The Use of Genetic Merit and Allelic Frequencies to Alter bTB Susceptibility and Disease Progression in Cow Herds

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ABSTRACT

The study aimed to characterize the relationship between Holstein cows' TLR2 gene polymorphisms and their susceptibility to tuberculosis. In total, 200 Tunisian Holstein cows were used, in wich 100 control and 100 cases. PCR-RFLP and direct sequencing PCR product were used to characterize genetic polymorphisms of TRL2. Univariate logistic regression analysis was performed to characterize the relationship between studied variables and the bTB status for analyzed animals. PROC LOGISTIC method was utilized to ascertain the genotypic and allelic frequencies that were associated with bTB status. The odds ratio (OR) of wild genotypes compared to muted genotypes was computed. Results showed that single intradermal tuberculin test for bTB was not affected by the farm or the age factors ($p > 0.05$). The allele frequency for studied animal was 72 % and 28 % respectively for G and T alleles. Statistical analysis confirms the significant ($p\ 0.05$) effect of the genotype at individual and herd level. The animal's vulnerability against bTB was most likely associated with the G allele and bTB can be observed in GG genotype 10 % more than TT genotype. The identification of specific SNP can be a sure way to improve the genetic merit of cow herds against bovine tuberculosis incidence. The use of genetic merit and allelic frequencies can alter bTB susceptibility and disease progression in cow herds.

INTRODUCTION

As per Zhang *et al*. (2019), the *Bacillus* genus is responsible for the chronic infectious disease tuberculosis, which can affect both animals and humains (Pant *et al*., 2023). *Mycobacterium bovis*, a member of the *Mycobacterium tuberculosis* complex, is the causative agent of bovine tuberculosis (bTB), a chronic infectious disease (le Roex *et al*., 2013; Bezos *et al*., 2023; O'Brien *et al*., 2023). Despite that bTB is widespread around the world and is the principal factor regarding economic losses in the livestock field, there aren't enough effective prevention and control measures in place, especially in developing countries (Proano-Pérez *et al*., 2011; Song *et al*., 2014; Wang *et al*., 2015; Barnes *et al*., 2023). *Mycobacterium bovis* can infect humans, animals, and many kinds of wildlife. bTB also poses a hazard to public health (Refaya *et al*., 2020; Ciaravino *et al*., 2021; Quadri *et al*., 2021). This disease primarily affects the lower and upper respiratory tracts throughout an incubation period of many weeks to months. Granulomatous pathologic alterations and overt clinical symptoms are associated with this illness (Chauhan *et al*., 2019; Banos, 2023; Rani *et al*., 2023). For effective bTB control, infected animals must be quarantined because they may has been infected for a long time before exhibiting clinical symptoms or lesions (Arnot and Michel, 2020; Çakir *et al*., 2022; Davey, 2023). Currently there is no effective vaccination against bTB, and the available treatment regimens are both expensive and not generally advised. Due to socioeconomic factors, social norms, and religious taboos, eradicating the disease through the slaughter of infected animals is especially challenging (Song *et al*., 2014; Ngwili *et al*., 2021; Petrovan *et al*., 2021; Ciaravino *et al*., 2023; Ramanujam and Palaniyandi, 2023). Selection of animal with the higher merit regarding the bTB resistance within herds

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could be a sure way and successful alternative methods given the challenge of decreasing the frequency of this illness using conventional measures, based on cumulative and permanent genetic gain (Marjamaki *et al*., 2021; Banos, 2023; Zubby, 2023). bTB depend to the animals genotype at both individual and herds level. The variation in the number of genes and their polymorphism increase or decrease susceptibility to bTB and alter disease progression. Toll-like receptors (TLRs) are a class of receptor molecules that are expressed on leukocytes, T and B cells, and some non-immune cells; their main role is to trigger the immune system in response to the presence of harmful bacteria and their virulence factors (Vlasova and Saif, 2021; Bastos *et al*., 2022; Lekki-Jozwiak and Bąska, 2024) . The toll-like receptor 2 (TLR2) recognizes *Mycobacterium* membrane components and links them to adaptor molecules like the myeloid differentiation factor (MYD88) and the toll-interleukin-1 receptor TIR, especially the TIR-domain-containing adapter- inducing interferon-β (TRIF) to activate MYD88 and TRIF-dependent signaling pathways in macrophages or other cells, leading to the secretion of inflammatory cytokines, chemokines, interferon, and antimicrobial peptides (Pahari *et al*., 2020; Thada *et al*., 2021; Hu and Spaink, 2022). This is thought to be how the toll-like receptor (TLR) starts host defense against tuberculosis (Mandala *et al*., 2020; Wicherska-Pawlowska *et al*., 2021; Kim and Shin, 2022; Donovan *et al*., 2023). Various marker systems can be used to study TLR polymorphism in cattle in the way to identify relationship between genetic and BTB resistance (Mandala *et al*., 2020; Wicherska-Pawlowska *et al*., 2021; Kim and Shin, 2022; Donovan *et al*., 2023). Different TLR gene mutations have been examined for their impact on disease resistance/susceptibility in other studies, and more recent research has examined their impact on economically significant traits of cattle, such as milk production and it quality (El-Hefnawy *et al*., 2020; Pal and Chakravarty, 2020; Kravitz *et al*., 2021; Mazzone *et al*., 2023; Usai *et al*., 2024). The present work aimed the characterization of TLR2 genetic polymorphism to alter bTB susceptibility and disease progression in cow herd's and to improve bTB resistance at North African level.

