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Gene expression of cytokines in the blood of domestics cats infected and not infected by *Feline alphaherpesvirus*

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ABSTRACT. The *Feline alphaherpesvirus 1* (FeAHV1) is commonly identified as the causative agent of respiratory and ocular disease in domestic cats. Studies on the expression of cytokine genes are relevant, as these genes can be used as molecular markers for the diagnosis of feline viral rhinotracheitis. In this sense, the aim of this study was to characterize, for the first time, gene expression profiles of cytokines in the blood of domestic cats infected with FeAHV-1 in Belem, state of Pará, Brazil from September 2020 to March 2021. Ocular secretion samples from 55 domestic cats (domiciled cats and shelter cats) were collected using sterile swabs, and subjected to deoxyribonucleic acid (DNA) extraction. These samples were evaluated using the conventional PCR technique to identify FeAHV1. Blood samples were also collected from 55 domestic cats for the expression study. After diagnosis of the virus, 12 blood samples were subjected to ribonucleic acid (RNA) extraction, of these samples, 6 were positive and 6 negative for FeAHV-

1, subsequently the gene expression of six cytokines and the endogenous GAPDH gene was performed in these samples using the RT-PCR technique. The results revealed that of the 55 cats, nine cats were positive for FeHV-1 infection. The gene expression of cytokines performed in 12 samples showed that the cytokines TNF-α and IL-6 were significant, TNF-α showed higher expression in negative samples for the virus and IL-6 higher expression in positive samples for FeHV-1. Cytokines IL-1β, IFN-α, IFN-β and IFN-γ did not show significance. The present study provided data on the behavior of the immune system in cats infected with FeAHV-1 and may contribute to future research on new forms of treatment for feline viral rhinotracheitis.

Key words: FeAHV-1; *Felis catus*; Immune system

INTRODUCTION

The need for new techniques for the prevention and diagnosis, as well as treatment of diseases are increasingly sought in veterinary medicine to improve the quality of life of animals (Araújo et al., 2010). In this sense, studies on diseases in domestic cats are essential, especially in relation to infectious diseases, such as feline viral rhinotracheitis caused by the *Feline alphaherpesvirus 1* virus (FeAHV1) which belongs to the order *Herpesvirales*, family *Orthoherpesviridae*, subfamily *Alphaherpesvirinae* and genus *Varricellovirus* (ICTV, 2023). This virus is implicated as a widespread and common cause of upper respiratory and ocular disease in cats (Lewin et al., 2020). In addition to infecting felines, viruses of the *Orthoherpesviridae* family affect other animal species such as mammals, birds and reptiles (ICTV, 2023).

Unlike tumor viruses that have multifunctional viral oncogenes that drive viral replication and oncogenesis, herpesviruses interfere with the host's innate immune response in many different ways, and this feature is important for the establishment of infection (Tian et al., 2018). Although several studies have been carried out in mouse models, the antiviral immune system of cats remains poorly investigated, and many questions remain unresolved, such as the behavior of cytokines in the face of FeAHV-1 infection (Capozza et al., 2021). Cytokines are proteins produced by innate and acquired immune cells, and mediate various functions of these cells and other cell types. Cytokine production takes place in response to microorganisms and other antigens, and they stimulate responses from cells involved in immunity and inflammation (Abraha, 2020).

A study evaluating the expression of cytokines in nasal tissue samples from cats with feline *alphaherpesvirus* was performed (Johnson and Maggs, 2005). And since then, studies evaluating the expression of cytokines in other animal species have been conducted, in pigs experimentally infected with *Suid alphaherpesvirus* (Verpoest et al., 2017), peripheral blood mononuclear cells (PBMC) from horses infected with *Equine alphaherpesvirus 4* (Hue et al., 2017) and in PBMC from chickens vaccinated with *Meleagrid alphaherpesvirus 1* (HVT) and challenged with the RB1B strain of *Gallic alphaherpesvirus 2* (Parvizi et al., 2015).

We identified FeAHV1 in domestic cats using the conventional PCR technique and subsequently determined, for the first time, gene expression profiles of cytokines: TNF-α,

IL-6, IL1-β, IFN-α, IFN-β, IFN- γ in the blood of FeAHV1 positive and negative cats in Belém, Pará state, Brazil.

MATERIAL AND METHODS

All procedures were conducted according to Conselho Nacional de Controle de Experimentação Animal (CONCEA) guidelines. This study was submitted and approved by the Commission for Ethics in the Use of Animals (CEUA) of the Universidade Federal Rural da Amazônia (UFRA) and granted approval number 6531300620 (ID 000203).

