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Bovine Genetic Variability and Therapeutic Response: Potential Application of Pharmacogenetics in Cattle

Jorge Luís de Figueiredo Salgado[1]*, Raquel Lima de Figueiredo Teixeira[1], Márcia Quinhones Pires Lopes[1], Michel José Sales Abdalla Helayel[2], Harrison Magdinier Gomes[1], Philip Noel Suffys[1], Roberta Olmo Pinheiro[3] and Adalberto Rezende Santos[1]

[1]Laboratory of Molecular Biology Applied to Mycobacteria, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil
[2]Department of Veterinary Collective Health, Fluminense Federal University, Niterói, Rio de Janeiro, Brazil
[3]Leprosy Laboratory, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil

Abstract

Brazil has the world's largest commercial cattle herd. However, the production system faces challenges due to various diseases, which have led to an increased demand for effective prophylaxis and treatment, especially for conditions like bovine mastitis. Despite the predominant use of different medications in livestock farming, their effectiveness is often hampered by the variability of individual animal responses and genetic variations. Thus, we aimed to investigate the variability of the *SLC15A2*, *SLC16A1* and *SLC22A6* genes in cattle. In humans, these genes are linked to the metabolic pathways of commonly employed mastitis treatments by Cloxacillin (CLO) and Ampicillin (AMP). The study encompassed 50 Girolando breed cows from Fazenda Nossa Senhora de Fátima in the Municipality of Silva Jardim, Rio de Janeiro, Brazil. Genotyping was performed by direct PCR sequencing of DNA from whole blood and the investigated regions in the three genes covering all exons except exon 10 of the *SLC22A6* gene. We identified 13 SNP including eight within *SLC22A6* (c.248C>T, c.277G>T, c.292G>T, c.315T>C, c.50T>A, c.50C>T, c.37C>T and c.44C>T) and five within *SLC16A1* (c.167G>A, c.242A>G, c.641C>T, c.644G>A and c.139C>T). Notably, no polymorphism was observed in *SLC15A2*. We here present a pilot that sheds some light on the potential of bovine pharmacogenetics and demonstrates a considerable variability in drug- metabolism associated genes in cattle.

Such studies pave the way for investigations of functionality of these SNPs and their potential associations with therapeutic responses in bovine mastitis treatment.

Keywords: Bovine • Mastitis • Genetic variability • Ampicillin • Cloxacillin • Pharmacogenetics

Introduction

Genetic variation is an important factor in drug response and can largely account for the observed interindividual variability in different treatments.

Single Nucleotide Polymorphisms (SNPs) are the most commonly observed genetic variations in DNA sequences and occur in at least 1% of the population They can occur in genes that encode drug target proteins or in enzymes involved in their metabolism and transport, potentially affecting pharmacokinetics (absorption, distribution, metabolism, and excretion) and pharmacodynamics (drug interaction with the target and the relationship between concentration and effect) [1].

Pharmacogenetics is a field that explores the intricate relationship between genetics and an individual's response to drugs by delving into how genetic variations impact an individual's reaction to medications, eventually improving drug therapies, minimizing adverse effects, and allowing treatment personalization. In animal pharmacogenetics, there is also investigation how genetic diversity can influence drug response, allowing veterinarians, researchers, and those involved in animal production to enhance the well-being of animals and optimize drug treatments in diverse scenarios.

Bovines are a conjuncture of species with significant economic importance that are utilized for various purposes, including meat and dairy production, understanding their pharmacogenetics carries substantial implications for veterinary medicine, livestock management and food safety.

Mastitis is an inflammatory and infectious disease of the mammary gland that can affect both humans and animals and when it manifests itself in its most aggressive form in cattle results in high losses for the entire dairy production chain [2-4]. The implementation of prevention and control measures for this disease in cattle is essential, but it is hampered by the variability of response to drug treatment, partly due to the genetic variability of cattle [5-7], partly due to variability of the ethiologic agent and partly due to the subjectivity in the antibiotic agent usage. The choice of drug is not guided by microbiological tests for isolation, identification of the etiologic agent and drug susceptibility testing. Consequently, despite the variety of drugs available on the market for treating this infection, both at subclinical and symptomatic levels, efficacy varies significantly.

