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Molecular characterization of the 3'-UTRsequence of the interferon-gamma gene and its expression profile in the ocular conjunctiva of amazon buffaloes

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Abstract

Interferon-gamma is an important innate immunomodulatory inflammatory cytokine, whose molecular characterization can demonstrate how this gene may or may not be conserved in populations throughout the evolutionary process. Thus, the study aimed to characterize the 3'-UTR of the Interferon gamma gene and determine its expression profile in the ocular conjunctiva of Amazon buffaloes. For this, blood and ocular conjunctival tissue samples were collected from two hundred and five healthy slaughtered buffaloes in the State of Amapa, Brazil. For the conventional polymerase chain reaction, primers for the 3'UTR region of the Interferon-gamma gene were used and the products were purified, sequenced and edited by comparing the corresponding sequences from buffalo species and other domestic, wild and human mammals. They were aligned and compared using genetic similarity analysis and phylogenetic analysis, using Network 5.0 program and MrBayes, version 3.1.2, respectively. Conjunctival samples were submitted to mRNA quantification of the interferon-gamma to profile gene expression relative to the endogenous gene GAPDH and β-ACTIN. The study demonstrates high-level interferon-gamma genetic similarity between ruminants, with a network of haplotypes forming groups of ruminants, primates, carnivores, bats, cetaceans and isolated individuals such as suiformes and rhinos. Ruminants and cetaceans have a close phylogenetic relationship, while there were different evolutionary distances between buffalo and other mammalian species. The interferon-gamma gene was expressed in all animals in relation to endogenous genes. The results served to better understand the immune system of Amazonian buffaloes and to determine the evolutionary profile in relation to other mammalian species.

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Introduction

The Brazilian buffalo population is approximately 1.4 million, with the North region having the largest herd, with 63.38%. In this region, where the Brazilian Amazon is located, the states of Para stand out as the largest Brazilian and Latin American producer, whose most of the herd is concentrated in Marajo Island and Amapa State representing the second largest population in the region and country (1). Although, buffalo production presents one of the best economic performances in the Amazon region, it has adverse environmental conditions such as high humidity,

temperature, rainfall that can lead to thermal stress and reduced immune response (2,3). This also results in the formation of wetlands favorable for the maintenance of microorganisms and some larval stages of parasites (4,5) and which, due to the buffalo's habit of remaining submerged in these areas, may be more susceptible to diseases of various natures (3,5), including eye infections (6). On the other hand, this swampy environmental so provides protection against certain pathogens and parasites, especially ticks that transmit disease (5). This scenario demonstrates that susceptibility and/or resistance to diseases are complex traits that can be influenced by genetic factors in buffaloes, linked to the environmental conditions in which they live (5,7). Therefore, molecular tools have made it possible to identify candidate genes involved in the mammalian immune system and to what extent these genes may or may not be conserved in populations throughout the evolutionary process (8). Na important innate immunomodulator of vertebrate mammals is Interferon-gamma (*IFN-γ*), a pro-inflammatory cytokine and unique member of the type II Interferon class, produced mainly by Natural killer (NK) cells, T and B lymphocytes, dendritic cells and macrophages, whose expression is triggered in response to microbial infections (9,10) and intracellular bacterial infections, especially by mycobacteria (11). The *IFN-γ* is also involved in neoplasm control, used on a large scale in human cancer immunotherapy (12). Molecular characterization and gene expression of a variety of cytokines that modulate innate immunity has been demonstrated in vertebrate species (13-15), including domestic buffalo (16,17) and African buffalo (18). The *IFN-γ* gene has already been characterized in the buffalo species, however, in the promoter region (8). However, there are no reports on the molecular details of the terminal untranslated region (3'-UTR) of this gene in the species, as well as its conservation levels and similarity with other mammalian species, only previous studies that demonstrated polymorphism at position 4667G> A, Genbank MH351208 (19). In this context, research based on the genetic characterization of buffaloes is necessary to improve the genes bank of the species, since mapping these molecular characters is an important preliminary step for many studies on evolutionary history, genetic similarity and diversity, immunological and performance traits (5). Thus, it is essential to characterize and determine the expression of genes related to the innate immune system of buffaloes that can serve as reference molecular markers for future phenotypic studies, in order to promote the selection of more resistant animals to pathogens in breeding programs and genetic improvement (20).