Samples

The study was carried out with samples of 55 cats seen at the Infectious Diseases clinic of the Veterinary Hospital of UFRA (HOVET) shelter and companion animal households in several neighborhoods in the city of Belém, state of Pará, Brazil, from September 2020 to March from 2021. For the gene expression study, blood samples were collected by cephalic venipuncture and an RNAlater™ stabilization solution (Invitrogen, Carlsbad, CA, USA) was used for storage. Ocular secretion samples were also collected using swabs for the investigation of the virus.

Molecular diagnosis

Genomic DNA was extracted from 55 samples of conjunctival secretions using the commercial gDNA extraction kit from Bio gene (Quibasa, Belo Horizonte, MG, BR) according to the manufacturer's recommendations. The FeAHV1 primers were designed according to the Thymidine Kinase UL23 gene sequences. The forward primer (5'-ACTTCCACGAGAACCTCCTG-3') and the reverse primer (5'-CCGGGCTTTGAAAACACTGA-3') amplify a target fragment of 316 base pairs.

The reaction for FeAHV-1 diagnosis was performed in a final volume of 20 µL containing 1x Taq Pol Master Mix 2x (Cellco), 2 µL of extracted DNA (approximately 50ng/µL) and 5 mmol/µL of each primer. DNA extracted from the Felocell® CVR-C vaccine (Laboratories Zoetis Ltda) was used as a positive control and ultra-pure water as a negative control. The amplification reactions were carried out in a Thermal cycler 2720 thermocycler (Applied Biosystems, Foster City, CA, USA), obeying the following conditions: 5 minutes at 94°C, 35 cycles at 94°C for 1 minute, 57.4ºC for 30 seconds, 72ºC for 30 seconds, and final extension at 72ºC for 7 minutes. The products obtained in PCR were submitted to electrophoresis in gel de agarose a 1,5%.

DNA sequencing

PCR products for the FeAHV-1 gene showed expected bands. Then, these PCR amplicons were purified (Qiagen, QIAquick PCR Purification Kit, Germantown, USA) and commercially sequenced (Omikka, Paulínia, SP, Brazil) using the Sanger technique. Sequencing performed from feline conjunctival secretions showed 100% identity for the *Felid alphaherpesvirus 1* gene with the sequences deposited in GenBank (taxonomy:10334).

RNA extraction and real-time PCR

RNA extraction was performed in 12 samples, six animals positive and six negative for FeAHV1, from 300 µL of blood using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) and processed according to the manufacturer's recommendations. These samples were selected according to the quality of the blood available. All RNAs were quantified in a NanoDrop ND-1000 spectrophotometer (Agilent, Santa Clara, CA, USA) to measure the RNA concentration that had its purity determined by the A260/A280 and A260/A230 ratios. All samples were diluted to a concentration of 20ng/µL.

Primer sequences were developed and designed using the Primer3 program (http://bioinfo.ut.ee/primer3-0.4.0/) for the genes Tumor necrosis factor-alpha (TNF-α), Interleukin-6 (IL-6), Interleukin-1-beta (IL-1β), Interferon-alpha (IFN-α), Interferon-beta (IFN-β), Interferon-gama (IFN-γ) and the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, which was used as an endogenous gene (table 1).

Table 1. Primer sequences used to analyze cytokine gene expression in cat blood.

Cytokines	Forward primer (5'-3')	Reverse primer (5'-3')	ID
TNF-α	GTGGAGCTGACAGACAACCA	AGCACATGTGTGGAAGGACA	493755
IL-6	CAAATGTGAGGACAGCAAGG	TGAACCCAGATTGGAAGCAT	493687
IL-1β	GACGGTTTTGTGTGTGATGC	TATGAGCCAGACAGCACCAG	768274
INF-α	AGACTCTCCCTCTACCTGCA	CTGCAAGGCTGTTGACGAAT	493648
INF-β	GGGATGGAATGAGACCACTG	TCCTCCATGATTTCCTCCAG	493849
INF-γ	CATTCAAAGGAGCATGGACA	TTGAGGAAGTCATCCCGTTT	493965
GAPDH	TCAAGAAGGTGGTGAAGCAG	TGGAAGAGTGGGTGTCACTG	493876

TNF-α: Tumor necrosis factor-alpha IL-6: Interleukin-6 IL-1β: Interleukin-1-beta IFN-α: Interferon-alpha IFN-β: Interferon-beta IFN-γ: Interferon-gama GAPDH: Glyceraldehyde-3-phosphate dehydrogenase ID: Unique identifier pb: base pairs.

Pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) were selected because they are the first cytokines produced in inflammatory processes. Interferon class cytokines (IFN-α, IFN-β and IFN-γ) were chosen because they are produced in response to viral stimuli, and the infectious agent object of this study is a virus. Both pro-inflammatory cytokines and interferons act in acute inflammation (Abraha, 2020).

The reactions were performed with the Power Sybr® Green RNA-to-CT one-step kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommendations, with a final volume of 20 µL, they were performed using the CFX96 TouchTM Real-Time Detection System (Bio-Rad, Hercules, CA, USA). Ct (threshold cycle) values were obtained, and relative gene expression values were determined using the $2^{-\Delta Ct}$ equation, where ΔCt corresponds to the difference between target gene Ct and endogenous gene Ct (Livak and Schmittgen, 2001).

Statistical analysis

Gene expression profiles of selected cytokines were tested for normality using the Kolmogorov-Smirnov test. Then, the Student t test was applied to differentiate the levels of expression of these cytokines between positive and negative animals for FeAHV1 through the SAS program (Free Version University Edition). The significance level was $P < 0.05$.

RESULTS

Of the 55 pet cats evaluated, 9 cats were positive for FeHV-1 and 46 cats were negative for the virus. The results of PCRs from negative and positive animals are shown in (Figure 1).

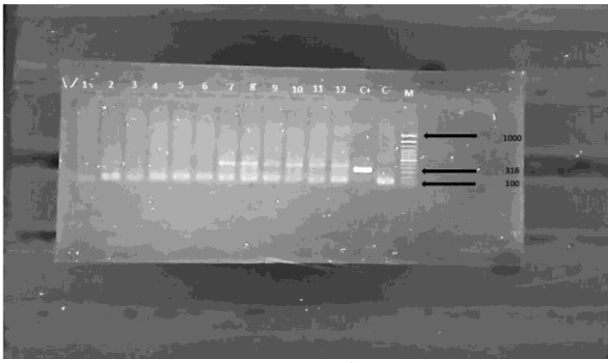

Figure 1. 1.5% agarose gel electrophoresis showing samples from FeAHV-1 negative cats (lanes 1 to 6), samples from FeAHV-1 positive cats (lanes 7 to 12) 316pb, positive control (c+), negative control (c-) and DNA ladder marker (M) (100pb) (ThermoScientific, USA).

All cytokines were detected and only TNF-α and IL-6 cytokines showed a significant difference ($P < 0.05$) in expression between FeHV-1 positive and negative animals. TNF-α cytokine gene expression the group of negative animals was 2 times greater than that observed in the group of positive animals. Different from the IL-6 cytokine gene expression, which was 2 times higher in the group of positive animals compared to the group of negative animals. The cytokines IL1-β, IFN-α, IFN-β and IFN-γ showed increased expression in the group of positive animals compared to the negative group, but this increase was not considered statistically significant ($P > 0.05$), these results are shown in (Figure 2).

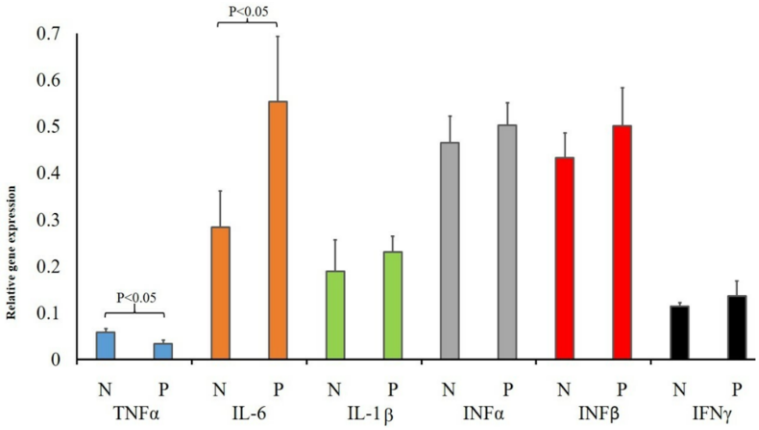

Figure 2. Relative gene expression levels of cytokines: TNF-α, IL-6, IL-1β, INF-α, IFN-β and IFN- γ in the blood of domestic cats. N: FeAHV-1 negative cats. P: FeAHV-1 positive cats. $P < 0.05$: significant difference observed in the gene between the two groups.