MATERIALS AND METHODS

The study was approved by the Ethical Review Committee of the National Agronomic Institute of Tunis, Tunisia. All measures were taken to minimize the pain and discomfort of animals during the conduction of this experiment.

Sample collection and DNA extraction

The standard method for detection of bovine tuberculosis, intradermal tuberculin skin test, was used to identify case and control individuals. The identification of target cows involves the measurement skin thickness after injecting bovine tuberculin intradermal. After the injection in the next 72 h if any subsequent swelling is spotted in the site of injection the decision concerning the tested animals is made. This study involved 200 Holstein cows, 100 of which tested positive for bTB (case individuals) and 100 of which tested negative (control individual). These cows were from five different Tunisian geographical regions. The single intradermal tuberculin test with bovine tuberculin was used to test for bTB. The cows' blood was drawn and kept in tubes containing 1.5% EDTA-2K. The Blood Genomic DNA Extraction Kit (innuPREF Blood DNA micro kit) was used to extract genomic DNA, which was then kept at -20°C.

Genotyping

The TLR2 gene polymorphism was analyzed using PCR-RFLP and direct sequencing of PCR products methods. Integrated DNA Technology's Oligo Analyzer software was used to create primers for the SNPs in the TLR2 (rs55617172) gene to amplify target loci. Table I lists the used primers and restriction enzyme. After optimizations, ideal PCR conditions, were used and generated PCR products were used for both restriction and sequencing parts. The 25 µl volume used for the PCR reaction contained 7.7 µl of PCR Master Mix, 2.5 µl of each primer, 4 µl of genomic DNA (50 ng/µl), and 8.3 µl of ultrapure water. The following steps were taken throughout the PCR reactions: A preliminary denaturation step at 95°C for 7 min; 30 cycles of 95°C for 40 s, 59.2°C for 40 s; 72°C for 40 s; and final annealing at 72°C for 12 min. PCR results were examined using electrophoresis on 2 % staining agarose gel. To find known mutations and restriction fragment length polymorphisms, *TLR2* gene amplifiers were digested with EcoRV (Table I). Both case and control samples were used for single-pass bidirectional sequencing analysis.

Table I. Primers and restriction enzymes used for the characterization TLR2.

Primers	Chromosomes	Primer sequence	Tm	Length (bp)	Enzyme
TLR2	17	3`-TTAAACTCCATCCCCTCTGG-5` 5`-TAAAGGGACCTGAACCAGG-3`	59.2°C	245bp	EcoRV

Statistical analysis

Statistical analysis

Analyzed data contains studied genotypes for TRL2 (GG, TG, TT), population (Farm) and age (primiparous and multiparous). Univariate logistic regression analysis was performed to characterize the relationship between studied variables and the bTB status for analyzed animals. PROC LOGISTIC method was utilized to ascertain the genotypic and allelic frequencies that were associated with bTB status. The odds ratio (OR) of wild genotypes compared to muted genotypes was computed.

The analysis of the bTB incidence (The single intradermal tuberculin test for bTB) was done using the following model:

$$Y_{ijkl} = \beta_i \ TLR2_i(Np_m) + \beta_j \ Ag_j(Np_m) + \beta_k \ farm_k(Np_m) + e_{ijk}$$

With:

Y_{ijkl}: depend of the bTB status.

TLR2: the fixed effect of the ith genotype of the TRL2 gene (i = GG, TG or TT) with same BTB status (case or control individuals)

Ag: the fixed effect of jth age (primaparous or multiparous)

farm: the fixed effect of the k^h farm (l = 1, 2,3,4 or 5)

e_{ijk}: residuals error

RESULTS AND DISCUSSION

Effect of non-genetic factors

Statistical analysis showed a non-significant effect of non-genetic factors. The single intradermal tuberculin test for bTB was not affected by the farm or the age factors (p>0.05).

