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Posted Date: 14 October 2025

doi: 10.20944/preprints202510.0938.v1

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Association of *SLC11A1* 3′UTR $(GT)_n$ Microsatellite Polymorphisms with Resistance to Paratuberculosis in Sheep

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Abstract

Paratuberculosis (Johne's disease), caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP), is a chronic enteric infection that significantly impacts small ruminant health and productivity. Genetic variation in host immune genes, particularly *SLC11A1*, has been implicated in resistance to intracellular pathogens. The aim of this study was to investigate whether polymorphisms in the 3′UTR $(GT)_n$ microsatellite of *SLC11A1* are associated with resistance or susceptibility to paratuberculosis in sheep, complementing existing SNP-based genome-wide association studies (GWAS) in cattle and goats. A total of 138 animals were genotyped, and a subset of 53 was analyzed for *SLC11A1* expression. Six alleles were identified, with $(GT)_{21}$ and $(GT)_{23}$ significantly enriched in resistant sheep ($p < 0.05$), while $(GT)_{22}$ and $(GT)_{24}$ were more common in sensitive animals. Overall allele distribution showed a significant genotype–phenotype association ($\chi^2 = 12.4$, $p = 0.006$, Cramér's $V = 0.38$). In contrast, no significant differences were observed in basal *SLC11A1* mRNA expression between groups or across genotypes. Our findings extend previous GWAS results in sheep by providing preliminary allele-level resolution of a functional microsatellite locus. Identification of resistance-associated alleles provides a foundation for genetic selection strategies that complement vaccination and management, supporting sustainable control of paratuberculosis in sheep.

Keywords: *Mycobacterium avium* subsp. *paratuberculosis*; Johne's disease; sheep; *SLC11A1* gene; microsatellite polymorphism; disease resistance

1. Introduction

Paratuberculosis, also known as Johne's disease, is a chronic enteric infection caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) that significantly affects ruminant livestock, particularly cattle, sheep, and goats [1,2]. The disease results in progressive weight loss, diarrhea, and eventual death, producing substantial economic losses for the livestock industry [3-5]. Prevalence studies have demonstrated wide distribution across Europe and worldwide, with evidence of endemicity in several countries [6,7]. The economic and animal welfare consequences of MAP infection highlight the urgent need for effective control strategies [8,9].

The pathogenesis of paratuberculosis is characterized by a prolonged subclinical phase during which MAP survives within host macrophages, evading immune detection and establishing chronic infection [10,11]. Pathological studies reveal a spectrum of granulomatous lesions associated with disease progression [12,13]. Experimental models in sheep and cattle have further delineated the immune response, confirming that both innate and adaptive immunity shape disease outcomes [14,15]. Vaccination has been used as a control measure, with variable success in reducing clinical

 doi:10.20944/preprints202510.0938.v1

signs and fecal shedding, though not completely eliminating infection [16-19]. However, vaccine-based strategies remain controversial due to diagnostic interference and incomplete protection [9,16].

Host genetics is a critical factor in determining susceptibility and resistance to MAP, as evidenced by variability among breeds and individuals [20-22]. Genome-wide association studies (GWAS) in cattle and sheep have identified quantitative trait loci (QTLs) associated with disease outcomes, many implicating genes involved in innate immunity and macrophage function [23-29]. Likewise, candidate-gene studies have associated polymorphisms in immune regulatory genes, including *SLC11A1*, *TLR2*, *CARD15/NOD2* and *IFNG*, with paratuberculosis risk [30-32]. Among these, the *solute carrier family 11 member 1* (*SLC11A1*) gene, a key regulator of macrophage antimicrobial activity, has emerged as a leading candidate across species [33-36]. *SLC11A1* encodes a divalent metal transporter expressed in phagosomal membranes, controlling the intracellular availability of iron and other cations essential for bacterial replication [36,37]. Functional studies have consistently linked polymorphisms in *SLC11A1* to altered susceptibility to intracellular pathogens, including *M. tuberculosis*, *Brucella*, and *Salmonella* [30,31,38-40].

In ruminants, a polymorphic microsatellite in the 3′ untranslated region (UTR) of *SLC11A1* has been a major focus of research due to its potential role in post-transcriptional regulation. In goats, specific $(GT)_n$ alleles are strongly associated with resistance to MAP, and functional assays confirm these variants modulate inducible gene expression upon pathogen challenge [41-44]. Similarly, associations between 3′UTR polymorphisms and disease susceptibility have been reported in cattle and buffalo [45,46]. These findings indicate the functional significance of this locus and its potential utility as a genetic marker for resistance.

