, 20].

As the use of antibiotics in livestock production is very common, a new challenge today is to reduce their use and treatments by increasing the natural ability of animals to resist infections. This strategy

is critically dependent on a better understanding of the host immune response at the early stages of infection. Indeed, the establishment, persistence, and severity of infection are clearly dependent on the rapidity and effectiveness of the host response against the invading pathogens. Furthermore, clearance of bacterial pathogens from the mammary gland is often ruled by responses that occur within immediate hours after initial infection [21].

Preliminary data in cattle suggest that PTX3 could play an important role in early mammary infection [8]. In this work we studied PTX3 pattern of expression in different organs and tissues from healthy goats and investigated the modulation of PTX3 during experimental IMI in mammary tissue, and during natural *S. aureus* IMI in milk and blood cells and milk fat globules (MFG).

2. Material and Methods

2.1.Animals and samples

In this study three groups of samples were used: a) organ and tissue samples from two healthy goats b) mammary archived tissues from six goats experimentally infected with *S. aureus* in a previous study by Cremonesi et al. 2012 [10], c) milk and blood samples from nine lactating goats.

Samples from a wide panel of tissues and organs (a) were taken from two healthy goats at the slaughterhouse. A piece of 0.5 cm^3 was taken from each organ and tissue after normal standard operation at slaughterhouse and immediately immersed in 3 ml of RNAlater (Life Technologies, Carlsbad, CA, USA), and stored at -20°C until use.

Mammary archived tissue samples from six goats, experimentally infected with *S. aureus* in a previous study [10], were analyzed 30 hours post infection (b).

Finally, from nine goats (6 healthy goats and 3 goats with clinical *S. aureus* mastitis) (c), located at Roccaforte Ligure (Alessandria, Italy), we collected 15 ml of milk and 20 ml of blood. The study complied with the recent Italian law on animal experimentation (D.Lgs. 26/2014) and ethics (Italian Health Ministry authorization n. 628/2016-PR). Before milk sampling, teat ends were carefully cleaned. First streams of foremilk were discharged, and then approximately 15 ml of milk was

collected aseptically into sterile vials. Milk was transported at 4°C to the laboratory and immediately processed. *S. aureus* infection was diagnosed following NMC procedure (NMC 2017). Blood was collected from the jugular vein in vacutainer tubes containing EDTA (BD, Franklin Lakes, New Jersey, USA), and transported to the laboratory at room temperature to avoid neutrophil activation.

2.3 Immunohistochemistry

Mammary tissue samples were fixed in 10% formalin for 24 hours and then embedded in paraffin. Tissue slices of 3 μm were prepared, and then affixed on the slide. The slides were immersed in two consecutive xylene (Merck KGaA, Frankfurt, Germany) baths for a total of 0 minutes . Slides were then immersed in different alcoholic solutions: 5 minutes in absolute alcohol (Diapath, Martinengo, Brescia, Italy), 20 minutes in methanol (Merck KGaA, Frankfurt, Germany) + H_2O_2 (36%, Merck KGaA, Frankfurt, Germany), 5 minutes in absolute alcohol, 2 minutes in 95% alcohol (Diapath, Martinengo, Brescia, Italy), 2 minutes at 70% alcohol, and two minutes in a Tris buffer (0.1 mM pH 7.6). Unspecific staining was blocked with 10% heat inactivated horse serum in Tris buffer for 30 minutes. The samples were then incubated 18 hours at 4°C with the primary antibody (polyclonal affinity-purified rabbit IgG against human PTX3) and washed 3 times for 3 minutes in Tris (0, 1 mM, pH 7.6; Merck KGaA, Frankfurt, Germany) before incubation of 30 minutes at room temperature with the biotinylated secondary antibody (anti-rabbit IgG, Dako, Glostrup, Denmark) diluted 1:200. Finally, after 3 washes of 3 minutes with Tris, slides were incubated with avidin-biotin reagent (Vectastain Elite ABC system, Vector, Burlingame, CA, USA) for 30 minutes followed by other 3 washes. Signal detection was made using 3-amino-9-ethylcarbazole (AEC, vector kit, Burlingame, CA, USA) as peroxidase substrate for 15 minutes, followed by a H&E stain (Sigma-Aldrich, St. Louis, USA). After wash in running water for 3 minutes, the slides were prepared with coverslip and stored at room temperature until analysis under the microscope.

2.4 Milk processing

The milk samples were centrifuged at 850 g for 10 minutes at 4°C; 500 µl of surface fat layer (Milk Fat Globules, MFG) were transferred with a sterile spatula in a 2 ml sterile tube containing 1.5 ml of TRIzol (Invitrogen, Life Technologies, Carlsbad, CA, USA). After a vigorous vortexing, the samples were kept at -80°C. The skimmed milk was eliminated and the cell pellet was washed in 5 ml of PBS (Sigma-Aldrich, St. Louis, USA). The pellet was then resuspended in 1 ml of RPMI (Invitrogen, Life Technologies, Carlsbad, CA, USA) supplemented with 10% FCS (Invitrogen, Life Technologies, Carlsbad, CA, USA). The cells ($0.5\text{-}2x10^6$) were lysed in TRIzol (Invitrogen, Life Technologies, Carlsbad, CA, USA) and then stored at -80°C.

