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ORIGINAL ARTICLE

Innate immunity gene expression profiles in conjunctival membrane biopsies from Amazonian buffalo

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ABSTRACT

Domestic buffalo production plays an economically important role in the Brazilian Amazon, but they are susceptible to many diseases favored by the tropical climate and annually flooded habitats, including ocular diseases. In this context, it is important to select genotypes that maximize innate ocular immunity in Amazonian herds. We aimed to characterise, for the first time, gene expression profiles of the innate immune system in the conjunctival membrane of buffalo. Ocular conjunctival tissue samples were collected from 60 clinically healthy slaughtered animals in the northern Brazilian state of Amapá. The samples were histologically processed for classification into three groups according to the quantitative degree of lymphoid tissue associated with the conjunctiva (discrete, G1; slight, G2; and moderate, G3 presence of lymphoid tissue). RT-PCR was used to quantify gene expression of inflammatory cytokine (IL6, IL10, TNFA, IFNG), Toll-like receptor 4 (TLR4), and Defensin beta 110 (DEFB110), relative to the endogenous GAPDH gene. G1 animals presented low expression for IL6, IL10, TNFA, and DEFB110, while G2 exhibited high expression for IL6, IL10, IFNG, and TLR4. All G3 animals showed high expression for all tested genes. These results suggest a greater resistance to pathogenic microorganisms of buffalos in the G3 group, and the proportion of lymphoid tissue associated with the conjunctiva may be related to the immune resistance of individuals.

KEYWORDS: *Bubalus bubalis*, lymphoid tissue, inflammatory cytokines, interleukin, TLR, DEFB110

Perfis de expressão de genes da imunidade inata em biopsias de membrana conjuntival de búfalos da Amazônia

RESUMO

A produção de búfalos domésticos desempenha um papel economicamente importante na Amazônia brasileira, mas eles são suscetíveis a muitas doenças favorecidas pelo clima tropical e habitats inundados anualmente, incluindo doenças oculares. Nesse contexto, é importante selecionar genótipos que maximizem a imunidade ocular inata em rebanhos amazônicos. Objetivamos caracterizar, pela primeira vez, perfis de expressão gênica do sistema imune inato na membrana conjuntival de búfalos. Amostras de tecido conjuntival ocular foram coletadas de 60 animais clinicamente saudáveis abatidos no estado do Amapá, norte do Brasil. As amostras foram processadas histologicamente para classificação em três grupos de acordo com o grau quantitativo de tecido linfoide associado à conjuntiva (discreta, G1; leve, G2; e moderada, G3 presença de tecido linfoide). RT-PCR foi utilizado para quantificar a expressão gênica de citocinas inflamatórias (IL6, IL10, TNFA, IFNG), receptor Toll-like 4 (TLR4) e Defensina beta 110 (DEFB110), em relação ao gene GAPDH endógeno. Os animais do G1 apresentaram baixa expressão para IL6, IL10, TNFA e DEFB110, enquanto G2 exibiu alta expressão para IL6, IL10, IFNG e TLR4. Todos os animais do G3 apresentaram alta expressão para todos os genes testados. Esses resultados sugerem maior resistência aos microrganismos patogênicos dos búfalos do grupo G3, e a proporção de tecido linfoide associado à conjuntiva pode estar relacionada à resistência imunológica dos indivíduos.

PALAVRAS-CHAVE: *Bubalus bubalis*, tecido linfoide, citocinas inflamatórias, interleucina, TLR, DEFB110

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INTRODUCTION

Domestic buffalo (*Bubalus bubalis* H. Smith) production is a globally important economic system for beef, milk, and mozzarella cheese production (Safari *et al*. 2018). Brazil has approximately 1.4 million buffalo, with the largest population in the northern region of the country, in the Brazilian Amazon. In this region, the states of Pará and Amapá house the largest herds (IBGE 2019).

Buffalo are considered phenotypically well-adapted to the Amazon region (Safari et al. 2018). They spend many hours of the day in wet areas to help regulate their body temperature, but which can also lead to reduced immune responses and greater susceptibility to a variety of diseases (Garcia 2013; Rehman *et al*. 2021). The susceptibility or resistance to diseases is influenced by genetic factors and effects linked to environmental conditions (Rehman *et al*. 2021; Javed *et al*. 2021). In addition, extensive flooding of pastures during the Amazon rainy season form favorable habitats for the maintenance of a wide variety of microorganisms (Butenschoen *et al*. 2011), including some parasites and vectors (Rehman *et al*. 2021).