Recent sheep GWAS have identified genomic regions associated with MAP resistance and have implicated the *SLC11A1* gene region among other candidates [28,29,47], however, SNP-based approaches are limited in their ability to detect microsatellite variation [48]. Therefore, although *SLC11A1* is known to be important in other species, the allelic variation of its 3′UTR $(GT)_n$ microsatellite and its association with paratuberculosis outcomes remain uninvestigated in sheep, leaving a defined knowledge gap.

This preliminary study aimed to determine whether specific, functionally relevant allelic variants of the $(GT)_n$ microsatellite in the ovine *SLC11A1* 3′UTR are associated with resistance or susceptibility to paratuberculosis, rather than to discover novel alleles. By genotyping this locus in a well-characterized sheep population with defined infection status, we sought to provide the first preliminary allele-level evidence for its role in sheep, thereby complementing the findings from previous GWAS. This work aims to identify possible genetic markers to support breeding programs for improved disease resistance.

2. Materials and Methods

2.1. Study population and sample collection

The study population comprised 138 adult sheep (Karagouniki, Boutsika, and Chios breeds) maintained at the Agricultural University of Athens, Greece. The flock consisted predominantly of Karagouniki sheep (n = 124), with smaller numbers of Boutsika (n = 5) and Chios (n = 9) animals. The flock has been monitored for paratuberculosis since 2014 using fecal real-time qPCR and blood ELISA [49] and has not been vaccinated against the disease. The animals are housed in an area isolated from other domestic and free-ranging species. Although qPCR testing regularly detects MAP infection in the flock, only a small number of clinical cases occur annually, which are promptly removed [49].

Sample testing was conducted in compliance with ISO17025 requirements (ISO/IEC 17025:2017) in connection with ELISA, DNA isolation, and qPCR. The analysis incorporated the recommended measures of quality assurance, including use of positive and negative controls, validation of the DNA quality, detection of PCR inhibitors, and confirmation of the specificity of the amplification product using sequence analysis.

 doi:10.20944/preprints202510.0938.v1

Whole blood samples were collected from the 138 adult animals of the flock and were processed for DNA isolation and sequence analysis of the 3′ UTR of the *SLC11A1* gene (n=138, 1 sample/animal). Two groups consisting of total 53 individuals were formed from these animals, based on the results of the qPCR and ELISA tests conducted during the 2 years that preceded the investigation (2020-2022). These groups, referred to as resistant (R) and sensitive (S), consisted of the individuals that reacted negatively to all the ELISA and qPCR tests that were conducted (group R, n=18), and of those that reacted positively to either of these tests at least 3 times (group S, n=35). All animals classified as S group and R group belonged exclusively to the Karagouniki breed. Individuals from the Boutsika and Chios breeds did not meet the inclusion criteria for either phenotypic group, as none showed consistent diagnostic reactivity over the monitoring period.

Samples of whole blood were collected in heparinized sterile blood collection tubes from the animals of the R and S group and were transported to the laboratory within 30min after collection, where they were stored at -80°C. These samples (n=53, 1 sample/animal) were processed for RNA isolation and RT-qPCR designed for the assessment of expression variations of the *SLC11A1* gene.

2.2. DNA and RNA isolation

DNA and RNA isolation was performed on whole blood (n=138) and buffy coat (n=53), respectively. In brief, isolation of DNA was conducted with Nucleospin Tissue DNA kit (Macheray-Nagel GmbH & Co. KG, Germany), whereas, with regards to the isolation of RNA, 5ml of whole blood samples were centrifuged at 600×g for 15min, within 30 min after collection. The sediment (buffy coat) was diluted in an equal volume of PBS-citrate (Sigma-Aldrich, USA) and then layered over Ficoll-Paque (Amersham Bioscienses, Sweden) solution. After centrifugation at 500×g for 40min, the mononuclear cells were collected and washed three times in PBS (Sigma-Aldrich, USA). RNA isolation was performed with the Nucleospin RNA Plus XS kit (Macheray-Nagel GmbH & Co. KG, Germany), whereas purification, with the RNA-Zol Direct Clean-Up kit (Fisher Molecular Biology, USA). All procedures were conducted according to the instructions of the respective manufacturers.

The quality and quantity of the DNA and RNA isolated was assessed for purity and integrity by submerged gel electrophoresis followed by image analysis using a Bio-Rad ChemiDoc XRS+ Molecular Imager (Bio-Rad Laboratories Inc., U.S.), and by optical density count at 260/280 nm, using a NanoDropTM 8000 spectrophotometer (Thermo Fisher Scientific Inc., U.S.). The presence of inhibitors in the DNA samples was assessed by a PCR assay targeting the housekeeping gene *β-actin* [50].