Therefore, the aim of this study is to characterize the 3'-UTR terminal region of the Interferon-gamma gene and determine its expression profile in the ocular conjunctiva of Amazon buffaloes.

Materials and methods

Ethics aspects

All procedures of this research were conducted in accordance with all animal welfare standards determined by CONCEA (National Council for the Control of Animal Experimentation) of Brazil and approved by CEUA (Commission for Ethics in the Use of Animals) of the Federal Rural University of Amazon (033/2015).

Samples collection

Blood and ocular conjunctival tissue samples were collected after the slaughter of two hundred and five healthy male and female buffalo animals, aged between 5 and 7 years, crossbred at the State Slaughterhouse of Santana, in the state of Amapa, under the State Inspection Service. Approximately 10 mL of blood was collected from each animal, which was stored in 15 mL polypropylene tubes and preserved in isothermal boxes containing commercial ice, in addition to an eyelid fragment containing ocular conjunctival tissue and introduced into a polypropylene container with 2 mL of RNAlater solution (Invitrogen, USA) and kept on ice. Then, the samples were transported to the Federal Rural University of the Amazon - UFRA, where they were processed and analyzed in the Laboratory of Serology and Molecular Biology.

PCR and DNA sequencing

DNA was extracted from two hundred and five blood samples obtained of 300 µL of venous blood using the phenol: chloroform: isoamyl alcohol (25:24:1) method according to the protocol of Sambrook *et al.* (21). For this, the samples were homogenized in 300 µL of homogenization buffer and 300 µL of lysis buffer, then 15 µL of proteinase K (10 mg/mL) were added and incubated at 56°C overnight. Subsequently, the DNA was purified by extraction with 600 µL of phenol: chloroform: isoamyl alcohol (25:24:1) and precipitated with 100 µL of sodium acetate (3M, pH 4.8), isopropyl alcohol (500 µL) and 70% ethanol (500 µl). The pellet was resuspended in 80 µL of TE buffer.

The DNA was subjected to polymerase chain reactions (PCR) to isolate and amplify fragments of the 3' untranslated terminal region (3'-UTR) of the *IFN-γ* gene using the following primer sequences: F: 5'-TCCTGTGACTATTTCACTTGACC-3' and R: 5'-TGACTAGATGGCATCATTTCATT-3' with a fragment size of 182 base pairs (NCBI GenBank accession no. OQ540766), obtained from a reference sequence (NCBI GenBank accession no.MH351208.1 of 461 base pairs). PCRs were realized with Taq DNA polymerase master mix red (Ampliqon, Odense, Denmark) for final volume of 25 µL, and all reagents and temperature conditions were according to the manufacturer's recommendations. The annealing temperature was 59°C for 30 seconds. PCR

products were separated on 1% agarose gel and stained with fluorochrome Gel Red.

The amplicons from 19 samples of the *IFN-γ* gene were purified using a DNA purification kit on an agarose gel band (Ludwig Biotech LTDA, Alvora, RS, BR), and subsequently submitted to DNA sequencing analysis, using BigDye Terminator v3.1 Cycle Sequencing Kit reagent (Invitrogen California/USA) in a final volume of 10 µL on an ABI 3500 Genetic Analyzer automatic sequencer (Applied Biosystems). The sequences obtained were edited using the BLAST system with the corresponding buffalo and other mammal reference sequences deposited in the NCBI GenBank and then aligned in the BioEdit 7.2 program using ClustalW multiple alignment (22).

Genetic similarity analysis and phylogenetic analysis

From the nucleotide sequence of the 3'-UTR of the *IFN-γ* gene of the buffalo species, a relationship was made between this same region of the gene and other different species of terrestrial, aquatic and aerial mammals. A polymorphic sequence at position g.4467G>A of the 3'-UTR of the Bubalus bubalis *IFN-γ* gene deposited at GenBank, with accession at NCBI GenBank No. MH351208.1, was used as a wild-type buffalo *IFN-γ* sequence and was compared with the sequences found in all individuals studied. The Network 5.0 program (Fluxus Technology Ltd.) was used to build the haplotype network based on the Median-joining algorithm (23) in order to establish the degree of genetic similarity of 26 mammalian sequences.