The most prevalent diseases of buffalo in the Amazon are brucellosis (Batista *et al*. 2019), tuberculosis (Carneiro *et al*. 2019), hemoparasitic diseases, mainly caused by *Babesia* spp. and *Theileria* spp. (Silveira *et al*. 2016), and trypanosomiasis (Pérez *et al*. 2020). Eye diseases are also frequent (Khalaf *et al*. 2021), and are related to habitat and environmental factors, age of animals, and management practices, which influence the composition of the conjunctival flora or ocular immunity reduction (Johns *et al*. 2011). Ophthalmic diseases cause damage to vision, reducing grazing duration, growth, and weight gain (Handool 2013).

To select genotypes with the best resistance to ocular diseases resistance, to maximize the health and reduce economic loss in buffalo herds, the expression of candidate genes related to the ocular innate immune system can be used as molecular markers for phenotipic research (Boichard *et al*. 2016). In humans, the determination of the expression of genes linked to innate ocular immunity, such as inflammatory cytokines, Toll-like receptors and antimicrobial peptides such as defensins, has been widely described (Redfern *et al*. 2011; Chen *et al*. 2018; Da Cunha *et al*. 2018). However, there is little information for other species (Varandas *et al*. 2020). In buffalo, there is still no information on this subject, and the aim of our study was to determine the expression profiles of genes related to the innate ocular immune system relative to the proportion of lymphoid cells in the ocular conjunctival membrane of buffalos in the Brazilian Amazon.

MATERIAL AND METHODS

Sample collection and ethical aspects

All aspects of this research were conducted in accordance with the animal welfare standards determined by CONCEA (Conselho Nacional de Controle de Experimentação Animal) of Brazil and approved in June 10, 2015 by the ethics commitssion on the use of animals (Comissão de Ética no Uso de Animais - CEUA) of Universidade Federal Rural da Amazônia (protocol # 033/2015 CEUA/UFRA).

We sampled 60 male and female, clinically healthy adult buffaloes of mixed breed aged between 5 and 7 years originating from extensive farming properties and destined for meat production in the city of Santana, Amapá state (Brazil) (0°2'39"S, 51°10'42"W).

Tissue samples of the conjunctival ocular membrane were collected about five minutes after the animals were slaughteried in the state slaughterhouse of Santana, linked to the state inspection service. Subsequently, two fragments of the conjunctival membrane of one of the eyes of each animal were sectioned. One of the fragments of about 5 cm was stored at room temperature in a polypropylene container containing 10% buffered formalin for histological analysis. The second fragment, of about 2 cm, was kept in a 2-ml polypropylene tube containing RNAlater solution (Invitrogen, Carlsbad, CA, USA) and later stored at -80 °C for the analysis of gene expression.

Histological processing and evaluation

Histological slides were prepared using the standard protocol of Prophet *et al*. (1992). The formalinized fragments were cleaved and processed in increasing concentrations of ethyl alcohol and xylol, followed by impregnation with paraffin, cleavage down to 4 µm thick and staining with hematoxylin and eosin (H&E).

The prepared slides were evaluated under a light microscope by a single pathologist. The samples were classified and grouped according to the presence and quantitative degree of conjunctiva-associated lymphoid tissue (CALT) as follows: discrete presence of lymphoid tissue (<10% lymphoid cells by high-power field - HPF), light presence (between 10% and 25% of lymphoid cells by HPF), moderate presence (between 25% and 50% of lymphoid cells by HPF) and high degree (> 50% of lymphoid cells by HPF). No case of high-degree presence was identified (Figure 1).