2.3. Sequence analysis of the ovine SLC11A1 gene

The sequence analysis of the 3′UTR of the ovine *SLC11A1* gene (GeneBank: U70255) was performed on the DNA isolated from the whole blood samples (n=138). The PCR assay incorporated into the amplification of the target region was performed as previously described (Table 1). Reactions were prepared in 20 μl containing 1× KAPA SYBR Fast qPCR Master Mix (Kapa Biosystems, USA), 200 nM of each primer, 10 ng bovine serum albumin (Thermo Fisher Scientific, USA), 2 μl template DNA, and nuclease-free water. Amplicons were sequenced on both strands using the BigDye® Terminator Cycle Sequencing Kit and PRISM® 377 DNA Sequencer (Applied Biosystems, USA).

Table 1. The primer sequences, product sizes, cycling conditions and relevant references for the assays targeting *IS900*, *SLC11A1* 3′UTR, *GAPDH*, *SLC11A1* mRNA, and *β-actin* used in this study.

Target	Primers (5′-3′)	Size	Thermal profile	Reference
IS900	F[1]: AATGACGGTTACGGAGGTGGT R[2]: GCAGTAATGGTCGGCCTTACC Pr[3]: TCCACGCCCGCCCAGACAGG	76 bp	95°C for 3min; 40 cycles of 95°C for 3sec, 60°C for 20sec, 72°C for 1sec; 43°C for 30sec	[51]
3′UTR *SLC11A1*	F: ACCTGGTCTGGACCTGTCTCATCA R: CATTGCAAGGTAGGTGTCCCCAT	346 bp	95°C for 3min; 35 cycles of 95°C for 10sec, 59°C for 20sec, 72°C for 1sec; 43°C for 30sec	[43]

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GAPDH	F: TTCCAGTATGATTCCACCCATG R: GCCTTTCCATTGATGACGAG	80 bp	42°C for 5min; 95°C for 15sec; 40 cycles of 95°C for 5sec, 52°C for 20sec, 72°C for 1sec; 43°C for 30sec	[52]
SLC11A1 mRNA	F: GGCTGTGGCTGGATTCAAAC R: ATGGTCAGCCAGAGGAGAATG	168 bp	42°C for 5min; 95°C for 15sec; 40 cycles of 95°C for 5sec, 57°C for 20sec, 72°C for 1sec; 43°C for 30sec	[43]
β-actin	F: TGTCTCTGTACGCTTCTGG R: GTGGTGGTGAAACTGTAGC	190 bp	95°C for 3min; 40 cycles of 95°C for 30sec, 55°C for 30sec, 72°C for 30sec; 72°C for 3min	[50]

[1] Forward; [2] Reverse; [3] Probe.

2.4. Gene expression analysis

Relative expression of *SLC11A1* was quantified using the One Step SYBR® PrimeScript™ RT-qPCR Kit II (Takara Bio Inc., Japan) as previously described (Table 1). Reactions contained 1× Takara buffer, 400 nM of each primer, 0.8 µl enzyme mix, 10 ng bovine serum albumin, 2 µl RNA, and RNase-free PCR grade water to a final volume of 20µl.

The relative quantification of the *SLC11A1* gene expression was performed using as reference the *GAPDH* gene whose amplification was conducted as previously described (Table 1). Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method [53].

2.5. Statistical analysis

Statistical analyses were conducted to assess (i) differences in allele frequency distributions of the (GT)n microsatellite region of the *SLC11A1* gene between resistant and sensitive phenotypic groups, and (ii) differences in relative gene expression levels of *SLC11A1* mRNA.

Allele frequencies were calculated as the proportion of individuals carrying each $(GT)_n$ repeat allele in the study population and in each subgroup. Associations between allele frequencies and phenotypic group (resistant *vs.* sensitive) were evaluated using Fisher's exact test for individual alleles and a chi-square test of independence for overall allele distribution. Effect sizes for contingency tests were estimated using Cramér's V.

Gene expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method, with *GAPDH* as the reference gene. Expression data were log-transformed for normality where appropriate. A two-tailed independent samples t-test was used to compare gene expression levels between groups. To assess the effect of genotype on gene expression, a one-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc tests was applied.

Assumptions of normality and homogeneity of variances for parametric tests were tested using the Shapiro–Wilk test and Levene's test, respectively. All analyses were performed using GraphPad Prism (v10) and IBM SPSS Statistics (v29.0). A p-value of <0.05 was considered statistically significant.