TRL2 gene polymorphism characterization

The 245 bp PCR products were digested with the EcoRV restriction enzyme. Genotypes were defined in regard of showed bands on 2 % stained agarose gel. The TT genotype was observed when 182 bp and 63 bp bands were revealed. The GG genotype (wild type) is characterized by 245 bp band. If 245 bp, 168 bp and 63 bp bands exist, it's characterized heterozygous individuals. Bhaladhare *et al*. (2016) found a similar profile (Fig. 1). However Kumar *et al*. (2019) found larger bands but they detected the same three genotypes, TT mutated homozygotes, TG mutated heterozygotes and GG wild-type homozygotes. Sequencing PCR product for analyzed cows, confirmed the set up genotypes. EcoRV restriction enzyme digested when T allele is exist (Fig. 2). Genotype frequencies for Wild type, heterozygote and mutated individuals were 56%, 32% and 12%, respectively. A polymorphism was characterized in the TLR2 gene's SNP (rs55617172) (Fig. 1). We found a significant difference (p < 0.05) between the rates of wild GG homozygotes in cases (64 %) and controls (48 %) when comparing the genotype frequencies of cases and control samples, indicating a likely relationship of this genotype with bTB susceptibility. In case, the heterozygote frequency was 26%, while in control, it was higher (38%). In cases, the genotypic frequency of TT was 10 % compared to 14 % in controls.
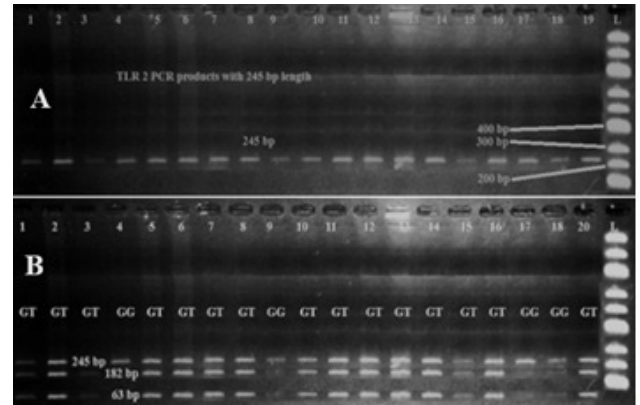

Fig. 1. TLR2 gene PCR products (A) and PCR-RFLP (B) electrophoresis profile (2 % stained agarose gel). GG, wild genotype; GT, heterozygote genotype; TT, mutated genotype.
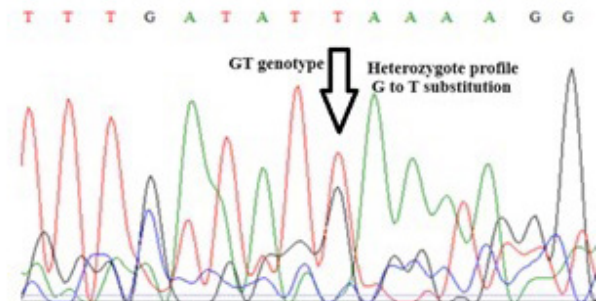

Fig. 2. Sequencing profile for heterozygote genotype (G to T substitution).

The allele frequency for studied animal was 72 % and 28 % respectively for G and T alleles. These results indicate the vulnerability of Tunisian dairy herds against tuberculosis pathogens. Statistical analysis confirms the significant (p 0.05) effect of the genotype at individual and herd level (Table II). The prevalence of the G allele rose in cases, confirming its link to the susceptibility against bTB. The observed odds ratio indicates that the incidence of bTB was 12 % when the genotype is homozygous GG than when it's heterozygous GT. The animal's vulnerability against BTB was most likely associated with the G allele and bTB can be observed in GG genotype 10 % more than

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TT genotype (Odds ratio = 9.8 % for TT).

Table II. Genotype affecting the susceptibility/ resistance against bovine tuberculosis in studied individuals.

SNP ID	Geno-type	Genotype frequency		p value	Odds ratio (95% CI)
		Case	Control		
rs55617172	G/G	64 %	48 %		1
	T/G	26 %	38 %	0.01	0,12
	T/T	10 %	14 %		0,098

Several study aimed to explore the association between TLR genetic polymorphism and bovine tuberculosis susceptibility. In the TLR2 locus Bhaladhare *et al.* (2016) were found a significant (p<0.01) association of genetic polymorphism with susceptibility/resistance to bTB. These authors found the same result of this study. The heterozygous individuals GT are more resistant compared to GG or TT homozygote genotypes. The same result regarding the association TLR gene polymorphism with tuberculosis incidence in buffalo were characterized (Alfano *et al.*, 2014). The identification of specific SNP can be a sure way to improve the genetic merit of cow herds against bovine tuberculosis incidence. The use of genetic merit and allelic frequencies can alter bTB susceptibility and disease progression in cow herds.

CONCLUSION

Results showed that single intradermal tuberculin test for bTB was not affected by the farm or the age factors (p>0.05). Genotype frequencies for Wild type, heterozygote and mutated individuals were 56 %, 32 % and 12%, respectively. The allele frequency for studied animal was 72% and 28%, respectively for G and T alleles. Statistical analysis confirms the significant (p 0.05) effect of the genotype at individual and herd level. The prevalence of the G allele rose in cases, confirming its link to the susceptibility against bTB. The observed odds ratio indicates that the incidence of bTB was 12 % when the genotype is homozygous GG than when it's heterozygous GT. The animal's vulnerability against bTB was most likely associated with the G allele and bTB can be observed in GG genotype 10 % more than TT genotype. The identification of specific SNP can be a sure way to improve the genetic merit of cow herds against bovine tuberculosis incidence. The use of genetic merit and allelic frequencies can alter bTB susceptibility and disease progression in cow herds.

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