2.5 Blood cells isolation

One ml of blood was lysed with 2 ml of ACK hypotonic solution (0.5M NH_4Cl, 10 mM $KHCO_3$, 200 µl 0.5M EDTA pH 8) after a 2-minute incubation at room temperature. Cells were washed twice in PBS (Sigma-Aldrich. St. Louis, USA), and the pellet was resuspended in 1 ml of RPMI (Invitrogen, Life Technologies, Carlsbad, CA, USA) supplemented with 10% FCS. 10^6 cells were resuspended in 1 ml of medium, and used for cytospin preparation, while 2-3 x 10^6 cells were lysed in TRIzol (Invitrogen, Life Technologies, Carlsbad, CA, USA) and stored at -20°C. The rest of the blood samples were diluted 1:2 in sterile PBS (Sigma-Aldrich. St. Louis, USA), and layered over a cushion of 10 ml of Histopaque-1.077 (Sigma-Aldrich. St. Louis, USA). After centrifugation at 800 g for 45 minutes at room temperature, the mononuclear cells layer was collected, washed twice in sterile PBS (Sigma-Aldrich, St. Louis, USA), and centrifuged at 600 g for 10 minutes at room temperature. The pellet of mononuclear cells (PBMCs) was resuspended in 2 ml of RPMI (Invitrogen, Life Technologies, Carlsbad, CA, USA) supplemented with 10% FCS. The PMNs layer was recovered and transferred into a sterile tube. Red blood cells were lysed using ACK hypotonic solution and washed again in sterile PBS (Sigma-Aldrich, St. Louis, USA). After centrifugation at

500 g for 8 minutes at room temperature, the PMNs pellet was resuspended in 2 ml of RPMI (Invitrogen, Life Technologies, Carlsbad, CA, USA) supplemented with 10% FCS. 5-8 x10^6 cells (mononuclear and PMNs) were lysed in TRIzol (Invitrogen, Life Technologies, Carlsbad, CA, USA) and then stored at -20°C. The purity of PMNs and PBMCs was assessed by differential count on cytospin samples stained with H&E. The percentage of purity of isolated PMNs resulted 91%, whereas the one of PBMCs was 76%.

2.6 RNA extraction (from tissues and from blood and milk cells)

Total RNA was extracted from all the samples lysed in TRIzol (mammary tissues, MFG, milk and blood cells; mononuclear and PMN cells) according to the manufacturer's protocol (Sigma–Aldrich, St. Louis, MO, USA). Finally, the RNA concentration and quality were determined using a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany) at 260/280 nm wavelength. The samples were stored at -80°C.

2.7 *Mammary tissue RNA extraction and array hybridization*

From tissue samples, total RNA was extracted following the protocol previously described [10]. After the assessment of the RNA quality and quantity by using the Bioanalyser 2100 (Agilent, Santa Clara, CA, USA), the RNA samples were hybridized on a custom array (Combimatrix CustomArray 90K, Seattle, WA, USA) following the protocols of the manufacturer, as previously described [10]. The hybridized arrays were scanned with a GenePix 4000B microarray scanner (Axon, Toronto, Canada) and the images (TIF format) were exported to the CombiMatrix Microarray Imager Software, to perform quality checks of the hybridizations and the spots on the slide. Data were extracted and loaded into the R software using the Limma analysis package from Bioconductor and systemic identification and grouping of differentially expressed genes into biological networks were performed using the software packages Ingenuity Pathway Analysis (Qiagen, Hilden, Germany).

2.8 Reverse-transcription and Real Time PCR

One µg of total RNA from each sample was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The samples were incubated in a 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA) according to the following cycle: 10 minutes at 85°C, 120 minutes at 37°C and hold at 4°C. The cDNA samples were stored at -20°C.

The cDNA obtained from each sample was used as a template for Real Time PCR in an optimized 25 µl reaction volume in MicroAmp optical 96-well plates, as previously described [22]. Each plate contained duplicates of each cDNA sample (3 µl), 2× Power Sybr Green PCR Master Mix (12.5 µl) (Applied Biosystems, Foster City, CA, USA) and the primers concentration was established at 300 nM each (0.3 µl of 10 µM solution).

The primer pairs for goat PTX3 were designed based on goat sequence GI:548451349, using Primer Express Software (Applied Biosystems, Foster City, CA, USA), and selected to produce amplicons spanning 2 exons [23]. Primers were purchased from ThermoFisher Scientific (Carlsbad, CA, USA); their sequences are listed in Table 1. We also employed primers for bovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene and for bovine IL-1β [24]. Bovine primers were used because they also specifically recognize goat sequences of GAPDH (GI:1062975189) and IL-1β (GI:1834304), respectively. A duplicate no-template control (NTC) was also included in each plate. Real Time quantitative PCR was carried out in the 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) at the following thermal cycle conditions, 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Quantitation was determined after the application of an algorithm to the data analyzed by the software of the 7000 Detection System (Applied Biosystems, Foster City, CA, USA).

The expression of PTX3 and IL-1β genes was normalized using the calculated GAPDH cDNA expression (mean) of the same sample and run.

2.9 Sequence analysis

For the analysis of the goat PTX3 nucleotide sequence we used free online Blast software (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and for the phylogenetic tree building we used the free software on the http://www.phylogeny.fr/simple_phylogeny.cgi web site [25].

2.10 Statistical analysis

To compare the expression of PTX3 in the two experimental groups (infected and non-infected) in the immunohistochemistry experiments we used the *t* Student (for normally distributed data; Shapiro-Wilk test) or Mann-Whitney (for not normally distributed data; Shapiro-Wilk test) test, considering statistically significant value of $p < 0.05$.

To evaluate the significance of the PTX3 and IL-1β RNA messenger expression increase in the infected mammary tissues compared to those non infected, we used the Mann-Whitney test, considering statistically significant value of $p < 0.05$.

For the biological function and network analysis, the p-value significance calculated by right-tailed Fisher's exact test was used.