3. Results

3.1.(. $(GT)_n$ repeat polymorphism frequencies

A total of 138 sheep were successfully genotyped for the $(GT)_n$ microsatellite in the 3'UTR of ovine *SLC11A1*. In the overall population (n=138), the $(GT)_{24}$ allele was the most frequent, observed in 44 individuals (31.9%), followed by $(GT)_{22}$ in 40 individuals (29.0%), $(GT)_{23}$ in 35 individuals (25.4%), and $(GT)_{21}$ in 17 individuals (12.3%). The rare alleles $(GT)_{25}$ and $(GT)_{26}$ were each found in only 1 animal each (0.7% each), (Figure 1).

For the resistant subgroup (n=18; animals repeatedly negative in diagnostic tests), $(GT)_{23}$ was most common with 33.3%, followed by $(GT)_{24}$ with 27.8%, $(GT)_{21}$ with 22.2% and $(GT)_{22}$ with 16.7%. In the sensitive subgroup (n=35; animals repeatedly positive in diagnostic tests), $(GT)_{24}$ was most

 doi:10.20944/preprints202510.0938.v1

frequent with 45.7%, followed by $(GT)_{22}$ with 42.9%, $(GT)_{23}$ with 8.6%, and $(GT)_{21}$ with 2.9%. The study population consisted predominantly of Karagouniki sheep (n = 124), with smaller numbers of Boutsika (n = 5) and Chios (n = 9). Diagnostic testing indicated that all animals classified as resistant (n = 18) and sensitive (n = 35) belonged exclusively to the Karagouniki breed. The Boutsika and Chios individuals did not show consistent positive or negative reactivity during the two-year monitoring period. Consequently, statistical comparisons among breeds were not possible, and all subsequent genotype–phenotype analyses were based on the Karagouniki subset. This composition reflects the structure of the monitored flock and supports the representativeness of the dataset for preliminary association testing [21,22]. Moreover, the number of animals classified as resistant (n = 18) was considerably smaller than that of sensitive (n = 35) individuals. This imbalance reflects the natural epidemiology of paratuberculosis, in which persistently negative animals are rare even in well-managed flocks due to the chronic, subclinical progression of infection [7,11,15,22]. Inclusion in the resistant group required consecutive negative results in both ELISA and qPCR testing over a two-year surveillance period, a stringent criterion that limited the number of qualifying animals. Although the small size of the resistant group reduces statistical power, the analysis still revealed significant genotype–phenotype associations ($\chi^2 = 12.4$, $p = 0.006$) and strong effect sizes for certain alleles, suggesting that the observed relationships are biologically meaningful.

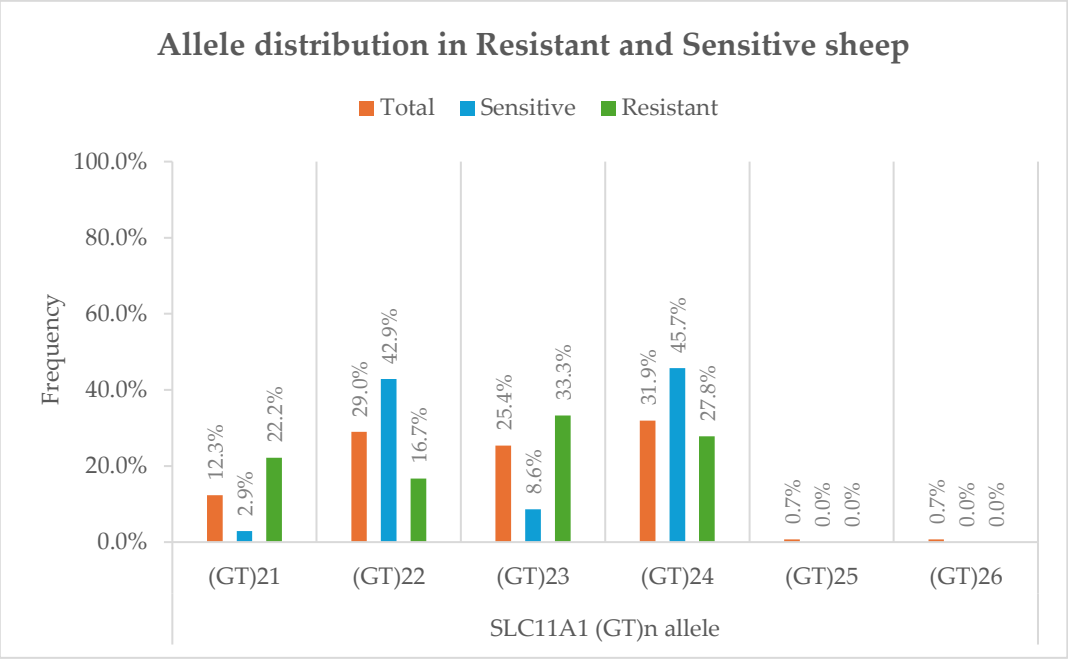

Figure 1. Distribution of ovine *SLC11A1* $(GT)_n$ alleles in the study population and in MAP-resistant (R) and MAP-sensitive (S) phenotypic groups.

