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Hemoparasites effect on interferon-gamma and miRNA 125b expression in *Bubalus bubalis*

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Abstract

This study aimed to determine and evaluate the effect of the g4467 G>A SNP on the expression profile of IFN-γ and miRNA 125b in dairy buffaloes (Bubalus bubalis) with and without hemoparasites. Molecular diagnosis was performed by Polymerase Chain Reaction (PCR) on 145 buffaloes for *Babesia* spp., *Trypanosoma vivax*, and *Anaplasma marginale*. All buffaloes were investigated for the presence or absence of the polymorphism and genotyped using the restriction enzyme. Real-time PCR (RT-qPCR) quantified the expression of IFN-γ and miRNA 125b. All buffaloes were negative for *Babesia* spp. and *A. marginale*, and only 12 were positive for *T. vivax*. The genotypes GG, GA, and AA were found in proportions of 3.4%, 2.1%, and 94.5%, respectively. The A allele was the most frequent (95.5%). The SNP showed deviation from Hardy-Weinberg equilibrium (HWE) ($P<0.05$) and a deficit of heterozygotes with FIS 0.759. All animals of the found genotypes expressed both genes, except for GG positive for *T. vivax*. IFN-γ expression was higher for GA and GG negative ($P<0.05$) and AA positive. However, miRNA 125b expression was lower for AA and GA positive and higher for AA, GA, and GG negative. AA-positive buffaloes for *T. vivax* may exhibit susceptibility due to higher IFN-γ expression and lower miRNA 125b. GG and GA-negative buffaloes exhibited higher expression in both, suggesting they have greater resistance to positive ones.

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Introduction

Bubalus bubalis is a financially valuable system for meat, milk, and dairy production (1), and the state of Pará stands out in breeding and the number of heads, with the largest herd in Brazil and Latin America. From a breeding perspective, the Amazonian environment still proves challenging with its tropical climate of high humidity, elevated temperature, and high rainfall, which favor the emergence of vectors, mainly ticks, one of the main transmitters of many pathogenic agents. In addition, the presence of vectors and agents affects the immune state of the hosts, making them resistant or susceptible to diseases (2,3). Hemoparasites are responsible for generating significant economic losses to production systems; thus, the use of molecular tools for selecting resistant animals and for more accurate diagnosis has become an effective alternative, as PCR has greater sensitivity and specificity capable of detecting the presence of the parasite in asymptomatic animals (4). These tools also allow for the identification of candidate genes of mammalian immunology. Among these genes, the pro-inflammatory cytokine IFN-γ synthesized by T and NK cells stands out, characterized as a considerable immunomodulatory molecule of the innate immune system

of vertebrates. IFN-γ expression is triggered by various factors, such as viral and intracellular bacterial infections, especially by mycobacteria (5,6).

Targeted research on the untranslated regions (UTRs) of cytokine genes is not well established, particularly for the IFN-γ gene in the buffalo species. Based on genetic determinations of buffalo and knowledge of the expression of immune system genes, these can be used as molecular markers in future research to enable the selection of animals resistant to parasites in genetic improvement programs. Therefore, the objective of this study was to detect the g4467 G>A SNP in the 3' UTR of the IFN-γ gene and investigate its action on its expression profile and miRNA 125b linked to the SNP sequence in negative and positive dairy buffaloes for *T. vivax*.

Materials and Methods

Ethics and sample collection

The Ethics Committee on Animal Use (CEUA) approved the study of the Federal Rural University of Amazon (0054/2009/CEUA/UFRA). A total of 145 crossbred dairy buffaloes aged between 3 and 6 years were sampled from dairy farms in the municipality of Bujaru, Belém (Brazil) (1°37'40''S, 48°13'21''W). 5 ml of blood was collected in tubes containing anticoagulant (EDTA). 1000 µL of blood were transferred to tubes with 300 µL of RNAlatter (Invitrogen) and subsequently stored at -80 ºC for gene expression analysis.

Molecular diagnosis

It was performed by the phenol method (7) and stored at -20°C. PCR was used for *Babesia* spp. and *Trypanosoma vivax*, with a final volume of 25 µL, comprising 12.5 µL of Taq Pol Master (Cellco), 2 µL of DNA, 0.5 µL of primers and 9.5 µL of ultrapure water. Primers BAB 1F and BAB 2R and Tvi2 and DTO156 were used, respectively (Table 1). The positive DNA controls were for *B. bigemina* and *T. vivax*, and ultrapure water was the negative control. The reactions were performed in a 2720 Thermal Cycler (Applied Biosystems). The products were analyzed on a 1.5% agarose gel for Babesia spp. and 2% for *T. vivax*.