3. Results

3.1 PTX3 nucleotide sequence is highly conserved among the species

Few data on goat PTX3 are available in literature and in the databases we only found a single predicted DNA sequence of goat PTX3 messenger (GI:548451349). This record is derived from a genomic sequence (NW_005100608.1) annotated using gene prediction method, Gnomon. This sequence is homologous to several sequences of the NCBI database belonging to different species (some of them being predicted from genomic sequences), showing a very high homology with ovine PTX3 (99%), bovine (92%), water buffalo (92%), bison (92%) and deer (91%). A lower homology was found with dolphin (86%), cat (84%), pig (84%), horse (84%), human (84%),

macaque (84%), mouse (76%), chicken (68%), xenopus (64%) and zebrafish (73% of a small portion of 100 bp) sequences.

The alignment between the unique genomic sequence (GI:541128986) for goat PTX3 and the predicted mRNA sequence, revealed that the gene of this molecule is on goat chromosome 1 and includes three exons as described in human and mouse [12].The phylogenetic study confirmed that PTX3 is a conserved molecule, present in fish, amphibians, birds and mammals. It is interesting to note that PTX3 of ruminants (cattle, goat, sheep, deer and buffalo), pigs and cetaceans (dolphin and orca) ha~~s~~ve a strong sequence homology (Figure 1). Goat PTX3 sequence shows a lower homology with cat, horse and primates (humans and macaque), but seems quite distant from dog and rodents (mouse and rat) (Figure 1). As expected the sequences of chicken and xenopus were the least homologous while still having some relationship with mammalian sequences (Figure 1), compared with the zebrafish one, that ~~is~~ was the outgroup, phylogenetically earlier developed (Figure 1).

3.2 PTX3 is ~~poorly~~ mainly expressed in tissues rich of endothelial, hematopoietic cells or extracellular matrix.

Given that few data are available in literature on the expression of PTX3 in goat [8], we first studied the expression pattern of its messenger in a large panel of organs and tissues from healthy animals.

As shown in Figure 2 A, the mRNA expression level of PTX3 is high in the bone marrow, mammary gland, aorta, rectum, pancreas and skin compared to the majority of the organs and tissues where the expression level is lower (lung, trachea, abomasum, salivary gland, bladder, spleen, caecum, adrenal glands, jejunum, colon, omasum, brain, rumen, kidney, testis, duodenum, lymph nodes, thymus, liver, heart and ileum).

Blood cells of healthy animals express discrete levels of PTX3 messenger. In particular we noted that PMNs are those with the higher expression, whereas circulating mononuclear cells expressed lower levels (Figure 2 B).

In milk samples from healthy animals, we observed discrete levels of PTX3 mRNA expression in both MFG (representing epithelial mammary gland cells) and milk cells (Figure 2 C).

3.3 S. aureus infection results in upregulation of PTX3 in the udder

Given the high levels of PTX3 expression in goat udder, we decided to study its modulation ~~in a~~ during previously performed experimental intra-mammary *S. aureus* infection model [10]. Six goats without any clinical sign of mastitis were inoculated with the pathogen in the left udder and with sterile PBS as control in the right one. Thirty hours post infection (pi), the animals were sacrificed and biopsies (3 biopsies from each half udder) from both half udders were taken and preserved for molecular and histological examinations [10].

PTX3 gene expression was analyzed by Real Time PCR in tissue samples taken at the end of the trial. In all the animals we observed an up-regulation of PTX3 in the infected udder tissue, compared to the contralateral inoculated with PBS (Figure 3 A).

The same tissues were subjected to immunohistochemical analysis with anti-hPTX3 polyclonal antibody. The control tissues presented positivity in the glandular epithelial cells, mainly in the apical cytoplasmic membrane, and in the infiltrating leukocytes (Figure 3 B); an average of 50-75% of the ductal epithelial cells resulted slightly positive, with a varying intensity of the different samples (Figure 3 C). In the infected tissues, the hyperplasia and inflammation were evident, but the expression of PTX3 in ductal epithelial cells didn't present signs of modulation (Figure 3 C). On the contrary, the apical positivity of glandular epithelial cells significantly increased in the infected tissues both in terms of positivity and intensity ($p = 0.0018$ and $p = 0.0001$ respectively) (Figures 3 C). Only in the infected udders we observed a massive presence of PMNs, where the majority of them (> 75%) was positive for PTX3 staining, with a varying intensity from cell to cell (Figure 3 C).

In the infiltrating leukocytes we observed a positivity of 50-75% of the cells, with high intensity of staining in both the experimental groups, with any modulation in the course of infection (Figure 3 C). The secretum remained intensely positive in both infected and non-infected udders (Figure 3 C).

The array hybridization analysis confirmed that PTX3 was significantly up-regulated in the infected tissues compared to the control ones. Moreover, twenty-one genes were found to be differentially expressed (p-value < 0.05 and log2 fold change > 1.5) between healthy and experimentally infected tissues. No genes showed a decrease in expression. The top ten genes (LTF, S100A2, RRAD, SOD2, PTX3, S100A9, IL18BP, C3, ICAM1, ANXA2) showed an important role in "Chemotaxis of cells", in "Inflammatory response" and in "Organization of cytoplasm" networks as predicted by Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Inc.).

IPA identified a series of functional categories differentially activated in infected tissues compared to the controls. The most representative one was "Cellular movement" (p = 7,48E-09) followed by "Cellular Growth and Proliferation" (p = 1,43E-04), "Cellular Movement, Immune Cell Trafficking" (p value = 9,22E-10), "Inflammatory Response" (p = 1,96E-07). Differentially expressed genes were mainly involved in functions such as migration and proliferation of cells, leukocyte migration and inflammatory response.