3.2. Genotype–Phenotype association

Despite the limited number of resistant animals (n = 18) described above, statistical analyses were performed to assess whether the observed microsatellite allelic variation in the *SLC11A1* 3′UTR was associated with MAP-resistance phenotype. Fisher's exact test revealed significant enrichment of the $(GT)_{21}$ allele in resistant sheep (22.2%) compared to sensitive sheep (2.9%) ($p = 0.040$, OR = 9.5, 95% CI: 1.00–89.5). Similarly, $(GT)_{23}$ was more frequent in resistant animals (33.3%) than in sensitive ones (8.6%) ($p = 0.048$, OR = 5.3, 95% CI: 1.00–28.7). Conversely, the $(GT)_{24}$ and $(GT)_{22}$ alleles were more prevalent among sensitive sheep (45.7% and 42.9%, respectively) than resistant sheep (27.8% and 16.7%, respectively), though these differences did not reach statistical significance ($p = 0.217$ and $p = 0.063$, respectively).

 doi:10.20944/preprints202510.0938.v1

When allele distributions were analyzed overall, a significant association was observed between genotype and phenotype ($\chi^2 = 12.4$, df = 3, p = 0.006), with Cramér's V = 0.38, indicating a moderate effect size.

3.3. SLC11A1 gene expression

Relative mRNA levels of *SLC11A1* were measured in 53 sheep of Karagouniki breed (18 resistant, 35 sensitive). The $2^{-\Delta\Delta Ct}$ method was used, normalized to *GAPDH*. A t-test comparing sensitive and resistant groups revealed no significant difference [mean ΔCt_S = 4.92, mean ΔCt_R = 4.75; t(51) = 0.63, p = 0.531; 95% CI: –0.39 to 0.73; Cohen's d = 0.17], (Figure 2). One-way ANOVA of expression levels across all genotypes also showed no statistically significant variation [F(2,50) = 1.42, p = 0.25]. Post hoc pairwise comparisons using Tukey's HSD confirmed the absence of significant differences.

In summary, analysis of the *SLC11A1* 3′UTR $(GT)_n$ microsatellite in 138 sheep revealed a significant association between specific alleles and the MAP-resistance phenotype. The alleles $(GT)_{21}$ and $(GT)_{23}$ were enriched among resistant animals, whereas $(GT)_{22}$ and $(GT)_{24}$ were more common in sensitive individuals. Despite the small number of resistant animals and the absence of breed-level comparisons, the statistical strength of the association ($\chi^2 = 12.4$, p = 0.006; Cramér's V = 0.38) suggests biological relevance. No significant differences were observed in basal *SLC11A1* mRNA expression between phenotypic groups, indicating that microsatellite variation may influence inducible rather than constitutive gene regulation. Collectively, these results provide preliminary allele-level evidence supporting a role for *SLC11A1* microsatellite polymorphisms in resistance to MAP infection in sheep.