RT-qPCR was used for *Anaplasma marginale*, with a final volume of 10 µL containing 5.0 µL of 2x Master Mix qPCR (Cellco), 1 µL of DNA, 0.5 µL of primers and 3.0 µL of ultrapure water. Primers AM-1 and AM-2 were used (Table 1). The positive control consisted of gBlock 10-1, while ultrapure water was used as the negative control. The reactions were performed in a CFX96 Touch Real-Time Thermal cycler (Bio-Rad).

Table 1: Sequences of primers, protocols, and references used for detection of hemoparasites in dairy buffaloes

Agent	Oligonucleotides (5'-3')	Size (pb)	Protocols	References
Babesia spp.	BAB 1F AAGTACAAGCTTTTTACGGTG BAB 2R CCTGTATTGTTATTTCTTGTC ACTACCTC	420-440	PCR – I	(8,9)
T. vivax	Tvi2 GCCATCGCCAAGTACCTCGCCGA DTO156 TTAGAATTCCCAGGAGTTCTT GATGATCCAGTA	177	PCR – II	(10)
A. marginale	AM-1 TTGGCAAGGCAGCAGCTT AM-2 TTCCGCGAGCATGTGCAT	95	RT-qPCR - III	(11)

PCR - I: initial denaturation at 95ºC for 2min, 45 cycles with denaturation at 94ºC for 30s, hybridization at 61ºC for 30s, extension at 72ºC for 45s, and final extension at 72ºC for 10min. PCR - II: initial denaturation at 94ºC for 2min, 35 cycles with denaturation at 94ºC for 30s, hybridization at 55ºC for 30s, extension at 72ºC for 30s, and final extension at 72ºC for 10min. RT-qPCR - III: enzymatic activation at 94ºC for 2min, 41 cycles with denaturation at 95ºC for 5s, Hybridization/Extension at 58ºC for 30s, and dissociation curve at 65-95ºC (Increment of 0,5ºC) for 5s.

Genotyping of the g4467 G>A polymorphism

Initially, PCR reactions were performed to isolate part of the 3′UTR of the IFN-γ gene using the primer pair F5'TCCTGTGACTATTTCACTTGACC3' and R5'TGACTAGATGGCATCATTTCATT3', based on GenBank ID 102416494, resulting in a 182 bp fragment. The reactions were carried out in a Thermal Cycler 2720 (Applied Biosystems), with a final volume of 20 µL, containing 10 µL of Master mix, 2 µL of DNA, 0.5/µL of primers, and 7.0 µL of ultrapure water, following the protocol: initial denaturation at 95°C for 3 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 59°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 1 min. The products were visualized on a 2.0% agarose gel. The PCR products were then subjected to BspLI (NIaIV) (Invitrogen) restriction enzyme digestion. Five µL of the PCR product, 0.35 µL of the restriction enzyme, 1.5 µL of the reaction buffer, and 8.15 µL of ultrapure water were used for the protocol: The restriction reaction conditions were 37°C for 3 h, according to the manufacturer's recommendations. The genotypes were visualized on a 3.0% agarose gel.

RNA extraction

Following the manufacturer's recommendations, 300 µL of blood was added to 700 µL of Trizol (Life Technologies).

RNAs were quantified on a BioDrop spectrophotometer (BioDrop) with an A280/A260 ratio.

Gene expression (RT-qPCR)

The IFN-γ and miRNA 125b (12) primers were investigated for their expression. GAPDH and LET-7a for *Bubalus bubalis* were constitutive genes (Table 2). The protocol followed the manufacturer's recommendations, with a final volume of 10 µL. The relative expression values were determined by the equation 2-ΔCt, where ΔCt is the difference between the target gene's Ct and the endogenous gene's Ct (13).

Statistical analysis

Allelic and genotypic frequencies, EHW (P> 0.05), and FIS were obtained using GenePop (14). The expression patterns were analyzed using SAS OnDemand, assuming non-normal distribution. The Kruskal-Wallis test was used with a significance level of 0.05.