Based on IPA analysis~~,~~ PTX3 gene, overexpressed in experimentally infected tissues, is involved in the mechanisms of bacterial phagocytosis by phagocytes (antigen presenting cells, neutrophils and myeloid cells) neutrophils response and regulation of the inflammatory response (Figure 4).

3.4 The infected udder affects the inflammation in the contralateral one

After observing different levels of PTX3 expression in the control udders, in order to find out if the infection in one of the two udders could affect the inflammatory response of the contralateral one, we analyzed by Real Time PCR the expression levels of IL-1β in both uninfected and infected udders. As shown in Figure 5, some control udders showed high levels of expression of the pro-inflammatory cytokine~~,~~ at the same level of some infected udders.

3.5 Natural S. aureus infection causes PTX3 modulation in blood and milk cells

In order to study the modulation of PTX3 messenger in milk and blood cells during *S. aureus* infection, we collected samples from healthy and naturally infected goats. PTX3 was up-regulated in blood and milk cells of animals affected by *S. aureus* natural infection, compared to cells of healthy animals. Comparison of blood and milk cells, and MFG from animals suffering from *S. aureus* mastitis to those from healthy animals revealed that PTX3 mRNA was significantly up-regulated during infection in the circulating blood PMNs, while in the mononuclear cells there was only an increase trend of expression (Figure 6 A). PTX3 was also up-regulated in milk cells of infected animals, while in MFG of infected animals there was an increasing trend of expression (Figure 6 B).

4. Discussion

Mastitis is an inflammation of the mammary gland, mostly due to bacterial infections. It represents an important issue in the field of dairy ruminants, being one of the major causes of economic losses.

In order to better understand the pathogenesis of *S. aureus* mastitis in goat, we investigated the modulation of PTX3 during the immune response of the mammary gland. PTX3 is known to be involved in the inflammatory response of the udder in goats, but no data is available about its pattern of expression in healthy tissues, what kind of cells produces it and how it is modulated during *S. aureus* infection [9, 10, 26].

e searched for goat PTX3 messenger sequence in the data bases and we only found a predicted sequence, based on goat genomic sequences. This unique sequence was highly homologous to the PTX3 messenger of other species, confirming that this molecule is highly conserved among the animals. In particular, and as expected, it showed high levels of homology with other ruminants (> 90%) compared to monogastric mammals (from 75% to 85%). The most homologous species

proved to be sheep with 99% of homology. Cattle, buffalo, bison and deer highlighted numerous stretches of highly conserved sequence compared to the goat. The multiple alignment of PTX3 of different species confirmed, again, the conservation of the molecule, suggesting that an evolutionary pressure aims to preserve its structure and function among the animals [12]. We also observed a clear sequence homology between ruminants (cow, goat, sheep, deer, and buffalo) and cetaceans (dolphin and orca) based on the phylogenetic analysis of PTX3 sequence. Our data are supported by the results of paleontological [27], morphological [28] and molecular [29] studies, where the order Cetacea (whales, dolphins) seems to be more closely related to the order Artiodactyla (cows, camels, and pigs) compared to other orders of ungulates, Perissodactyla (horses), Hyracoidea (hyraxes), Proboscidea (elephants), and Sirenia (sea cows). In particular Graur et al. [29] proposed a Ruminantia/Cetacea clade.

The PTX3 gene is located on goat chromosome 1 and includes three exons. The number of exons proved highly conserved, as evidenced by publications in PTX3 of human and mouse [12].

Once we identified the sequence of the messenger, we designed specific primers to study the expression pattern of PTX3 in a large panel of healthy goat tissues and organs. We found levels of constitutive expression in tissues rich of hematopoietic cells of the myeloid lineage(bone marrow), tissues rich in endothelial cells(aorta and rectum) and in tissues rich of extracellular matrix (skin). Our results are in line with the expression pattern described for humans and mouse [17]. These data confirm, together with the sequence alignment analysis, that PTX3 is highly conserved during evolution, both in sequence and pattern of expression.

The high level of PTX3 expression in the udder is demonstrated also in other species (human and mouse) suggesting the protective role of this pentraxin in mammary gland, in order to fight infections and the onset of mastitis, as well as to transfer defense from the mother to the newborn [11].

In the remaining healthy tissues we observed low levels of PTX3 expression, accordingly to the fact that this molecule is induced during inflammation (stimulation with IL-1β, TNF-α, TLRs ligands) [3, 30, 31, 33].

PTX3 mRNA expression in blood cells of healthy animals is evident in both mononuclear cells and PMNs, the latter having a higher level of expression. This data is in contrast with the expression pattern in leukocytes in human and mouse and could represent a peculiarity of goats [16]. In infected animals we observed a significant increase in its expression in PMNs and an upward trend in mononuclear cells. In milk from healthy goats we found discrete levels of PTX3 expression in both MFG and somatic cells. These levels significantly increase in somatic cells of infected animals, probably because of an increase in PMNs, the major producers of PTX3 in goats.

The level of mRNA expression in MFG represents its expression in mammary epithelial cells [9]. Based on this, our results on PTX3 mRNA expression (whole mammary tissue and MFG) show a correspondence with the PTX3 protein expression we observed by immunohistochemical analysis. In healthy udder tissues, PTX3 is mainly expressed by glandular epithelial cells and very little from the epithelial cells of the ducts. The fact that PTX3 production is mainly in the glandular epithelial cells could be functional to the organism in certain physiological phases (like in colostrum for protection to the neonate) or in pathologic conditions (like deep bacterial infections), acting as a protective molecule [11]. In glandular epithelial cells PTX3 is expressed in the apical portion facing the lumen; this would explain the levels found in milk fat globules that originate precisely from the apical part of the above cells.