To establish the evolutionary relationship between 19 sequences of these mammalian species, a phylogenetic dendrogram was assembled from an unrooted tree. Bayesian inference was implemented in MrBayes, version 3.1.2, using Monte Carlos simulation through Markov chain of two simultaneous runs of four chains of 10,000,000 generations with every 500 trees being sampled (24). The first thousand trees were discarded as burn-in and the posterior probability of each node was calculated from the remaining trees, examined initially in TreeView X (25). The bootstrap test was used to assess the reliability of the molecular phylogeny, with the value reached from the times the set was restored. Subsequently, the tree was visualized and edited using Fig Treeversion 1.4.4.

RNA isolation and qRT-PCR

The RNA extraction was carried out with TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to manufacture recommendations. Gene expression analyzes were performed using real-time quantitative polymerase chain reaction (qRT-PCR) using the 12 samples, in triplicate, selected based on the highest degrees of purity (A260/A280 ratio > 1.9). Primer sequences were developed and designed using the Primer3 program for the *IFN-γ* gene (*IFNG*), and two constitutive genes, the *GAPDH* and *β-ACTIN*(*ACTB*) for *Bubalus bubalis* were used as control (endogenous) (Table 1).

Table 1: Sequence of primers designed according to exon and Genbank identity (ID) for expression analysis of the *IFN-γ* (*IFNG*) gene and the endogenous genes *GAPDH* and *β-ACTIN* (*ACTB*) in buffalo

Genes	Sequence Primers	Exon	GenBank ID
IFNG	F: 5'-CAGATCATTCAAAGGAGCATGGA-3' R: 5'-GTCCTCCAGTTTCTCAGAGCT-3'	3	102416494
GAPDH	F: 5'-ACCCAGAAGACGGTGGATG-3' R: 5'-CCGTTGAGCTCAGGGATGA-3'	7	102404028
ACTB	F: 5'-GACATCCGCAAGGACCTCTA-3' R: 5'-GGGCAGTGATCTCTTTCTGC-3'	5	102413719

The samples were subjected to qRT-PCR to quantify mRNA expression of the selected gene using the one-step Power Sybr® Green RNA-to-CT™ kit (Applied Biosystems, Foster City, CA, USA), according to the recommendations from the manufacturer, to a final volume of 10 µL. All reactions were performed on the CFX96 Touch™ Real Time Detection System (Bio-Rad, Hercules, CA, USA).

Initially, the cDNA synthesis reaction was performed from reverse transcriptase (Applied Biosystems, Foster City, CA, USA), where they were subjected to a temperature of 43ºC for 30 minutes. Then, real-time qPCR was performed under the following conditions: initial denaturation temperature of 94°C for 2 minutes, followed by 40 cycles containing a denaturation temperature of 94°C for 15 seconds and hybridization and extension at 60°C for 1 minute for the detection of the Ct curve. Finishing with the conditions for determining the Melt curves at an initial temperature of 65°C and a final temperature of 95°C, with an increase of 0.5°C every 5 seconds.

The Ct (threshold cycle) values were obtained and relative gene expression values were determined using the equation $2^{-\Delta Ct}$, where ΔCt means the difference between target gene Ct and endogenous gene Ct (26).

Statistical analysis

Relative gene expression results were tabulated and submitted to the normality test using Kolmogorov-Smirnov method. Descriptive statistics were used, including median

and a Boxsplot was generated. The significance level considered was set 0.05.

Results

A 182 bp fragment was amplified corresponding to the 3'-UTR of the *IFN-γ* gene from a buffalo population (Figure 1). All analyzed sequences (access from ACTGene Analisis Moleculares Ltda, ID 85677BBF) were identical for all individuals, with no genetic diversity in the studied population. Furthermore, no SNP was detected at position g.4467G>A or at other positions of the 3'-UTR of the *IFN-γ* gene, however, there was a small diversity with the polymorphic wild sequence of *Bubalus bubalis* used as reference (access in the NCBI GenBank MH351208.1). The homologous percentages between buffalo sequences and other mammalian sequences are specified in Table 2.