Table 2: Sequences of primers used according to GenBank ID for gene expression analysis in dairy buffaloes

Genes	Oligonucleotides (5' - 3')	Genbank ID
IFN-γ	F: CAG ATC ATT CAA AGG AGC ATG GA R: GTC CTC CAG TTT CTC AGA GCT	102416494
miRNA 125b	F: GCA GTC CCT GAG ACC CT R: CCA GTT TTT TTT TTT TTT TCA CAA GT	351208
GAPDH	F: ACC CAG AAG ACG GTG GATG R: CCG TTG AGC TCA GGG ATGA	102404028
LET-7[a]	F: GCA GTG AGG TAG TAG GTTG R: GGT CCA GTT TTT TTT TTT TTT TAA CTA TAC	030971.1

Results

Molecular diagnosis of hemoparasites

None of the 145 buffaloes were positive for *Babesia* spp. and *A. marginale*. Only 12 were positive for *T. vivax*, corresponding to approximately 8.27% (12/145) of the population.

Investigation of the g4467 G>A SNP in the IFN-γ Gene

PCR-RFLP products generated digestion patterns of electrophorese bands. The G allele is the undigested PCR product, with a fragment of 182 bp. In contrast, the A allele generated a digestion pattern with two fragments, one of 101 bp and the other of 81 bp. Therefore, genotyping of the GG homozygote resulted in a single band of 182 bp; for the GA heterozygotes, the enzyme only digested the A allele, generating bands of 182 bp, 101 bp, and 81 bp, and for the AA homozygote, the enzyme digested both alleles, generating bands of 101 bp and 81 bp. The SNP in the present study is part of a miRNA 125b target region. When the A (mutant) allele is present in the IFN-γ mRNA sequence, miRNA 125b loses specificity for pairing compared to the G allele in the sequence. At the end of genotyping, all buffaloes, the genotypes AA, GG, and GA, were found in all negative animals for the three hemoparasites analyzed. Still, the genotypes GG and AG were observed in *T. vivax* positive animals, and the genotype AA was not found.

Genetic diversity of the population

The genotypic frequencies of GG, GA, and AA for the g4467 G>A SNP were 3.4%, 2.1%, and 94.5%, respectively, with deviations from HWE (P<0.05) and a deficit of heterozygotes, which was evidenced by the positive F_{IS} value (0.759). In the population, the mutant A allele of the SNP (G→A) had a higher frequency (95.5%) compared to the wild-type allele (4.5%) (Table 3).

Table 3: Genetic diversity of the dairy buffalo population for the SNP g4467 G > A in the 3' UTR of the IFN-γ gene

SNP	alleles		Observed Genotypes			F_{IS}	EHW
g4467 (G → A)	G 0,045	A 0,955	GG (0,034)	GA (0,021)	AA (0,945)	0,759	0.000

EHW: Hardy-Weinberg equilibrium (P>0.05); F_{IS}: inbreeding coefficient of the F statistic.

Gene expression profile patterns

Negative and positive animals for T. vivax with the three genotypes found for the g4467 G>A SNP in the IFN-γ Gene, except for the positive GG, exhibited distinct expression levels for both IFN-γ and miRNA 125b. The expression of the IFN-γ gene exhibited higher levels for the negative GA genotypes, with statistical differences only between the negative GG and AA genotypes (P<0.05). On the other hand, miRNA 125b exhibited lower levels of expression for the positive AA and GA genotypes and higher levels for the negative AA, GA and GG genotypes, with statistical differences between AA and GA and GG (P<0.05)(Figure 1).

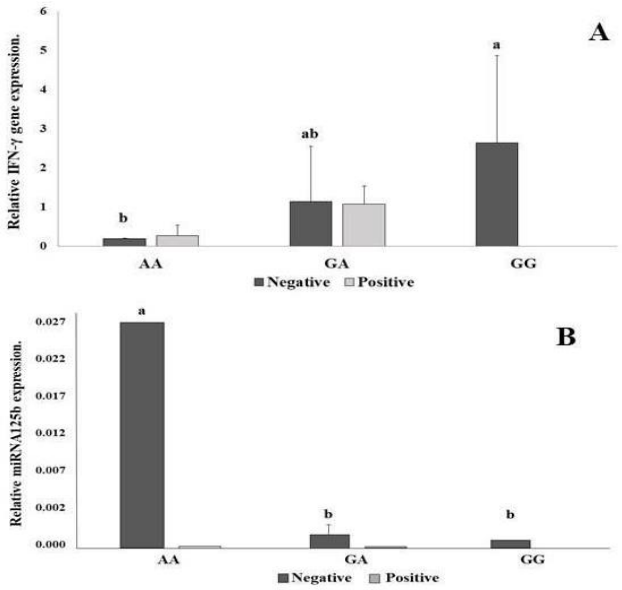

Figure 1: Relative expression of the IFN-γ gene (A) and miRNA 125b (B) in the different genotypes (AA, GA, and GG) of buffaloes from the Amazon region negative and positive for *T. vivax*. According to equation 2-ΔCt. Different letters above the bars indicate significant pairwise comparisons according to Tukey's test (P< 0.05).