RNA extraction

RNA extraction was performed using the 2-cm samples. Around 100 mg of conjunctival tissue were macerated in liquid nitrogen and treated with TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's recommendations. Subsequently, the RNA was diluted in ultrapure water and stored at -80 °C. After extraction, RNA

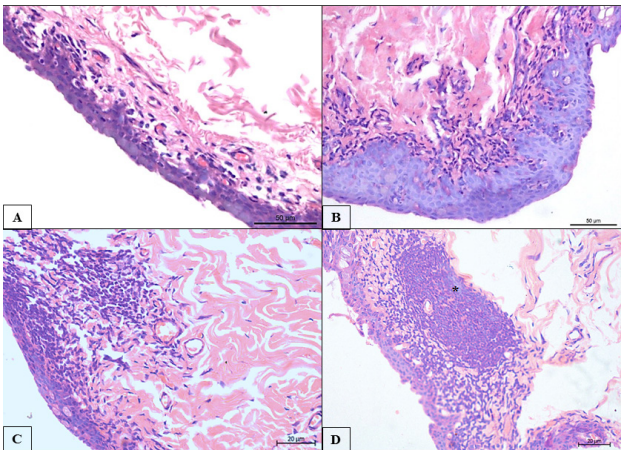

Figure 1. Ocular conjunctival membrane of buffalos in the Amazon. Photomicrographs showing different categories of conjunctiva-associated lymphoid tissue (CALT) amount in the epithelial and subepithelial layers: A – discrete; B – slight; C – moderate; D – lymphoid tissue with follicle arrangement (*). Stained in H&E (hematoxylin and eosin). Scale bar = 200µm. This figure is in color in the electronic version.

was quantified using the BioDrop Touch Duo™ UV-Vis spectrophotometer (BioDrop, Cambridge, England, UK), using absorbances of 260 and 280 nm and a A280/A260 ratio. Samples with A280/A260 ratio > 1.9 were considered adequate for the analysis.

Gene expression patterns

For the gene expression analyses, the samples were categorized into three groups according to the histological analysis: G1 (discrete lymphoid tissue) (20 samples), G2 (light lymphoid tissue) (30 samples), and G3 (moderate lymphoid tissue) (10 samples). No sample with high degree of lymphoid tissue was identified. All the samples presented high degree of purity (A260/A280 ratio > 1.9).

Primer sequences were developed and designed using the Primer3 program (http://bioinfo.ut.ee/primer3-0.4.0/) for the genes Interleukin 6 (IL6), Interleukin 10 (IL10), Tumor Necrosis Factor alpha (TNFA), Interferon gamma (IFNG), Toll-like 4 Receptor (TLR4), and Defensin beta 110 (DEFB110). A constitutive gene, the GAPDH for *Bubalus bubalis* was used as a control (endogenous) (Table 1).

The samples were then submitted to Real-Time Polymerase Chain Reaction (RT-PCR) to quantify the mRNA expression of the selected genes using the Power Sybr® Green RNA-to-CT™ one-step Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommendations for a final volume of 10 µL. Each sample was analyzed in triplicate. All reactions were performed on the CFX96 Touch ™ Real-Time Detection System (Bio-Rad, Hercules, CA, USA). The Ct (threshold cycle) values were obtained, and relative gene expression values were determined using the equation $2^{-\Delta Ct}$, where ΔCt is the difference between target gene Ct and endogenous gene Ct (Livak and Schmittgen 2001).

Table 1. Primer sequences designed according to exon and Genbank identity (ID) for buffalo gene expression analyses.

Gene	Primer sequences (5`-3`)	Exon	GenBank ID
IL6	F:GCTCTCAGGCTGAACTGTAGG R:TGCAGTCCTCAAACGAGTGG	5	001290980.1
IL10	F:GGGGTGTCTACAAAGCCATGA R:TGCATCTTCGTTGTCACGTA	5	001290845.1
TNFA	F:ACTGAGGCGATCTCCCTTCT R:GGTCAACATCCTGTCTGCCA	4	006041931.1
IFNG	F:CAGATCATTCAAAGGAGCATGGA R:GTCCTCCAGTTTCTCAGAGCT	3	102416494
TLR4	F:TTTATTCCTGGGGTGGCCAT R:CTCTGGATGAAGTGCTGGGA	1	102407022
DEFB110	F:CTGTTGGAAATACTTTGTCACCA R:TTTCCTCTGTACCATATTGTGCA	2	102395067
GAPDH	F:ACCCAGAAGACGGTGGATG R:CCGTTGAGCTCAGGGATGA	7	102404028

IL6: Interleukin 6; IL10: Interleukin 10; TNFA: Tumor Necrosis Factor alpha; IFNG: Interferon gamma; TLR4: Toll like 4 Receptor; DEFB110: Defensin beta 110.