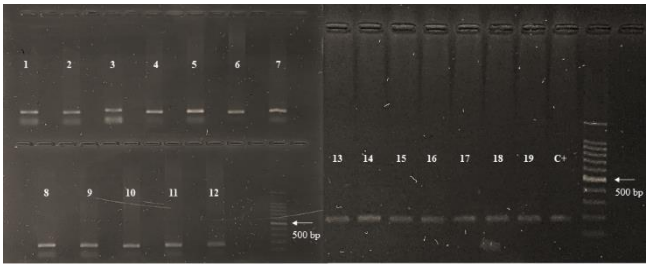

Figure 1: Agarose gel-stained Safe dye for a 182 bp fragment of the 3'-UTR region of the *IFN-γ* gene obtained from 19 buffalo blood samples from the Amazon region. Molecular mass standard Ladder from 100 to 1000 bp (CellcoBiotec).

Table 2: Homologous percentages of buffalo *IFN-γ* 3'-UTR sequence with other mammalian sequences

Species	Identity (%)	NCBI accession number
Bubalus bubalis	99.45	MH351208.1
Bos indicus	96.70	XM_019960052.1
Cervus elaphus	92.47	X63079.1
Physeter catodon	90.56	XM_007106255.2
Orcinus orca	90.00	XM_004281863.2
Camelus ferus	87.71	XM_006189690.2
Camelus dromedarius	86.59	XM_031462226.1
Vicugna pacos	86.03	XM_006205835.2
Sus scrofa	87.50	X53085.1
Aotus nancymaae	85.00	XM_012444968.1
Homo sapiens	83.33	NM_000619.3
Pteropus vampyrus	83.15	XM_011373848.1
Suricata suricatta	83.07	XM_029954582.1
Rousettus aegyptiacus	82.97	XM_036237134.1
Saimiri boliviensis	82.78	XM_003926543.2
Ceratotherium simum simum	82.12	XM_004429381.2
Puma yagouarondi	80.43	XM_040491841.1
Panthera pardus	79.68	XM_019455038.1

Genetic similarity analysis

The haplotype dendrogram (Figure 2) revealed seven different groups of mammals, which included ruminants, cetaceans, suiformes, perissodactyls, bats, carnivores and primates. Domestic and wild ruminants of the Bovidae, Cervidae and Camelidae families showed high similarity between species. Haplotype 1 was the haplotype that revealed the highest frequency of species, to which five of the seven bovine species analyzed belong, including the buffalo sequence studied and a sequence from a cross between *Bos taurus* and *Bos indicus*.

The wild sequence of the *Bubalus bubalis* and *Bos mutus* species, both bovines, belonged to haplotypes 2 and 3, respectively, showing only one point of mutation between individuals belonging to haplotype 1. The deer, however, showed considerable similarity with the bovids, followed by camelids, as they exhibited a greater number of mutation points when compared to deer. In addition, haplotype 6 was the second with the highest frequency, which was repeated twice because it presents both species of camelids (*Camelus ferus* and *Camelus dromedarius*).

Cetaceans showed greater genetic similarity with ruminants when compared to other groups of mammals, followed by human and non-human primates, while carnivores, bats and the isolated species of perissodactyl exhibited less genetic similarity and higher amounts of mutation points.

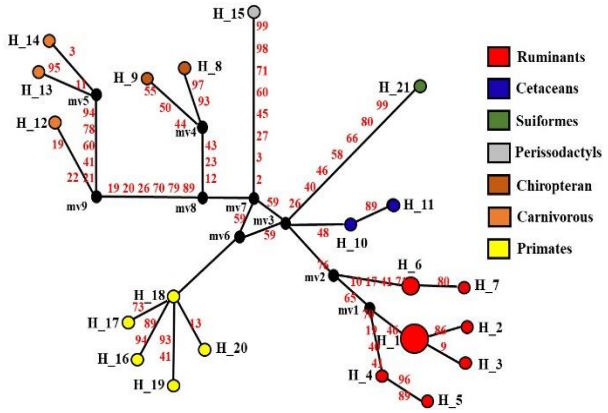

Figure 2: Haplotype network of mitochondrial DNA controller region sequences demonstrating the genetic similarity, mutation points and haplotype frequency of different genetic groups of terrestrial, aerial and aquatic mammals. The circles represent the haplotypes found and their sizes are proportional to the frequency of individuals that present them. The numbers in red are the mutation points and the colors refer to the genetic groups to which the individuals belong.

Phylogenetic analysis

The phylogenetic analysis (Figure 3) the bootstrap values were 0.9631 at the node of the two *Bubalus bubalis*

sequences, 0.862 at the node between the *Bubalus bubalis* and *Bos indicus* sequences and 0.9987 between the species of the Bovidae family and other ruminants of the Cervidae family, proving the confidence level of the test. *IFN-γ* sequences could be classified into distinct groups of mammals.