Finally, we studied how PTX3 expression, both as mRNA and as a protein, is modulated during *S. aureus* infection. The messenger is up-regulated in a statistically significant manner in the infected udder when compared to the respective healthy contralateral one. This finding is in agreement with other studies in the literature, where an increase of PTX3 messenger was observed in the course of mammary infection [9, 10]. In the early stage of the infection, PTX3 gene expression confirmed its involvement in the leukocyte trafficking, particularly in the recruitment of neutrophils in the

infected tissue. PTX3 showed the characteristic of innate immune protein since it stimulates the phagocytosis of bacteria, initiating the classical pathway of complement activation. The protein is significantly up-regulated in glandular epithelial cells, but not in the ducts, in macrophages or in secretum, where its expression remains at the same levels of healthy tissues. Only in infected tissues, we observed a massive presence of PMNs in the alveoli. The PMNs expressed varying levels of PTX3, probably depending on their degree of activation and degranulation. These results suggest that the early PTX3 response to invading *S. aureus* is mainly mediated by glandular epithelial cells and neutrophils and less by alveolar macrophages.

In control udder tissues we observed highly variable levels of PTX3 mRNA, in some cases similar to infected tissues. This prompted us to investigate whether the experimental infection in one of the udders could influence the inflammatory condition of the healthy contralateral one. We analyzed the expression levels of IL-1β as a marker of inflammation. Healthy udders with IL-1β levels comparable to those infected were the same ones that expressed higher levels of PTX3 and their contralateral udders were those with the highest values of IL-1β and PTX3. This shows that the monolateral infection causes intense inflammatory response, probably followed by a systemic response that influences the inflammatory status of the contralateral udder, where the mastitis agent is absent. The systemic response is also supported by the fact that circulating PMNs and mononuclear cells of goats affected by *S. aureus* mastitis show an up-regulation of PTX3.

5. Conclusion

Our observations confirm the potential of PTX3 as an early marker of mastitis. In fact, it is a secreted protein that could be dosed both in blood serum/plasma and in skimmed milk/whey. PTX3 is also modulated (up-regulated) in the course of mammary infection both as a messenger and as a protein. Further studies are needed to see if the modulation is induced only by certain pathogens or if it is present in any type of infection. It will also be necessary to understand if there is a correlation

between the number of somatic cells (since this is one of the most used parameters as mastitis index) and the increase of PTX3 in milk.

~~Moreover~~ Finally, exogenous PTX3 could also be developed as therapeutic tool in the control of intra-mammary infections [32].

In conclusion PTX3 seems a good candidate as a biomarker or biological agent in the fight against mammary infections in dairy ruminants, but further studies are needed to set up its use as a diagnostic tool or a therapeutic drug.

Declarations of interest: none

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FIGURE CAPTIONS

Figure 1. Phylogenetic tree of PTX3 mRNA

Phylogenetic tree obtained after PTX3 mRNA sequences alignment of several species, using the free software on the http://www.phylogeny.fr/simple_phylogeny.cgi web site. This cladrogram represents a Neighbour-joining tree without distances corrections (Dereeper A., Guignon V., Blanc G., Audic S., Buffet S., Chevenet F., Dufayard J.F., Guindon S., Lefort V., Lescot M., Claverie J.M., Gascuel O. Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res. 2008 Jul 1;36(Web Server issue):W465-9. Epub 2008 Apr 19).

Figure 2. PTX3 mRNA pattern of expression

The PTX3 messenger expression was analyzed in different organs and tissues by qPCR. A) expression of PTX3 in a wide panel of organs sampled from two healthy goats. B) expression of PTX3 in whole blood, PMNs and mononuclear cells from 6 healthy goats. C) expression of PTX3 in milk fat globules (MFG) and cells from 6 healthy goats. The gene expression level of the target gene was normalized to GAPDH and the results are presented as Arbitrary Units (AU). Results are expressed as mean ± 1 standard error in all the panels.

Figure 3. Modulation of PTX3 expression during experimental *S. aureus* intramammary infection

Six healthy goats were experimentally infected in the left udder with *S. aureus*, whereas the contralateral udder was infused with sterile PBS as control. Thirty hours post infection the animals were sacrificed and biopsies were taken from both udders. A) PTX3 mRNA expression of not infected and infected udders was investigated by qPCR. The gene expression level of the target gene was normalized to GAPDH and the results are presented as Arbitrary Units (AU). The samples from the two udders of the same animal are linked. Mann-Whitney test. B) Immunohistochemistry of PTX3 in control (bar=50 micron) and infected udders (upper panel bar=18 micron; lower panel bar=50 micron). C) Immunohistochemistry positivity score graphs (0.5= <25%; 1= 25-50%; 2= 50-75%; 3= >75% of positive cells). Student's T or Mann-Whitney test. D) Immunohistochemistry intensity score graphs (0.5= weak; 1= slight; 2= moderate; 3= intense). Student's T or Mann-Whitney test.

Figure 4: Ingenuity Pathways Analysis (IPA) for PTX3 gene.

Network analysis for "Categories with Diseases and Functions". The gene is labelled in red, indicating upregulation. Discontinuous line represents indirect interaction with "activation" (orange), "inhibition" (blue), "findings inconsistent with the state of downstream molecules" (yellow) and "non-predicted" effects (grey).

Figure 5. Expression of IL-1β during experimental *S. aureus* intra-mammary infection

The IL-1β messenger expression was analyzed in not infected and infected udders by qPCR. The samples from the two udders of the same animal are linked. The gene expression level of the target gene was normalized to GAPDH and the results are presented as Arbitrary Units (AU). The samples from the same animal are linked. Mann-Whitney test.