Statistical analysis

The normal distribution of the gene expression variables was assertained with the Kolmogorov-Smirnov test. All data were compared among lymphoid-tissue categories using one-way ANOVA, and pairwise differences between groups were tested with the post-hoc Tukey test. The significance level was set at 0.05.

RESULTS

Gene expression patterns

G1 samples had low gene expression for the majority of genes analyzed, with high gene expression only for IFNG and TLR4 (Figure 2). G2 samples showed high expression for IL6, IL10, IFNG, and G3 showed high expression for all genes (Figure 2).

The average relative expression for gene IL6 was significantly higher ($F = 8.37$, $df = 6$, $P = 0.0184$) in G3 than G1, however, there was no significant difference between G3 and G2 or between G2 and G1. For IL10, relative gene expression differed significantly among all groups, with G3 showing the highest values ($F = 25.72$, $df = 6$, $P = 0.0011$). For IFNG and DEFB110 relative gene expression in G3 was significantly higher than in G2 and G1 ($F = 87.60$, $df = 6$, $P < 0.0001$; $F = 13.81$, $df = 6$, $P = 0.0057$, respectively). There were no significant differences in relative gene expressions among groups for TNFA and TLR4.

DISCUSSION

This study demonstrated, for the first time, mRNA expression levels of cytokine inflammatory genes and other factors related to innate immunity in ocular conjunctiva of buffalos. The genes tested in this study have all been widely evaluated in humans for their genotypic characterization

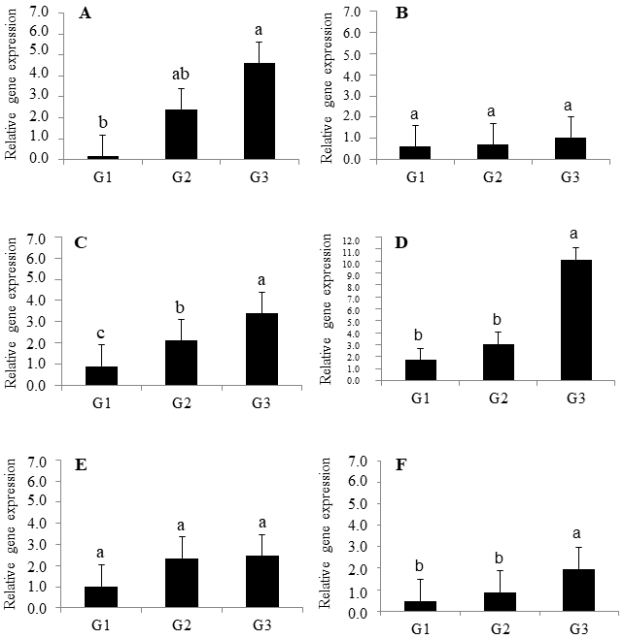

Figure 2. Relative gene expression of the IL6 (A), TNFA (B), IL10 (C), IFNG (D), TLR4 (E) and DEFB110 (F) genes in groups with discrete (G1), slight (G2) and moderate G3) amount of conjunctiva-associated lymphoid tissue (CALT) in the epithelial and subepithelial layers of the ocular conjunctival membrane of buffalos from Amapá, Brazil, according to the equation $2^{-\Delta Ct}$. Different letters above the bars indicate significant pairwise comparisons according to the Tukey test ($P < 0.05$).

of innate immunity (Garreis *et al*. 2010; Da Cunha *et al*. 2018; Rajaee *et al*. 2018), as well as in other animal species (Tirumurugaan *et al*. 2010; Turchetti *et al*. 2015; Ateya *et al*. 2017; Varandas *et al*. 2020). However, prior to this study, there was no research on buffaloes in relation to these genes in ocular tissues, only in other tissues (Vahanan *et al*. 2008; Shi *et al*. 2017; Batra *et al*. 2019).

Cytokines IL6, IL10, TNFA, and IFNG were also determined in water buffalo through serum levels and gene expression in liver tissue after experimental infection with *Fasciola gigantica* (Shi *et al*. 2017). Previous studies have analyzed cytokine gene expression from dogs with allergic conjunctivitis (Varandas *et al*. 2020), and gene expression in the human eye (Garreis *et al*. 2010; Redfern *et al*. 2011; Chen *et al*. 2018; Da Cunha *et al*. 2018).