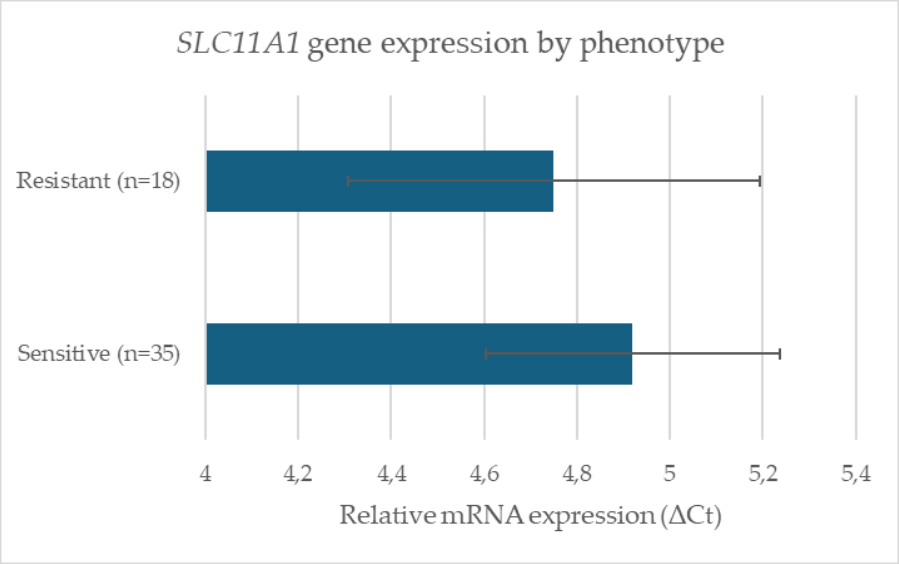

Figure 2. Comparison of *SLC11A1* gene expression levels between MAP-resistant and MAP-sensitive sheep. Relative mRNA expression was quantified by RT-qPCR and reported as ΔCt values (normalized to *GAPDH*). Bars show the mean ΔCt for each group (Sensitive, n = 35; Resistant, n = 18). Error bars indicate the 95% confidence interval of the mean (Resistant: 4.29–5.21; Sensitive: 4.60–5.24). An independent samples t-test found no statistically significant difference between groups (t(51) = 0.63, p = 0.531, Cohen's d = 0.17).

4. Discussion

Paratuberculosis imposes significant production losses in sheep flocks, including reduced productivity and increased mortality [3,22]. Serological surveys continue to highlight widespread infection [7], and economic reviews emphasize its financial impact [4]. Genetic selection for resistance, as suggested by our results, could reduce prevalence and losses, complementing vaccination and management measures [8,9].

Advances in biomarker discovery and molecular diagnostics may improve early detection and monitoring of MAP infection [54,55]. Our genetic findings contribute to these approaches by

 doi:10.20944/preprints202510.0938.v1

providing early evidence that specific *SLC11A1* alleles are associated with disease resistance in sheep. The present study provides additional insights into the role of *SLC11A1* 3′UTR $(GT)_n$ microsatellite polymorphisms in resistance to paratuberculosis in sheep. We identified significant enrichment of the $(GT)_{21}$ and $(GT)_{23}$ alleles in resistant animals, and higher prevalence of $(GT)_{22}$ and $(GT)_{24}$ in sensitive animals, though not always reaching statistical significance. These findings contribute to a growing body of evidence implicating *SLC11A1* as a candidate gene modulating susceptibility to MAP and other intracellular pathogens in livestock and humans [33,36,38]. To place our findings in context, Table 2 summarizes reported associations between *SLC11A1* polymorphisms and resistance/susceptibility across species.

Table 2. Comparative summary of reported associations between *SLC11A1* polymorphisms and disease-related phenotypes in livestock and humans.

Species	Variant/Region Analyzed	Association with Resistance/Susceptibility	Notes	References
Sheep	Genetic influences (preliminary, candidate-based)	Suggested possible genetic effect on Johne's disease susceptibility	Early evidence, not locus-specific	[21]
Sheep	GWAS (SNPs across genome)	Regions associated with MAP resistance; included *SLC11A1*	SNP-based, no microsatellite resolution	[29]
Sheep	GWAS (antibody response to MAP)	Regions linked to immune response; *SLC11A1* implicated	High-resolution genomic mapping	[28]
Sheep	Retrospective SNP analysis	Identified associations near *SLC11A1* with MAP resistance	Based on FFPE DNA, SNP focus	[47]
Sheep	3'UTR $(GT)_n$ microsatellite	$(GT)_{21}$ and $(GT)_{23}$ associated with resistance; $(GT)_{22}$ and $(GT)_{24}$ with susceptibility	Association found despite no difference in basal expression	This study
Goats	3′UTR $(GT)_n$ microsatellite	Shorter alleles enriched in resistant goats	Consistent with ovine findings	[43]
Goats	Functional analysis, 3′UTR microsatellite	Variants affected inducible expression under MAP challenge	Demonstrated functional mechanism	[41]
Goats	3′UTR microsatellite	Specific alleles associated with reduced paratuberculosis incidence	Validated earlier results	[44]
Cattle	Candidate gene SNPs (*SLC11A1*, *TLR4*, *IFNG*)	Associations with MAP susceptibility	Population-specific variation	[56]
Cattle	SNPs in *SLC11A1*	Associated with MAP infection risk	Consistent across populations	[46]
Cattle	SNPs in *SLC11A1* and others	Linked with breeding values for MAP traits	Large-scale genomic approach	[57]
Cattle	SNPs in *SLC11A1*	No association with MAP infection	SNPs polymorphic variants showed no allele/genotype differences between cattle	[58]
Cattle	*SLC11A1* SNP rs109453173	Associated with resistance (GG genotype/G allele protective; CC/CG linked to susceptibility)	Case–control study; suggests potential resistance marker	[59]
Buffalo	3′UTR microsatellite	Allelic variation influenced *MCP1* mRNA after *Brucella* challenge	Functional immune effects	[45]
Pigs	*SLC11A1* polymorphisms	Associated with immune traits	Cross-species evidence of functional role	[60]
Humans	*SLC11A1* SNPs and promoter variants	Associated with tuberculosis susceptibility	Strong parallels with livestock	[38,61]