Several studies have investigated the presence of genetic variants in genes specifically related to drug metabolism [8-10], drug transport [11,12] and pharmacokinetics in cattle [13]. However, none have been investigated for the presence of polymorphisms in drug transporter genes with therapeutic response. Although several antibiotics are used in the treatment of bovine mastitis the choice of a specific antibiotic depends on several factors, such as the agent causing the infection, bacterial sensitivity, and the severity of the disease. Even so, the effectiveness of the most common used drugs, Ampicillin (AMP) and Cloxacillin (CLO), used for the treatment of mastitis caused by Staphylococcus aureus, varies considerably. Contrary to human mastitis, the influence of genetic factors on this variation is still unknown for bovines.

***Address for Correspondence:** Jorge Luís de Figueiredo Salgado, Laboratory of Molecular Biology Applied to Mycobacteria, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil, Tel: + 5521982345729; E-mail: salgado@ioc.fiocruz.br; jorgeluis.fsalgado@gmail.com*

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In humans, AMP/CLO are transported *via* peptides of the SLC (Solute Carrier) family, primarily involving the genes *SLC15A2* [14,15], *SLC16A1* [16], *SLC22A6* [17] and ABC [18,19]. Given the lack of literature data pertaining to these genes in cattle, we have drawn upon the available human data and employed bioinformatics tools to pinpoint their orthologs within the bovine context. The primary objective of this study was to evaluate the genetic variability of these genes in the Girolando breed. We explore this through a SNP discovery through direct PCR sequencing of the gene coding sequences.

Materials and Methods

Study population and sample collection

In an observational descriptive study model the variability of the drug transporter genes *SLC15A2*, *SLC16A1* and *SLC22A6* in cattle was evaluated. Blood samples were collected from a total of 50 Girolando cows at the Nossa Senhora de Fátima dairy farm, located in the municipality of Silva Jardim, Rio de Janeiro, Brazil. The entire process of sample selection and collection was directly coordinated by veterinary professionals at the farm. The study animals, ranging from 36 to 72 months of age, were categorized as adult cows. Employing a vacutainer system (BD Vacutainer®) containing sodium citrate as an anticoagulant, 3mL of blood was drawn from each animal's coccygeal complex. The collected samples were homogenized through inversion and subsequently stored at a temperature of -20ºC.

DNA extraction

DNA was extracted from 200 µL of blood using the commercial kit "QIAamp DNA Blood®" (QIAGEN), following the manufacturer's instructions. The quality and quantity of the extracted DNA were assessed using the Qubit 3.0 model (Invitrogen™ Qubit™ 3 Fluorometer).

Strategies for PCR amplification of target sequences

After the identification of the *SLC22A6*, *SLC15A2* e *SLC16A1* genes in the bovine genome (https://www.ncbi.nlm.nih.gov/gene), a set of primers were designed for the amplification and sequencing of nine exons of the *SLC22A6* gene (NCBI Reference Sequence: NC_037356.1), four exons of the *SLC16A1* gene (NCBI Reference Sequence: AC_000160.1) and three exons of the *SLC15A2* gene (NCBI Reference Sequence: AC_000158.1). Primers were designed using primer3plus software (http://www.bioinformatics.nl/cgi bin/primer3plus/primer3plus.cgi) based in the reference sequences obtained.

PCR amplification and sequencing of the different targets

Samples were amplified by conventional PCR using an exon-specific set of primers (Table 1). For amplification of the different exons of *SLC22A6*, *SLC15A2* e *SLC16A1* genes, the PCR and cycling conditions were determined as follows: a total amount of 20 ng of genomic DNA was added to a reaction mixture with a final volume of 50 µL, containing 2 mM $MgCl_2$, 200 µM dNTPs, 1X PCR buffer, 1U of taq DNA polymerase (Invitrogen™), and 20 µM of each primer. Samples were incubated at 94 °C for 5 min, followed by 35 cycles of 94°C for 1 min.; 59 °C, for 1 min. and 72 °C for 90 sec. The final extension took place at 72 °C for 5 minutes. Especially for the exon 3 of *SLC16A1*, because of the size of the amplified product (1000 bp) to be sequenced, two internal oligonucleotides were used generating two overlapping fragments.

Evaluation of the PCR product was achieved by electrophoresis on a 1% agarose gel followed by ethidium bromide (0,5µg/mL) staining. The PCR products were purified with ChargeSwitch® PCR Clean-UP Kit (Invitrogen, USA), according to the manufacturer's recommendations and subsequently used for direct Sanger sequencing. The sequencing reaction was performed using the aforementioned primers and the ABI PRISM Big Dye Terminator Kit v. 3.1 Kit (PE Applied BioSystems) according to the manufacturer's recommendations on an ABI PRISM 3730 DNA Analyzer (PE Applied BioSystems).