From a histological perspective, most animal species have lymphoid tissues associated with the conjunctiva, however, there are few lymphocytes or lymphoid aggregates in the normal conjunctival membrane of rodents (Forrester *et al*. 2010), similar to what we observed in our buffalo samples, which had mostly a discrete to mild degree of CALT in the mucosa.

The high levels of gene expression, including the main inflammatory cytokines, in G3 individuals suggests their greater resistance to pathogenic microorganisms when compared to individuals from G1 and G2. The relatively higher levels of lymphoid tissue associated with the conjunctiva in G3 can be explained by the production of cytokines, mainly by lymphocytes and macrophages (Kak *et al*. 2018). They are the main cells of the lymphoid tissue associated with the ocular conjunctiva (Forrester *et al*. 2010). These genetic characteristics must be considered when the phenotypic factors that interfere with the individual's immune response are excluded from variables, such as the involution of lymphoid tissue with advancing age (Mastropasqua *et al*. 2017). The expression of cytokines in the conjunctiva of dogs is directly associated with the type of cells that produce them, which was observed when healthy dogs were compared with dogs affected by allergic conjuctivitis (Varandas *et al*. 2020). However, the variation in gene expression in our study was observed in clinically healthy animals with different proportions of lymphoid tissue in the ocular conjunctiva.

Beta defensins are also produced in large proportion by macrophages, in addition to other cell types related to the immune system, such as mucosal epithelial cells and dendritic cells (Garreis *et al*. 2010). These characteristics are presumed to be the reason for the high expression of the DEFB110 gene in G3 individuals compared to G1 and G2. In contrast, TNFA and TLR4 genes showed the same levels of expression among groups, with TNFA being the only analyzed cytokine that did not show higher expression in G3. These results are probably due to the higher expression of IL10 in G3, which resulted in a suppressive effect on macrophages and lymphocytes producing TNFA, as IL10 has anti-inflammatory action (Lyer and Cheng 2012). In the case of TLRs, it is assumed that, because they are receptors present especially in epithelial cells (Tirumurugaan *et al*. 2010), including the conjunctiva and cornea (Redfern *et al*. 2011), their expression is independent of the number of cells that make up the lymphoid tissue.

In the last two decades, there has been progress in the identification and quantification of gene expression of TLRs in farm animals such as cattle (Ateya *et al*. 2017), sheep (Chang *et al*. 2009), goats (Tirumurugaan *et al*. 2010) and buffalos (Vahanan *et al*. 2008; Shi *et al*. 2017), showing their important role in disease control as these receptors are implicated in resistance levels of the whole organism, particularly in epithelial tissues (Novák 2014). The TLR4 gene showed relatively high average expression in our samples, as did the IL6, and IL10 genes in most samples. IL6 was tested as a vaccine adjuvant for bovine infectious keratoconjunctivitis and showed high efficiency in the ocular treatment of infected cattle (Di Girolamo *et al*. 2012).

IFNG was expressed in the conjunctival membranes of all groups. IFNG gene expression was also observed in *B. bubalis* in the liver tissue after experimental infection with *Fasciola gigantica*, Cobbold, 1855 (Shi *et al*. 2017) and in bovine species (*Bos indicus* L. *and Bos taurus* L.) after vaccination

for *Brucella abortus* (Schmidt, 1901) Meyer and Shaw, 1920 (Verma *et al*. 2017).

We were not able to determine degrees of resistance or susceptibility to diseases in the animals evaluated, since no field studies were conducted for the evaluation of ocular innate immunity genotypes in association with the phenotype of immunological status within the buffalo population studied.

CONCLUSIONS

Gene expression evaluation served as a basis for defining the innate immunological processes that occur in the conjunctival membrane of buffalos with no apparent ocular clinical signs and may contribute to future phenotypic research related to disease resistance or susceptibility in this species. Our study demonstrated that Toll-like receptors and inflammatory cytokines, especially interleukins and gamma interferon, are present in the ocular conjunctival epithelium mucosa and can play an important role in protecting the ocular surface of buffalos. This information reveals that inflammatory cytokine genes and toll-like receptors can be applied as molecular markers in buffalo genetic improvement programs to select animals more resistant to ocular pathogens. While beta defensin and tumor necrosis factor were poorly expressed genes in the conjunctival membrane, further studies are needed to assess the expression of these genes in animals with conjunctival acute inflammatory conditions to determine their contributions to innate immunity in buffalo eye mucosa.

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