Figure 6. Modulation of PTX3 expression during natural *S. aureus* intra-mammary infection

The PTX3 messenger expression was investigated in blood and milk from 4 healthy and 3 naturally infected goats by qPCR. A) PTX3 expression in blood PMNs and mononuclear cells. B) PTX3 expression in MFG and milk cells. The gene expression level of the target gene was normalized to GAPDH and the results are presented as Arbitrary Units (AU). The samples from the same animal are linked. Mann-Whitney test.

Fig 1

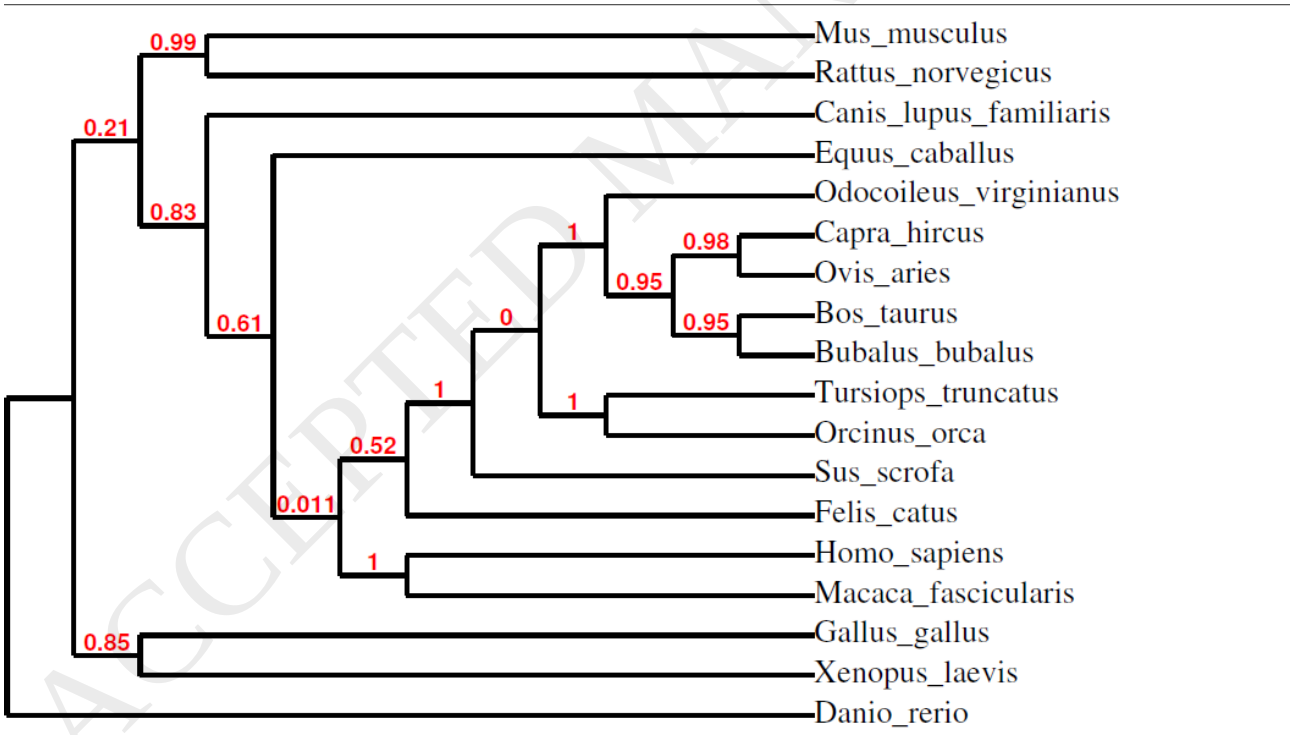

Fig 2

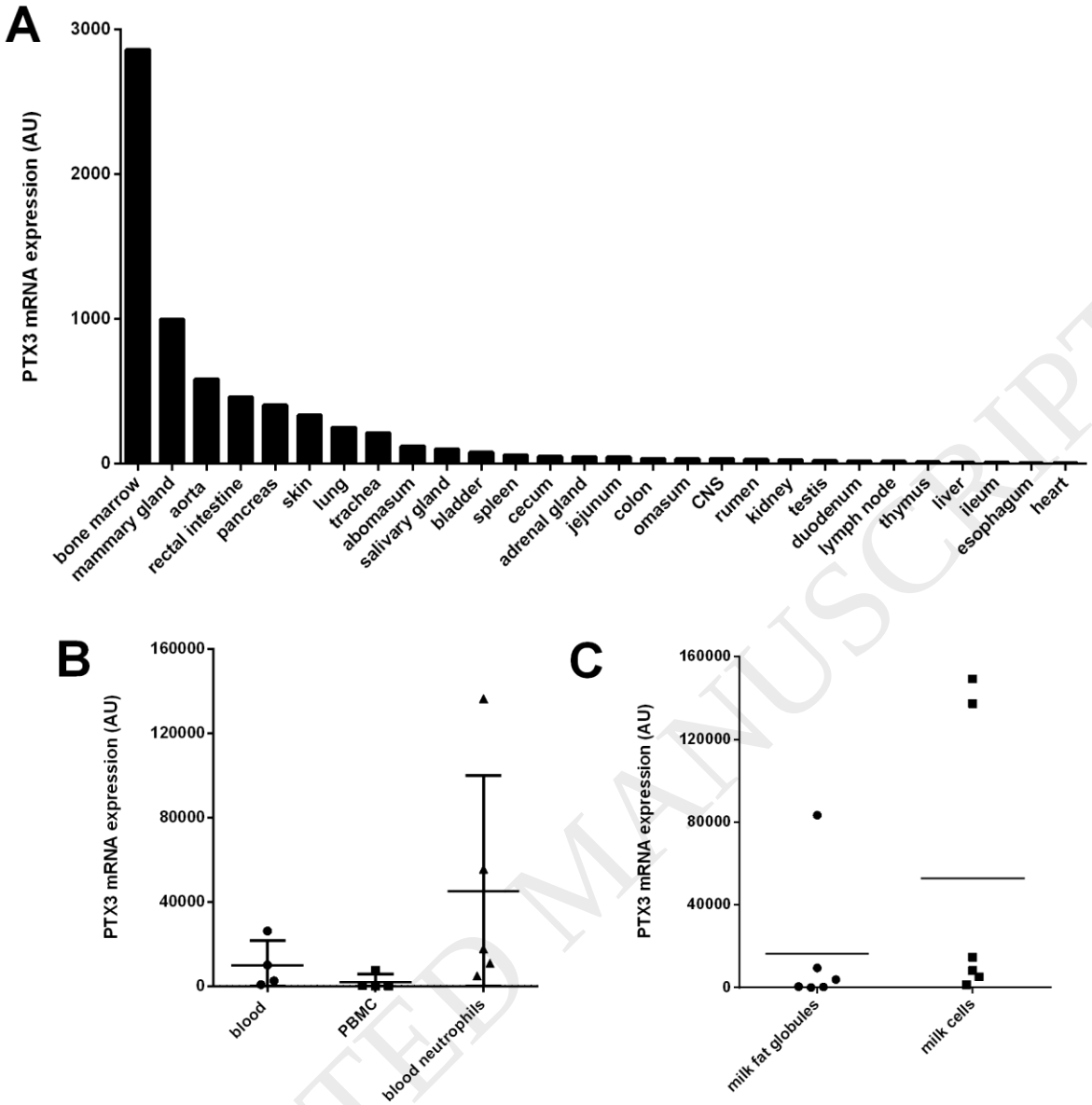

Fig 3

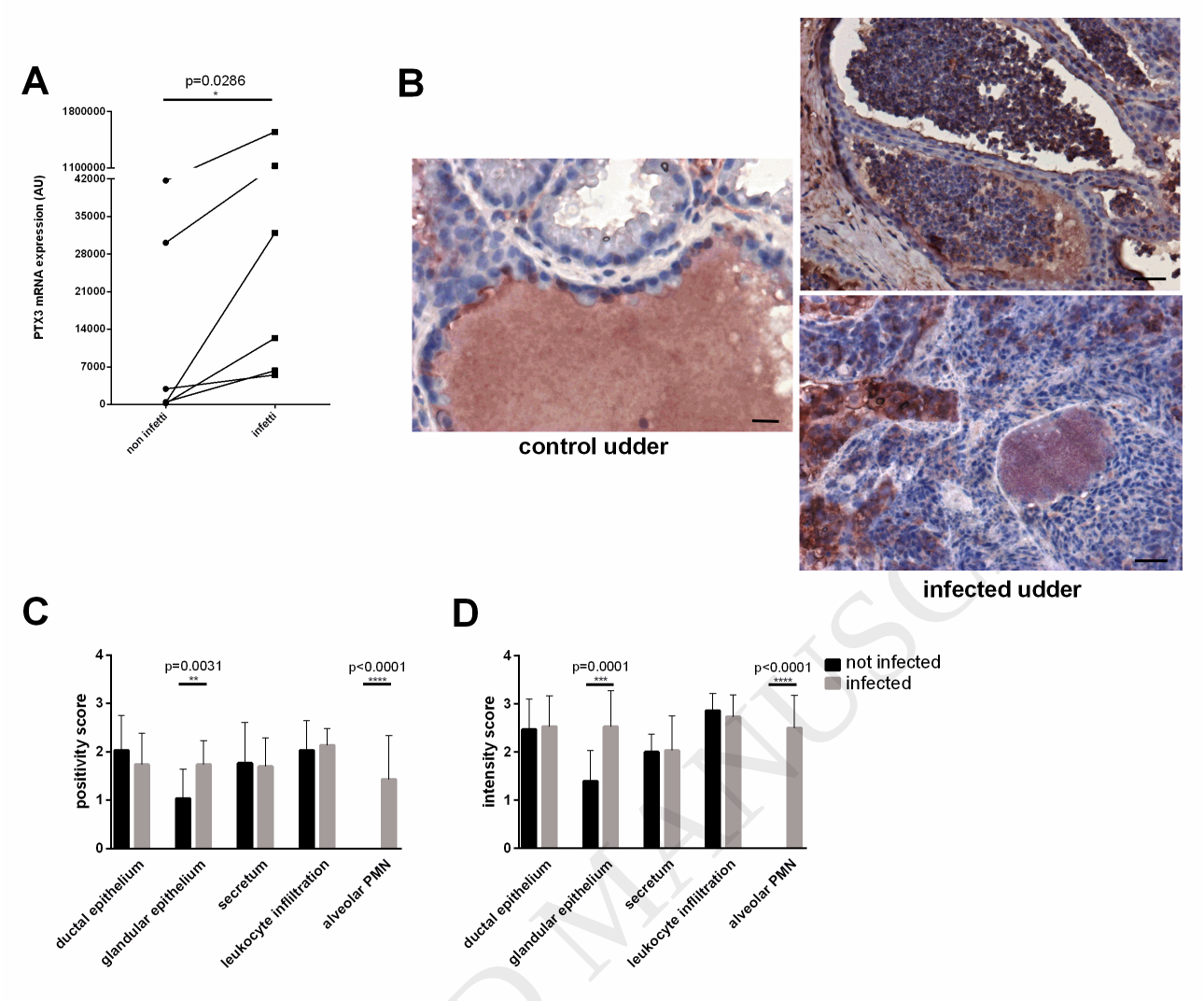

Fig 4

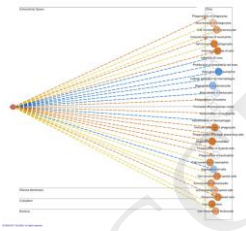

Fig 5