Sequence analysis and statistics

Sequence data from each sample underwent analysis to identify SNPs by alignment against the reference sequences of the genes: *SLC22A6*, *SLC15A2* and *SLC16A1* using SeqScape v.2.6 software from Applied BioSystems.

To determine the frequencies of polymorphisms and genotypes and to assess deviations from the Hardy- Weinberg equilibrium, we employed the Hardy-Weinberg exact balance test [20] with the assistance of SNPassoc v.2.0.Haplotype reconstruction was carried out using the Bayesian method, which is implemented in PHASE v.2.1.1. This method was used to ascertain the most likely haplotype pairs, ensuring unambiguous genotyping and determination of the most likely allele pairs [21,22].

Results

Animals

A total of 50 vaccinated, adult Girolando breed cows, which were registered, were included in the study. All the animals were adults, not in the lactation period, and were free from mastitis.

Genotyping

DNA samples isolated from blood of each animal underwent PCR

Table 1. Primer descriptions for PCR-amplification and sequencing of 10 exons from the *SLC22A6* gene, 5 exons from the SLC16A1 gene and 3 exons from the *SLC15A2* gene.

Gene	Exon	Product Size (bp)	Left Primer	Right Primer
SLC22A6	1	674	TTCAGTTCCAGGAGCGACTT	TTTCTTGACCCTGCAACTCC
SLC22A6	2	413	GGAGTTGCAGGGTCAAGAAA	GCCTGGGACCGTGATGTAT
SLC22A6	3	436	CTAACTGGGGCTGGACTGAG	AGGACGAAGGCTGTATGCTG
SLC22A6	4	595	GCAAAAACACACAGCAAGGA	AGGAGGCTGGAGAATTGGAT
SLC22A6	5	497	AACCAAGACCCCATGTTGAG	TGTGGTCAGCTCCTTCTTCA
SLC22A6	6	429	TCAATGGGAAGCAGGAAGAG	ATGCCTTTCACAGCTGGTCT
SLC22A6	7	500	TGCATGTTAAATGTCAGAAGAAA	AGCAAGAGAGGTTCGGACAA
SLC22A6	8	298	ACCCATGAGCACCTTGGTC	CCCTTTGACATGTGCCCTA
SLC22A6	9	297	CAGACGGGCTTGGGAATG	GGGAAGAGGAGGCCTAGGTT
SLC22A6	10	679	GGGGTCCTTGTCCCTGTGT	CGTGTGCAGGAACTGGAAT
SLC16A1	1	598	GGGAAAAACTTACAAAGCCTGT	CAAAGCAAGAGTTGTCATTGTTA
SLC16A1	2	361	CATTGCCTTTGTGTGTCTGC	TGCTTGTCAAAATGATAATCAGC
SLC16A1	3	1000	TGGTTAAGTAATGCAAAATATGTCTC	TCCCAATCTTCTCTGCCTGT
SLC16A1	3	230 (INTERNAL)	GCAAATACAGATCTCATTGG	AGCCTTCTCACTAGAGTAAT
SLC16A1	4	600	ACCCCAAATCAGTGTGACAT	GGCTACTGGTAAGGAGTGAAACA
SLC15A2	1	399	CCCATGTATTGTTAGTTAACCAGTG	TTTAGATCTGGTTTATAGGTTCATTCT
SLC15A2	2	374	AGGGACCATTGCTTCTTCAC	AAATCCCACCAGGCATTTTT
SLC15A2	3	500	CCTTTCACCCTCACAACTCC	TGGAAAAACTGATAATGAAGAGATAAA

amplification and genotyping through sequencing. Upon analyzing the resulting sequences, eight SNPs were identified in the *SLC22A6* gene, distributed across the exons as follows: four SNPs within exon 1 (c.248C>T, c.277G>T, c.292G>T, c.315T>C), one SNP within exon 3 (c.50T>A), one SNP within exon 4 (c.50C>T), one SNP within exon 5 (c.37C>T), and one SNP within exon 8 (c.44C>T). Genotype and allele frequencies are presented in Table 2. The minor allele frequencies ranged from 0.02 (less common) for SNPs c.277G>T, c.292G>T, and c.44C>T to 0.22 (most common) for SNPs c.248C>T and c.315T>C.

For the *SLC16A1* gene, five SNPs were identified, with one SNP located in exon 1 (c.167G>A) and four SNPs situated in exon 3 (c.139C>T, c.242A>G, c.641C>T and c.644G>A). Minor allele frequencies ranged from 0.01 (less common) for SNPs c.139C>T and c.167G>A, to 0.59 (higher than the normal allele) for SNP c.242A>G (Table 3). No polymorphism was identified in the *SLC15A2* gene.