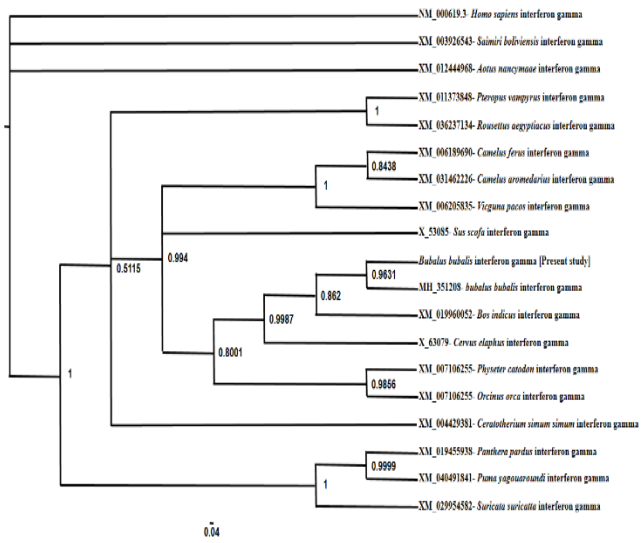

Figure 3: Phylogenetic tree based on IFN-gamma sequences showing the relationship between buffalo and other mammalian species. Phylogenetic relationships were reconstructed by the neighborhood method using ClustalW multiple alignment and bootstrapped 1000 times.

The analysis shows an evolutionary relationship between domestic and wild ruminants, as the species of the Bovidae and Cervidae families grouped in the same clade. Bovids (*Bubalus bubalis* and *Bos indicus*) show high proximity, especially between the studied and wild buffalo sequences, revealing a region of the *IFN-γ* gene highly conserved among domestic ruminants. The species of Cetaceans, which are marine animals and not ruminants, also belong to this same clade, demonstrating considerable homology between these animals.

On the other hand, other ruminants of the Camelidae family are part of a group distinct from bovids and cervids, demonstrating less evolutionary relationship between these ruminants. The swine species formed a distinct and isolated clade, but close to the buffaloes, while the rhinoceros (*Ceratotherium simum simum*), bats and carnivores formed the groups with the greatest phylogenetic distances, respectively. Non-human and human primates constitute the outgroups.

Interferon-gamma gene expression in all evaluated animals is represented on relative expression interval in relation to endogenous genes of a minimum of 0.0151 and a maximum of 1.5789, with a median of 0.5995 (Figure 4).

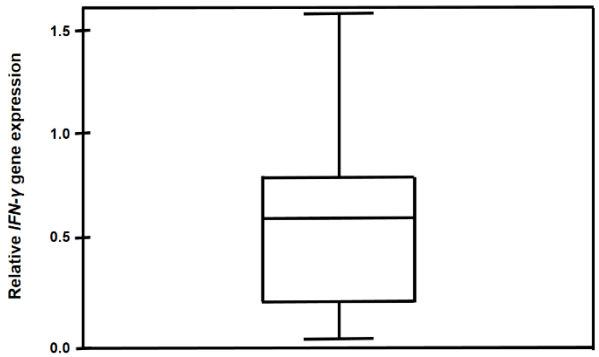

Figure 4: Relative gene expression of the *IFN-γ* gene in the based-on conjunctiva-associated lymphoid tissue (CALT) of conjunctival membrane samples of buffalo eyes from the Amazon region. The relative expression interval was represented in relation to endogenous genes with a median of 0.5995.

Discussion

Molecular characterization and gene expression of cytokines has been used in several studies in veterinary medicine (14,15), including in domestic and wild buffaloes (16-18), especially for the use of these cytokines as vaccine adjuvants (27) and in association with susceptibility or resistance to a variety of infections in animals (7,19). In this study, we analyzed the buffalo *IFN-γ* gene in the 3'-UTR, which is known to play post-transcriptional regulatory roles in mRNA gene expression (28), whereas Premraj *et al.* (8) characterized this same gene, however, in the promoter region, a regulatory region and considered highly polymorphic for most cytokine genes (8,29). In other species of ruminants, studies regarding the characterization of the *IFN-γ* gene were carried out in African buffalo (18), goats (14), as well as in other mammals (13) and a diversity of fish species (15,30).