Discussion

Approximately 8.3% of the buffaloes in the present study were positive and asymptomatic for *T. vivax*, indicating that these animals may have developed previous protection against some acute infection or had prior and continuous exposure to the parasite, which allowed for control of parasitemia through the host's immune response (15). The presence of hematophagous insects such as flies and horseflies on the property, which are important vectors of trypanosomiasis, also increases the possibility of mechanical transmission of *T. vivax* within the herd (16).

In buffaloes, the infection is characterized by low parasitemia, which favors the establishment of an equilibrium between the host and the parasite, allowing the disease to remain asymptomatic for a long time, typifying the chronic phase of the disease (17). The fact that the buffaloes in the present study were asymptomatic may be due to their trypanotolerance combined with protective immunity sustained by reinfections, given that the Amazon region is endemic for *T. vivax* infection. In studies conducted in Venezuela, most of the buffaloes positive for *Trypanosoma* sp. showed low parasitemia and manifested chronic infections (15).

The BspLI (NlaIV) enzyme was able to distinguish genotypes for the SNP rs43382671 A>G in the HDAC9 gene involved in the regulation and development of muscle tissues in Canchim cattle. In this study, the mutant allele G had a frequency of 79.81%, and the GG genotype had the highest frequency at 61.27% for the SNP. Additionally, the SNP had a significant effect on the additive genetic value (AGV) (P<0.05) for the loin eye area (LEA) (18). This is in line with the present study, as it was also able to distinguish genotypes for the g4467 G>A SNP in the IFN-γ gene, being the first study to use it in buffaloes.

The same SNP showed deviation from Hardy-Weinberg equilibrium (HWE), with a deficit of heterozygotes based on the positive FIS value, indicating that the reduction of these individuals may be due to factors such as selection effects, population grouping, and consanguineous mating, which decrease the number of alleles in a population, increasing the number of homozygous individuals (19).

The SNPs found in the 3' UTR influence post-transcriptional mRNA translation by modifying miRNA binding sites. In buffaloes, the SNPs in the 3'UTR of the IFN-γ gene have been linked to susceptibility to bovine tuberculosis (bTB) (12). The expression of IFN-γ observed in *T. vivax*-negative buffaloes evaluated in this study was similar to that of IFN-γ found in Canchim cattle with low parasitemia for *B. bovis* and *B. bigemina* (20). This expression may occur due to the role of IL-10 being unable to inhibit regular mRNA expression of IFN-γ, which may be a consequence of immune control mechanisms, added to loss or modified RNA processing (21).

The gene expression profiles of various cytokines were studied in peripheral blood mononuclear cells (PBMC) from cattle considered resistant (N'Dama) and susceptible (Boran) to African trypanosomiasis, with a 4.3-fold increase in IFN-γ expression observed in the resistant cattle, allowing the animals to more effectively control the infection through the Th1 immune response responsible for regulating the release of IFN-γ (22). Therefore, it can be inferred that the asymptomatic AA-positive buffaloes detected in the study presented higher levels of IFN-γ expression to control parasitism, conferring susceptibility to *T. vivax* infection compared to negative genotypes. On the other hand, regarding the negative GA heterozygote and GG homozygote, both may confer resistance to *T. vivax* infection in buffaloes since they presented higher levels of IFN-γ expression and possess the G allele in the sequence, which enhances its pairing with miRNA 125b, making it express more.

Allied to studies on gene expression are miRNAs, which can bind to the target sequence of mRNAs, acting in the regulatory process and, therefore, are considered potential candidates as infection biomarkers (23). However, in production animals, studies focused on miRNA expression have mainly concentrated on functions in regulating production characteristics such as muscle progression and hypertrophy, increase in adipose tissue, and fertility (24-26).

Once identified as capable biomarkers of immunity, it was demonstrated that the expression of miRNA 125b was reduced in healthy cows but tended to increase in cows with

mastitis in 14 genes of the Nuclear Factor kappa B (NF-κB) complex involved in regulating the expression of many genes linked to the inflammatory response (27). In the present study, the opposite was observed, where buffalo of the AA and GA genotypes, positive for *T. vivax*, exhibited lower levels of miRNA 125b expression when compared to the three negative genotypes, which exhibited higher levels. Thus, reduced miRNA 125b expression in positive animals may suppress expression in the NF-κB signaling pathway, generating immunological imbalance and making these animals more susceptible.