Table 2. Genotype and Minor Allele Frequencies (MAF) of the SNPs identified in the *SLC22A6* gene.

		SLC22A6		
SNP	**Genotypes**	**N= 50**	**Minor Allele**	**MAF (%)**
c.248C>T	CC	$29^{58\%}$	T	0.22
	CT	$20^{40\%}$		
	TT	$1^{2\%}$		
c.277G>T	GG	$48^{96\%}$	T	0.02
	GT	$2^{4\%}$		
	TT	0		
c.292G>T	GG	$48^{96\%}$	T	0.02
	GT	$2^{4\%}$		
	TT	0		
c.315T>C	TT	$29^{58\%}$	C	0.22
	TC	$20^{40\%}$		
	CC	$1^{2\%}$		
c.50T>A	TT	$47^{94\%}$	A	0.03
	TA	$3^{6\%}$		
	AA	0		
c.50C>T	CC	$35^{70\%}$	T	0.16
	CT	$14^{28\%}$		
	TT	$1^{2\%}$		
c.37C>T	CC	$45^{90\%}$	T	0.05
	CT	$5^{10\%}$		
	TT	0		
c.44C>T	CC	$48^{96\%}$	T	0.02
	CT	$2^{4\%}$		
	TT	0		

MAF: Minor Allele Frequency

Table 3. Genotype and Minor Allele Frequencies of the SNPs identified in the *SLC16A1* gene.

SNP	**Genotypes**	**N=50**	**MA**	**MAF (%)**
c.167G>A	GG	$49^{98\%}$	A	0.01
	GA	1^{2}%		
	AA	0		
c.242A>G	AA	$15^{30\%}$	G	0.59
	AG	$11^{22\%}$		
	GG	$24^{48\%}$		
c.641C>T	CC	$48^{96\%}$	T	0.02
	CT	2^{4}%		
	TT	0		
c.644G>A	GG	$34^{68\%}$	A	0.19
	GA	$13^{26\%}$		
	AA	$3^{6\%}$		
c.139C>T	CC	$49^{98\%}$	T	0.01
	CT	$1^{2\%}$		
	TT	0		

MA: Minor Allele; MAF: Minor Allele Frequency

Haplotypes frequencies

Haplotype identification in the *SLC22A6* and *SLC16A1* genes was accomplished using PHASE v2.1.1 software. Seven distinct haplotypes were identified for *SLC22A6*, and seven for *SLC16A1*. Tables 4 and 5 displays these haplotypes and their individual frequencies ranging from 0.01 to 0.55 for *SLC22A6* and from 0.01 to 0.40 for *SLC16A1* respectively.

Excluding the wild-type haplotypes for both genes, the most common haplotypes were number6 (TGGCTCCC) for *SLC22A6* and the number 1 (GCGCG) for *SLC16A1*.

Discussion

With the sequencing of the bovine genome and the development of next-generation sequencing techniques, knowledge about bovine genetic variability in the world has grown significantly, making it possible to conduct studies for a better understanding of this diversity in different breeds [23,24].

Currently, six different SNP chips are available for cattle genotyping, offering a range of low to high density options across two distinct platforms. Five of these SNP chips are provided by Illumina (San Diego, CA), including the Golden Gate Bovine3K BeadChip (Bovine3k), Infinium BovineLD BeadChip (BovineLD), Infinium BovineSNP50 v.1 BeadChip (BovineSNP50v.1), Infinium BovineSNP50 v.2 BeadChip (Bov ineSNP50v.2), and Infinium BovineHD BeadChip (BovineHD). Additionally, Affymetrix has also an Array, the Axiom GenomeWide BOS 1 Bovine Array, which includes 648, 875 SNP probes (Axiom Bos1) [25].

Exploring the functional relevance of DNA variants in livestock genomics including bovine populations [26], although not always exclusively focused on pharmacogenes offers insights into SNP analysis and the implications of genetic variants in livestock breeding and production [27].

In this context, studies examining the association of genetic markers in genes involved in pharmacokinetics,and consequently, their influence on therapeutic responses to different drugs, hold significant importance in herd management. Such research aims to propose and implement more effective therapies to reduce losses and enhance the productive quality of cattle. It is important to note, however, that research in bovine pharmacogenetics is still in its early stages, with much remaining to be explored to gain a comprehensive understanding in the field and its relationship to drug responses in cattle.