The buffalo *IFN-γ* gene is composed of four exons and three introns, similar to the *IFN-γ* genes of all vertebrate species (31,32) as it is the ancestral interferon gene organization of the type II (*IFN-γ*), considered universal from cartilaginous fish to mammals. Whereas type I interferons can vary from an organization of five exons and four introns in bony fish and some amphibians to an organization of four exons and three introns in cartilaginous fish, reptiles, birds, mammals and some amphibians. This variation can occur within *IFN* genes, due to retro transposition events that occurred throughout the evolutionary process of species, which gave rise to intron loss or exon fusion, as well as genes without introns (32).

The results allowed the determination of the genetic similarity of the studied buffalo population with all identical sequences between the animals. Therefore, it was possible to observe an absence of genetic variability in this population,

which may be due to the low sample size evaluated or the high selection pressure that allows the fixation of alleles in a population (33), in this case, of genes related to immune status, even with interference from the environment. Based on the analysis of the genetic similarity and phylogeny parameters for the *IFN-γ* gene, a very close similarity between species of ruminants stands out, which is in line with what was described by Premraj *et al.* (8) in relation to this same *IFN-γ* gene. This author also reveals a greater homology of buffalo sequences with bovine sequences when compared to other ruminants, similar to what was observed in the studied sequences.

These characteristics have also been determined for other genes involved in inflammatory responses and innate immunity, such as interleukins (16). The analysis of genetic similarity from the repetition of haplotypes is analogous to the phylogenetic relationship between the ruminants of the Bovidae (buffalo and cattle) and Cervidae families, being characterized as a highly conserved region in the group. Reports based on molecular DNA markers point out that mitochondrial DNA (34) and nuclear DNA (35) are the most widely used to estimate phylogenetic studies on several genes from buffalo populations and other mammals.

Although the similarity analysis showed some distance between cetaceans and some ruminants to which they belonged to different groups, the phylogenetic analysis found that both belong to the same clade, corroborating the reports by Foote *et al.* (36) from the phylogeny of the genome of cetaceans and ungulate ruminants, revealing that they belong to the same order of Cetartiodactyls, with a probable common ancestor. On the other hand, our study demonstrated considerable diversity and distance between ruminants and other groups of mammals, with many points of mutation between haplotypes. From an evolutionary point of view, it was possible to verify that the 3'-UTR sequence of the studied gene is poorly conserved when comparing multiple groups of mammals, possibly because it is a terminal region of the gene that is very susceptible to mutations, due to its low frequency of introns, which are highly conserved (37).

Furthermore, the natural selection process to which these species have been subjected could directly influence the frequency of genotypes linked to the immunity of these animals. This is probably due to the most varied environments in which mammals have inhabited over thousands of years (aerial, aquatic and most terrestrial habitats) with diverse regions and climates. In addition, the diversity of pathogens and their interactions with the host linked to the habits and behaviors of each species, which may influence the way in which the immune response can behave among different groups of mammals (5).

Our study, most of the evaluated buffalo had low gene expression for the interferon-gamma gene, a pro-inflammatory cytokine that regulates mammalian innate immunity (32), however, some animals also demonstrated high expression profiles. These results can be explained because the expression of a gene can vary considerably according to the tissue analyzed, the individual's immune status, the presence of polymorphisms, the stage of development or state of a disease (38,39). These results did not determine degrees of resistance or susceptibility to diseases in the evaluated animals, since field studies were not carried out under the same conditions to evaluate these genotypes and association with the immunological status within this buffalo population. However, studies in humans have shown that mutations alter the 3'-UTR sequence and are related to severe disease (37). This is reinforced because single nucleotide polymorphisms (SNPs) are mechanisms that affect the expression level and activity of genes in buffaloes (40), including inflammatory genes such as cytokines, being considered regulators that carefully control inflammatory reactions and innate immune. Therefore, they influence the type of inflammatory response in each individual, and may have a collaborative role in resistance against pathogens or an opposite role in the susceptibility of individuals to diseases (5,7,19,41).