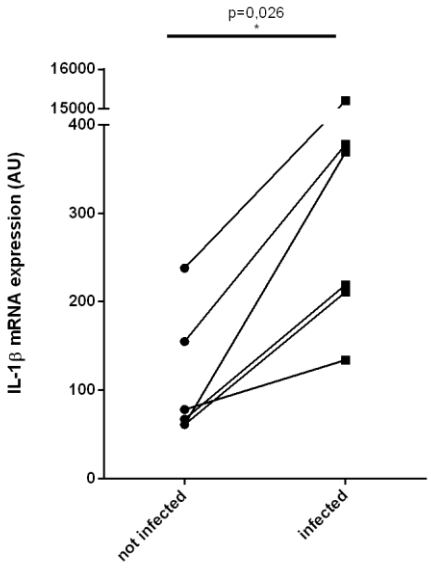

Fig 6

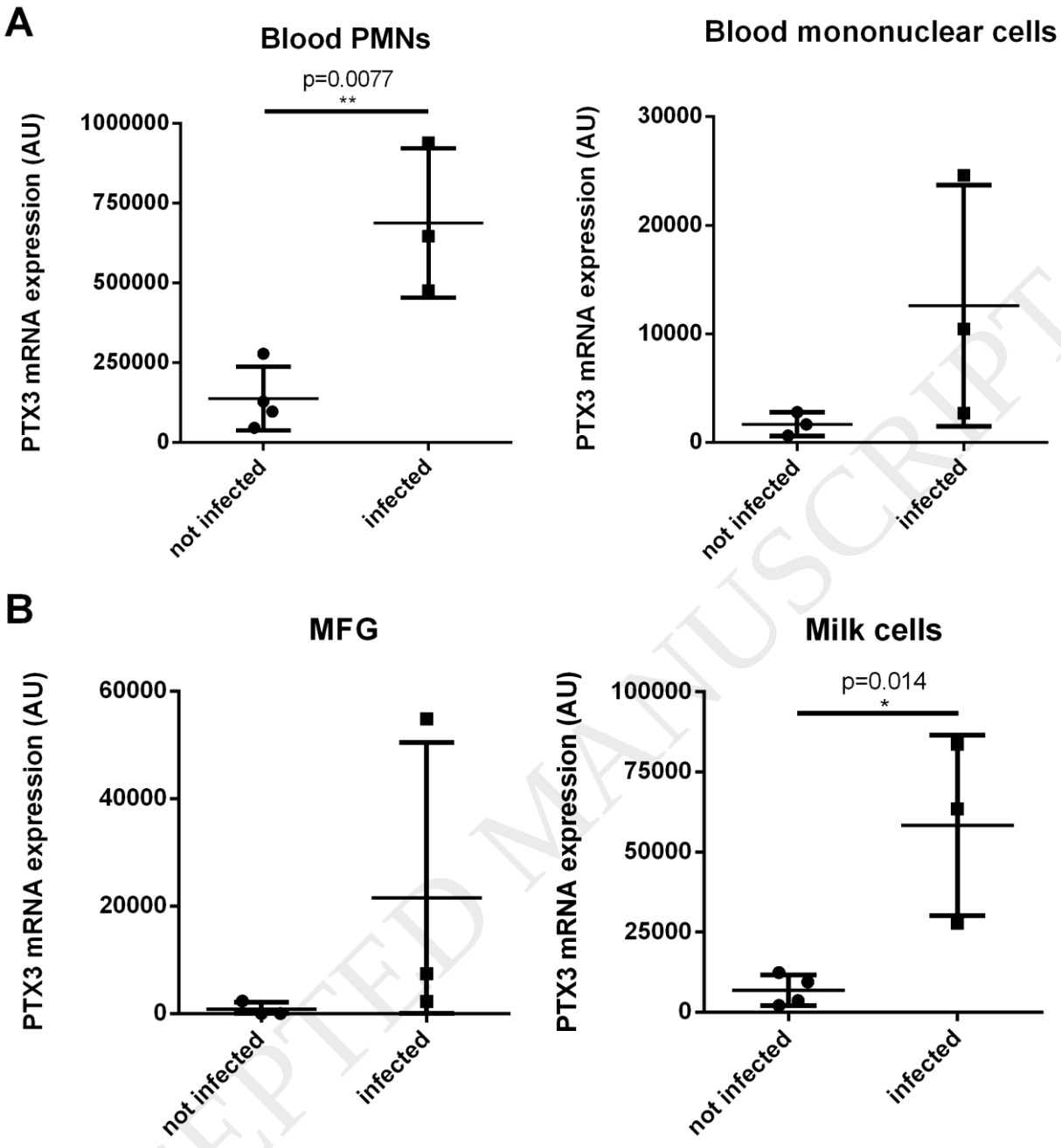

Table 1. Primer sequences.

Primer	**Sequence (5'-3')**
Goat PTX3 for	TCCATCCCACTGAGGACCC
Goat PTX3 rev	TCTCCAGCATGGTGAAGAGCT
bIL-1β for	CTGTTATTTGAGGCTGATGACC
bIL-1β rev	TTGTTGTAGAACTGGTGAGAAATC
GAPDH for	GGCGTGAACCACGAGAAGTATAA
GAPDH rev	CCCTCCACGATGCCAAAGT