Common drug transporter gene families that play a significant role in drug-carrier function include the ATP-Binding Cassette (ABC) transporters and the Solute Carrier (SLC) superfamily of transporters. These genefamilies have been extensively studied in various species, including bovines, for their role in drug transport and it is widely recognized that significant genetic variation exists in nearly every drug transporter. This genetic variation can lead to various outcomes, affecting aspects such as the expression, stability, folding, localization, and degradation of the transporter protein. Furthermore, they can influence the transport characteristics, substrate affinity, binding, and transport kinetics of the transporter [27].

The economic impact of pathological disorders, such as mastitis, is of crucial concern for the dairy sector. The recommended treatment, similar to human mastitis, yields variable outcomes. To address the lack of available data concerning the variability of pharmacogenes, and in particular transporter genes, this study focused on a partial mapping of the coding regions of bovine orthologs corresponding to human genes *SLC15A2*, *SLC16A1* and *SLC22A6*, which are involved in the transport of ampicillin and cloxacillin in Girolando cattle. The Girolando breed is the result of crossbreeding Holstein cattle with the Gir breed is prevalent in most Brazilian dairy farms and has undergone genetic improvement, with the objective of sustainable milk production in tropical and subtropical regions [28]. We detected SNPs in *SLC16A1* and *SLC22A6*), *SLC22A6* being the most polymorphic and *SLC15A2* being 100% conserved.

Table 4. Haplotype frequencies Identified in *SLC22A6* gene.

		***SLC22A6* Haplotypes**								**N**	**Frequency**
		Exon 1				**Exon 3**	**Exon 4**	**Exon 5**	**Exon 8**		
N	**Alleles**	**c.248C>T**	**c.277G>T**	**c.292G>T**	**c.315T>C**	**c.50T>A**	**c.50C>T**	**c.37C>T**	**c.44C>T**		
1	CGGTTCCC									55	0.55
2	CGGTTCTC							X		5	0.05
3	CGGTTTCC						X			15	0.15
4	CGGTACCC					X				1	0.01
5	CTTTACCT		X	X		X			X	2	0.02
6	TGGCTCCC	X			X					21	0.21
7	TGGCTTCC	X			X		X			1	0.01
									Total	100	1

Table 5. Haplotype frequencies identified in *SLC16A1* gene.

	***SLC16A1* Haplotypes**							
		Exon 1	**Exon3**					
	Alleles	**c.167G>A**	**c.139C>T**	**c.242A>G**	**c.641C>T**	**c.644G>A**	**N**	**Frequency**
1	GCGCG			X			38	0.38
2	GCGCA			X		X	17	0.17
3	GCGTG			X	X		2	0.02
4	GCACG						40	0.4
5	GCACA					X	1	0.01
6	GTGCA		X	X		X	1	0.01
7	ACGCG	X		X			1	0.01
						Total	100	1

Table 6. Description of the four SNPs already described and identified within *SLC22A6* coding region.

Coding Region	Protein Region	Genomic Region	Alleles	MA	MAF	Consequence Type	Variant ID
c.248C>T	p.Ser83Phe	g. 42271244	G/A	A	0.081	missense_variant	rs524446512
c.277G>T	p.Gly93Cys	g.42271215	C/A	A	0.043	missense_variant	rs381152264
c.292G>T	p.Gly98Cys	g.42271200	C/A	A	0.046	missense_variant	rs378269413
c.315T>C	p.Cys105Cys	g.42271177	A/G	G	0.086	synonymous_variant	rs437302202

MA: Minor Allele; MAF: Minor Allele Frequency

Using the Bovine Genome Variation Database (BGVD), which is a useful tool for in-depth analysis in bovine biology and livestock by integrating data from other databases such as NCBI, UCSC Genome Browser, AnimalQTLdb, AmiGO 2 and KEGG, we found that in the *SLC22A6* gene, 35 SNPs have been described in the coding region, among which 4 SNPs out of the 13 described by us (Table 6). In the *SLC16A1* genes, 22 SNPs have been described, and in the *SLC15A2* gene, 2 SNPs, all in the coding region.

This database contains information about 60,439,391 SNPs (single nucleotide polymorphisms), 6,859,056 indels (insertions/deletions), and 76,634 CNV regions (copy number variations) derived from 432 animals. It uses 54 cattle breeds in 6 groups of ancestral cattle, excluding the Girolando hybrid [29].