DISCUSSION

Our study demonstrated the presence of FeHV-1 DNA through PCR in samples of conjunctival secretions from pet cats. These animals lived in places with clusters of cats, which was a favorable factor for infection, they were not vaccinated and all positive animals showed clinical signs of FeHV-1 infection. Positive results of FeHV-1 infection indicate that the cat is either being infected by the virus or has recently been vaccinated against the disease. And clinically reestablished cats are latent carriers of the virus and can eliminate it continuously (GASKELL et al., 2007). In this sense, the cats in this study were animals that had FeHV-1 infection, as they showed clinical signs of the disease.

This study demonstrated, for the first time, mRNA expression levels of cytokine genes related to feline herpes virus type 1 infection in the blood of domestic cats. Other studies, evaluating cytokine genes in the face of infections caused by *Alphaherpesvirus*, were conducted in other animal species (Parvizi et al., 2015; Hue et al., 2017; Verpoest et al., 2017).

FeAHV-1 DNA was detected from nasal tissue samples in 21 adult shelter cats by viral isolation and traditional PCR, and the expression of some cytokines was evaluated by RT-PCR, increases in the expression of IL-6 and TNF-α cytokines were identified in samples in which FeHV-1 was detected compared to samples in which it was not (Johnson and Maggs, 2005). These results corroborate the findings in this study regarding the increased expression of IL-6 in the group of animals positive for FeAHV-1, and disagree regarding the expression of TNF-α, as the authors reported a greater expression in the group of positive animals to FeAHV-1, different from what was observed in our study.

After experimental infection by EHV-4, moderate expressions of TNF-α and IL-6 were observed in equine PBMC cells (Hue et al., 2017), disagreeing with what was observed in our study, where we identified increased expression of IL -6 and decreased TNF-α expression in FeAHV-1 infected cats. High expressions of TNF-α (22 times) and IL-6 (40 times) were identified in the nasal mucosa of 15-week-old pigs experimentally infected with pseudorabies virus, strain NIA3 (Verpoest et al., 2017), corroborating with the increased gene expression of IL-6 that we observed in the group of cats infected with the feline herpesvirus, and disagreeing with the increase in TNF-α, since we identified a decrease in this cytokine in the group of infected cats.

In the study that evaluated the gene expression of chickens immunized with herpesvirus of turkeys (HVT), and stimulated with the RB1B strain of *Gallid herpesvirus 1*, increases in IL-6 expression were identified in the group infected with the RB1B strain and vaccinated with HVT, 4 days after infection (Parvizi et al., 2015). Although this increase was not considered statistically significant when compared to the control group, corroborate what was observed in our study, in relation to the increase in IL-6 expression, in the group of infected cats by FeAHV-1.

Activated macrophages, above all, are responsible for the production of IL-10 which inhibits the production of other cytokines such as TNF-α, IL-1, IL-12 and chemokines by macrophages, and also participates in the blockade of accessory functions of macrophages in T cell activation (Abraha, 2020). The action of IL-10 on activated macrophages aims to terminate responses and lead the immune system to its resting state as the microbial infection is suppressed (Abraha, 2020). In our study, we observed a decreased expression of TNF-α in the group of cats infected with FeAHV-1, this decrease may be

related to the production of IL-10, but as we did not measure this cytokine, we cannot state that the decrease in TNF -α was for this reason.

IL-6 acts on acute phase reactions, immune responses, hematopoiesis and transmission of defense signals in the face of pathogen invasion or tissue damage (Narazaki and Kishimoto, 2018). Gene-targeted mouse studies have demonstrated the relevance of IL-6 in repressing local and systemic acute inflammatory mechanisms after exposure to endotoxin, in addition to participating in reducing vulnerability to viral, bacterial and fungal infections (Smith and Maizels, 2014). We detected a significantly increased expression of IL-6 in samples from cats infected with FeHV-1, which may indicate that these animals had a recent viral infection, as this is a cytokine that acts in the acute phase of the inflammatory and infectious response.

Although the studies (Parvizi et al., 2015; Hue et al., 2017; Verpoest et al., 2017) developed were carried out in other animal species, and with other herpesviruses, they direct us to the behavior of the immune system in the face of infection by viral agents from the same family.

CONCLUSION

The present study provided data on the behavior of the immune system in cats infected with FeAHV-1 and may contribute to future research on new forms of treatment for feline viral rhinotracheitis. Our study demonstrated that the inflammatory cytokine IL-6 is expressed in the blood of cats infected by FeAHV-1, and this data indicates that inflammatory cytokine genes can be used as molecular markers in the diagnosis of viral infection in cats. The cytokines IL1-β, IFN-α, IFN-β and IFN-γ despite having shown greater expression in cats carrying the virus, the expression did not show statistical significance, requiring further studies, this time involving a greater number of cytokines in the assessment.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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