In research conducted with African buffalos (*Syncerus caffer*), resistance to trypanosomiasis was achieved through evolution due to the synthesis of specific antibodies to the variant surface glycoprotein (VSG) and the formation of H_2O_2 trypanocide molecules that cause parasite elimination in the blood (28). As this is an endemic hemoparasite disease in the Amazon region, studies focused on the evolution of the Bubalus bubalis species, focusing on resistance to trypanosomiasis, should also be based on VSG genes. Therefore, developing research on potential immunity markers associated with miRNAs directly related to trypanosomiasis in buffalos is extremely necessary, given that these markers are more established for other infectious diseases.

Conclusion

This was the first study with the Bubalus bubalis species to use the BspLI (NlaIV) restriction enzyme. The IFN-γ and miRNA 125b genes showed distinct expression profiles between the genotypes of buffaloes, negative and positive for *T. vivax*. *T. vivax* positive buffaloes with the AA and GA genotypes may be considered susceptible to infection due to higher levels of expression of IFN-γ and lower levels of miRNA 125b. Negative GG genotype buffaloes demonstrated greater resistance to *T. vivax* infection by presenting higher levels of expression of both IFN-γ and miRNA 125b due to the presence of the G allele strengthening the pairing between them. However, future studies involving SNPs, expression of other cytokines, and VSG genes related to resistance or susceptibility in buffaloes may progressively and importantly confirm that these animals are indeed capable of immunologically responding efficiently to *T. vivax* infection so that they can be selected based on their genetic makeup.

Conflict of interest

This manuscript's authors declare no conflict of interest regarding the writing process or data analysis.

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تأثير الطفيليات الدموية على تعبير كل من الانترفيرون كاما والحامض الريبوزي الرسولي ١٢٥ب في جاموس الماء

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الخلاصة

تهدف هذه الدراسة الى تحديد وتقييم تاثيرg4467 G>A SNP على ملف تعريف التعبير للانترفيرون كاما والمايكرو الحامض النووى الرايبوزي في الجاموس الحلوب*Bubalus bubalis* مع وبدون طفيليات الدم .تم اجراء التشخيص الجزيئي بواسطة تفاعل البلمرة المتسلسل (PCR) على ١٤٥ جاموس لطفيليات *Babesia spp., Trypanosoma vivax, and Anaplasma marginale*. تم فحص جميع الجاموس بحثا عن وجود او عدم وجود تعدد الاشكال والانماط الوراثية وذلك بواسطة استخدام انزيم القطع. تفاعل البوليمرايز بالوقت الحقيقي حدد كمية التعبير عن الانترفيرون كاما-IFN والمايكرو الحامض النووي الرايبوزي miRNA 125b وكانت جميع الجاموس سلبية بالنسبة الى طفيلي *Babesia spp* والانابلازما *Anaplasma marginale* و١٢ فقط كانت موجبة لطفيلي *Trypanosoma vivax*. تم العثور على الانماط الجينية GG, GA, AA بنسبة ٣,٤% ,٢,١ % و٩٤,٥% على التوالي. كان الاليل A الاكثر شيوعا بنسبة ٩٥,٥% واظهر SNP انحرافا عن توازن هاردي وينبرج (P<0.05) (HWE) ونقص بالزيجوت المتغايرة مع FIS 0.759. جميع الحيوانات ذو الانماط الجينية التي تم العثور عليها عبرت عن كلا الجينين، باستثناء GG الموجب لـ*T.vivax*. كان تعبير الانترفيرون كاما اعلى بالنسبة GA و GG سلبيا (P<0.05) و AA ايجابيا. ومع ذلك كان التعبير المايكرو الحامض الرايبوزي miRNA 125b اقل بالنسبة لـ AA و GA ايجابيا واعلى بالنسبة لـ AA و GA و GG سلبيا. قد تظهر الجاموس AA الايجابي لـ *T.vivax* حساسية بسبب ارتفاع تعبير الانترفيرون كاما وانخفاض مايكرو الحامض النووي الرايبوزي miRNA 125b. اظهر الجاموس السالبة GG و GA تعبيرا اعلى في كليهما، مما يشير الى ان لديهم مقاومة اكبر تجاه تلك الايجابية.