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Evidence from goats has consistently demonstrated an association between *SLC11A1* polymorphisms and resistance to MAP. Korou et al. (2010) reported that shorter alleles at the 3′UTR microsatellite were enriched in resistant goats [43], findings later supported by functional analyses showing altered inducible gene expression [41,42]. Similarly, Abraham et al. (2017) identified specific *SLC11A1* alleles associated with reduced incidence of paratuberculosis in goats [44]. Our observation that the alleles $(GT)_{21}$ and $(GT)_{23}$ are linked to resistance in sheep aligns with earlier exploratory evidence of genetic influences on Johne's disease in sheep [21].

In cattle, *SLC11A1* polymorphisms have been associated with MAP susceptibility, though results vary across populations. Pinedo et al. (2009) found associations between *SLC11A1*, *TLR4*, and *IFNG* variants with MAP infection [56], while Ruiz-Larrañaga et al. (2010) identified SNPs in *SLC11A1* correlated with infection risk [46]. More recent GWAS studies also highlight *SLC11A1* and other innate immunity genes as candidates [25-27,57]. Functional evidence further supports the role of *SLC11A1* in macrophage control of MAP, as macrophages with certain genotypes show differential ability to limit bacterial replication [62]. Our results in sheep are consistent with these observations, however the absence of significant difference in *SLC11A1* expression between groups suggesting that the microsatellite may influence gene inducibility or protein function rather than baseline transcription.

Other livestock species also reinforce the role of *SLC11A1*. In buffalo, 3′UTR polymorphisms influenced *MCP1* mRNA expression in response to *Brucella* challenge [45]. In pigs, *SLC11A1* polymorphisms were linked to immune traits [40,60] and disease susceptibility [31]. In goats, additional work showed effects of 3′UTR alleles on gene function during MAP infection [41]. These cross-species findings highlight that, although *SLC11A1* mechanisms are generally conserved, the specific alleles conferring resistance may vary, necessitating species-specific investigations.

Our findings should also be interpreted considering previous GWAS in sheep, which identified genomic regions associated with MAP resistance or antibody response and highlighted the contribution of *SLC11A1* among other innate immune genes [28,29,47]. Although GWAS approaches are powerful for detecting broad genomic regions, they often fail to capture functional variation at microsatellite loci [48]. By directly characterizing allelic diversity at the $(GT)_n$ microsatellite in the 3′UTR of *SLC11A1*, our study complements GWAS findings and provides preliminary evidence for the role of this functional polymorphism in shaping resistance to MAP in sheep.

Our results must be considered in the broader framework of host genetics in MAP infection. GWAS studies in cattle and sheep have consistently identified loci associated with paratuberculosis, involving genes related to DNA repair, stress response, and innate immunity [23-26,28,29]. In cattle, associations with *CARD15*/*NOD2* [63,64], *TLR2* [65], and other innate receptors further emphasize polygenic influences [59,66]. Our findings add *SLC11A1* 3′UTR polymorphisms to this complex genetic landscape in sheep, consistent with reviews emphasizing multifactorial control of MAP resistance [67,68]. Notably, while we found significant genotype–phenotype associations, effect sizes were moderate, suggesting that *SLC11A1* contributes to but does not fully explain resistance.

One of the important aspects of our findings was the absence of significant differences in *SLC11A1* mRNA levels between resistant and sensitive groups. This observation contrasts with some functional studies in goats and buffalo, where 3′UTR variants affected gene expression [41,45]. However, our results are consistent with the notion that basal expression in blood cells may not capture functional differences that manifest only upon pathogen challenge [11,42]. Indeed, macrophage assays in cattle demonstrate that genetic differences may influence intracellular control of MAP rather than constitutive transcription [62]. This limitation suggests that the *SLC11A1* microsatellite in sheep may influence induced expression or protein function rather than constitutive transcription, therefore future research is important to include functional assays under MAP challenge and protein-level expression studies.

It should also be noted that our study population study population comprised three sheep breeds (Karagouniki, n=124; Boutsika, n=5; Chios, n=9), as breed-related genetic variation has been reported to influence MAP susceptibility in sheep [20–22]. However, as only Karagouniki sheep met

 doi:10.20944/preprints202510.0938.v1

the strict diagnostic criteria for inclusion in the sensitive or resistant phenotypic groups, we could not perform a breed-specific analysis. The low numbers of Boutsika and Chios sheep simply reflect their limited presence in the monitored flock. Nevertheless, this information has been retained for completeness, and future investigations including balanced multi-breed cohorts will be essential to determine whether the associations observed in Karagouniki sheep are consistent across other genetic backgrounds.