The frequencies of the minor alleles among the eight SNPs within the *SLC22A6* gene ranged from 1% to 22%, with the highest frequency observed for SNPs c.248C>T and c.315T>C, both considered to have a very high frequency. In the case of the *SLC16A1* gene, the variation in minor allele frequencies ranged from 1% to 59%, with the SNP c.242A>G showing a higher frequency compared to the normal allele.

The analysis of the distribution of these 4 SNPs of the *SLC22A6* gene among the 54 breeds of cattle in the BGVD database showed the absence of the SNPs c.315T>C, c.292G>T, and c.277G>T in Gir and Holstein breeds. In the Gir breed, the MAF of the c.292G>T and c.277G>T SNPs was low, however, surprisingly, for the SNP c.315T>C, the frequency was 22%.

Although there are several possibilities to explain the emergence and increase in frequency of a specific SNP in a hybrid cattle population. The exact explanation would depend on additional informations, such as the reproductive history of the hybrid population, detailed genetic data from ancestral populations and the selective management practiced, among other factors. However, we most likely consider a Founder Effect as the greatest probability to explain this significant increase in frequency in the hybrid population since the frequency data for the c.315T>C SNP were based on the genotyping of only 45 animals of the Holstein breed and 3 of the Gir breed, and may not have been detected due to sampling limitations.

In relation to the SNP c.248C>T, the MAF was 15 times higher among Gir in comparison to c.248C>T in Holstein and 22% Girolando, however, the same effect can be attributed due to the small sample size of the Gir breed.

Domestic cattle (*Bos taurus*) are divided into two subspecies: *Bos taurus taurus* (taurine cattle of European origin) and *Bos taurus indicus* (zebu cattle of Asian origin). Crossbreeding between individuals of both subspecies is common, occurring in herd genetic improvement programs and on properties where breeding is natural and uncontrolled. These hybrids, such as the Girolando breed (resulting from crossbreeding Holstein (5/8) and Gir (3/8), which we used in our study, are widely employed to combine the productivity of taurine cattle with the ruggedness and adaptability to tropical environments seen in zebu cattle [30].

Analyzing SNPs with high frequencies can be valuable for undestanding the genetic history and adaptation of populations. It can also help in identifying variants associated with specific traits. For instance, a high SNP frequency would be, among others, the result of a Positive Selection, if the SNP is linked to a trait or characteristic that provides a significant advantage [31,32] of a Genetic Drift, in small or isolated populations in which the frequency of an SNP may fluctuate randomly from generation to generationdue to genetic drift. In some circumstances, this drift can increase the frequency of an SNP to a

high level [33] and Environment-Dependent Selection Pressure, in dynamic or changing environments, various alleles may be favored at different times. Frequent environmental shifts can result in the selection of different alleles at different times, ultimately leading to the prevalence of multiple alleles at high frequencies [34]. Further studies, including larger sample sizes and representatives of pure breeds (Holstein and Gir), should be conducted to provide a more comprehensive and well-founded explanation.

Conclusion

Seven distinct haplotypes were identified for *SLC22A6* and *SLC16A1* genes, with frequencies ranging from 1% to 40% for *SLC16A1* and from 1% to 55% for *SLC22A6*. Among the potential factors that could account for these high frequencies, we emphasize the role of Linkage Disequilibrium (LD). These SNPs may be in strong LD with each other, indicating that they tend to be inherited together. Even if each individual SNP has a lower frequency, the combined haplotype can have a higher frequency if it is frequently inherited as a unit (35), and Functional Diversity. The presence of multiple haplotypes with relatively high frequencies may suggest functional diversity in the gene. Each haplotype might have distinct effects on drug transport, possibly in response to different drugs or physiological conditions, making them advantageous in different contexts. To understand the exact mechanisms and functional significance of these haplotypes, further genetic and functional studies, such as association studies, functional assays, and evolutionary analyses, will be necessary. These studies can help uncover the specific roles of these haplotypes in drug transport and their implications for cattle treatment and drug responses.

Author Contributions

JLFS - Performed the experiments and wrote the manuscript.

RLFT - Performed the sequence analysis and manuscript revision.

MQPL - Performed sequencing experiments and manuscript revision.

MJSAH - Performed the animal selection and sample collection.

HMG - Did the manuscript revision.

PNS and ROP - Provided laboratorial support.

ARS - Conceptualized the study, proofread the manuscript

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Data Availability Statement

The experimental protocol was approved by the Ethics Committee on the Use of Animals ECUA/IOC – Oswaldo Cruz Foundation number (L-016/2021).

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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