Polymorphisms have already been demonstrated in inflammatory cytokine genes in production animals such as buffalo (19), cattle (41), poultry (42) and sheep (43). Iannaccone *et al.* (19) found a new SNP in buffaloes, specifically at position g.4467G>A of the 3'-UTR sequence for the *IFN-γ* gene that affects the target sequence for miR-125b, conferring susceptibility to tuberculosis in this species. This same mutant sequence was deposited in GenBank by the same authors and was used as a wild-type buffalo *IFN-γ* sequence for comparison with the sequence found in our study.

In addition, no SNPs were found in the 3'-UTR sequence of the *IFN-γ* gene in the analyzed sequences, supposedly due to the low number of evaluated animals that represented a population or the little change in the environment in which they live that could influence the genetic variability of buffaloes of the Brazilian Amazon. An evolutionary hypothesis was determined by Luo *et al.* (35) to explain the fixation of genetic polymorphisms and genetic variability among domestic buffalo populations in the world and which is related to ancient geographic events. This can be exemplified by the genetic divergence that occurred between the river buffalo (Asian) and the swamp buffalo (African), which coincided with the migration of these animals across borders and the consequent genetic mixture between the two subspecies. After geographic separation, it is assumed that there was a greater flow of genes and mutations between buffaloes in a population, determining this divergence.

Conclusion

The stud demonstrates high phylogenetic homology of cDNA sequences from the 3'-UTR region of Interferon-gamma gene between ruminants and cetaceans, and great

genetic similarity within the ruminants, with few mutation points between these species, especially buffaloes and cattle. The gene was expressed in the conjunctival membrane, characterizing the action of interferon gamma in the ocular tissue of buffaloes, even in healthy animals. Therefore, the characterization of the 3'-UTR sequence of the interferon gamma gene will contribute to the understanding of the innate immune system of buffaloes and to determining how much this gene has remain conserved in these populations. Further studies in vivo are needed to assess the expression this immunomodulatory molecule in animals with conjunctival acute inflammatory conditions to determine their contributions to innate immunity, on susceptibility and/or resistance to diseases in buffaloes.

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Conflict of interest

All authors and involved institutions in this research declare that there is no conflict of interest.

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التوصيف الجزيئي لتسلسل 3'-UTR لجين إنترفيرون كاما وإظهاره في ملتحمة العين في جاموس الأمازون

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الخلاصة

إنترفيرون كاما هو سيتوكين التهابي مناعي فطري مهم، التي يمكن أن يوضح توصيفها الجزيئي كيف يمكن أو لا يمكن الحفاظ على هذا الجين في المجموعات طوال العملية التطورية. تهدف الدراسة إلى توصيف 3'-UTR لجين إنترفيرون كاما وتحديد ملف تعبيره في ملتحمة العين لجاموس الأمازون. تم جمع عينات الدم وأنسجة ملتحمة العين من مائتين وخمسة جاموس مذبوح سليم في ولاية أمابا، البرازيل. بالنسبة لتفاعل البلمرة المتسلسل التقليدي، تم استخدام البادئات لمنطقة 3'-UTR لجين إنترفيرون كاما وتم تنقية المنتجات وتسلسلها وتحريرها من خلال مقارنة التسلسلات المقابلة من أنواع الجاموس وغيرها من الثدييات المستئنسة والبرية والانسان. تمت محاذاتها ومقارنتها باستخدام تحليل التشابه الجيني وتحليل النشوء والتطور، باستخدام برنامج Network 5.0 و MrBayes ، الإصدار ٣,١,٢، على التوالي. فحصت عينات من الملتحمة باستخدام القياس الكمي للإنترفيرون - كاما لمعرفة التعبير الجيني بالنسبة للجين الداخلي GAPDH و β-ACTIN. توضح الدراسة التشابه الجيني عالي المستوى بين المجترات، مع شبكة من الأنماط الفردية التي تشكل مجموعات من المجترات وآكلات اللحوم والخفافيش والحيتان والأفراد المعزولين مثل suiformes ووحيد القرن. المجترات والحيتان لها علاقة تطورية وثيقة، في حين كانت هناك مسافات تطورية مختلفة بين الجاموس وأنواع الثدييات الأخرى. تم التعبير عن جين إنترفيرون جاما في جميع الحيوانات المتعلقة بالجينات الداخلية. نتائج هذه الدراسة توصلت الى فهم أفضل للجهاز المناعي لجاموس الأمازون وتحديد المظهر التطوري المتعلق بأنواع الثدييات الأخرى.