Another limitation was the small cohort of resistant animals (n=18). This is characteristic of paratuberculosis, as completely resistant individuals are uncommon even in infected flocks [7,11,15,22]. Our classification required consistent negative results over two years, making this a stringent and naturally small group. Similar challenges in identifying persistently negative animals have been reported in longitudinal and experimental infection studies [14,15,21]. Although the small sample size limits statistical power, the strong magnitude of certain associations (e.g., OR = 9.5 for $(GT)_{21}$) implies a real biological effect. Future research with larger populations is needed to validate these findings [20–22,67].

Finally, while this study focused on *SLC11A1*, other genes such as *TLR2*, *CARD15/NOD2*, and *IFNG* have also been associated with MAP resistance in cattle and sheep [56,59,63,65,66,69,70]. Integrating *SLC11A1* with other candidate genes such as *TLR2*, *CARD15/NOD2*, and *IFNG* will be essential to capture the full genetic landscape of resistance [59,66,69,70]. A more holistic understanding of genetic resistance will require integrating multiple loci into genomic selection strategies.

Evidence from tuberculosis research provides strong parallels. Human studies consistently link *SLC11A1* polymorphisms to tuberculosis risk [38,39], and similar findings exist in cattle [32,71-73] and goats [30]. Experimental infection models also demonstrate that *SLC11A1* influences macrophage activation and pathogen control [33-35].

The evolutionary conservation of *SLC11A1* highlights its importance in resistance to intracellular pathogens [33,74]. Comparative studies across species demonstrate both shared mechanisms and population-specific effects [75,76]. For example, in goats, cattle, pigs, and humans, *SLC11A1* variants are associated with disease outcomes, though the specific alleles differ [31,38,60,72]. Our identification of $(GT)_{21}$ and $(GT)_{23}$ as resistance alleles in sheep fits within this comparative framework, suggesting conserved mechanisms with species-specific variations.

Taken together, our findings underscore the potential of integrating genetic selection into paratuberculosis control. While vaccination provides partial protection [16,17], and management can reduce spread [8], genetic resistance offers a sustainable long-term strategy. The identification of resistant alleles in sheep aligns with broader efforts in cattle and goats to leverage genetic variation for disease control [28,29,77]. However, practical application requires further validation and consideration of breed-specific differences, diagnostic challenges, and integration with other control measures [9,11,70].

5. Conclusions

In conclusion, this study provides early evidence that polymorphisms in the 3′UTR $(GT)_n$ microsatellite of *SLC11A1* are associated with resistance and susceptibility to paratuberculosis in sheep. Specifically, the $(GT)_{21}$ and $(GT)_{23}$ alleles were enriched in resistant animals, while $(GT)_{22}$ and $(GT)_{24}$ were more common in sensitive animals. Our findings extend previous GWAS in sheep [28,29,47] by offering preliminary allele-level resolution at a functional microsatellite locus that is not captured by SNP arrays. They are also consistent with studies in cattle, goats, and buffalo [41-46,56,57] supporting the conserved role of *SLC11A1* in host defense across ruminants.

We acknowledge the limitations of our study, including the relatively small number of resistant sheep and the inability to perform breed-stratified analyses. Nevertheless, the strength of the observed associations suggests biological relevance. Future studies involving larger and more balanced cohorts, functional assays under pathogen challenge, and integration of additional candidate genes including *TLR2*, *CARD15/NOD2*, and *IFNG* [56,59,63,65,66,69,70] will be essential to

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confirm and extend these findings. Altogether, our results highlight that *SLC11A1* microsatellite polymorphisms may contribute to the genetic architecture of resistance to paratuberculosis in sheep and could eventually serve as markers to support breeding programs aimed at sustainable disease control.

Author Contributions: Conceptualization, J.I.; methodology, A.M. and A.K.P.; software, A.M.; validation, A.M.; formal analysis, A.M. and A.K.P.; investigation, A.M.; resources, J.I.; data curation, A.M.; writing—original draft preparation, A.M., A.K.P. and J.I.; writing—review and editing, A.M. and J.I.; visualization, A.M.; supervision, J.I.; project administration, J.I. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Protocol No. 77/03.09.2025 of the Research Ethics Committee (REC) of the Agricultural University of Athens.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author(s).

Conflicts of Interest: The authors declare no conflicts of interest.

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