# WATER BUFFALO (*BUBALUS BUBALIS*) SUSCEPTIBILITY TO BOVINE TUBERCULOSIS IS INFLUENCED BY G.4002C>T POLYMORPHISM IN INTERLEUKIN-10 GENE

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## ABSTRACT

Outcome of bovine tuberculosis (bTB), an infectious disease caused by *Mycobacterium bovis*, is influenced by host genetic background. For this reason, polymorphism discovery association studies are a powerful tool for selective breeding helping disease control. Interleukin-10 (IL-10) is a regulatory cytokine produced by different cells and fine-tune immune response to bTB. Thus, in this research, we evaluated the role of the single nucleotide polymorphism g.4002C>T in IL-10 gene for susceptibility to bTB in Mediterranean water buffalo. We characterized 184 animals grouped in cases and controls and demonstrated that homozygous subjects TT are about 3 times more susceptible to bTB compared to CC homozygous. Indeed this polymorphism is responsible of amino acid substitution p. (Thr 175 Met) in the primary protein sequence which affects protein secondary structure. This polymorphism might represent a valid tool for marker assisted selection against bovine tuberculosis in water buffalo.

**Keywords**: *Bubalus bubalis*, buffalo, gene polymorphisms, interleukin-10, bovine tuberculosis, Italy

## INTRODUCTION

Interleukin-10 (IL-10) is a regulatory cytokine produced by different innate and adaptive immune cells during bovine tuberculosis (bTB) (Dorhoi and Kaufmann, 2016) an infectious disease caused by *Mycobaterium bovis*. *M. bovis* is the causative agent of bovine tuberculosis (bTB), an infectious disease endemic in many countries (Marassi *et al*., 2009; Medeiros *et al*., 2010; Laisse *et al*., 2011) where is responsible of economic losses and i still considered a risk factor for humans (Humblet *et al*., 2009; Laisse *et al*., 2011). The interaction of *M. bovis* with its hosts is long-dated (Alvarez *et al*., 2009) and is therefore plausible assuming that their co-evolution (reciprocal adaptation) influenced the genomes of the pathogen as well as that of its hosts. The genetic makeup of the host may therefore reasonably plays a crucial role in the resistance to the pathogen. Selective breeding for disease-resistant genotypes represents therefore an approach supporting disease control (Bishop and MacKenzie, 2003; Persson and Vance, 2007). For example, SNPs located within the LRR domain of Toll-like receptors 2 (TLR2), Toll-like receptors 4 (TRL4) and Tool-like receptors 9 (TRL9) confer resistance to *M. bovis* (Alfano *et al*., 2014).

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Moreover, the same study identified three genetic polymorphisms - located in the solute carrier family 7 member 13 (SLC7A13); the Interleukin-1 alpha (IL1a); and the Deleted Malignant Brain Tumours 1 (DMBT1) genes - associated with *M. bovis* infection in the African buffalo (Roex *et al*., 2013). Furthermore, also variability in non translated regions have been associated with susceptibility to bTB. Indeed we recently have reported that an intronic polymorphism in Tumor Necrosis Factor-a (TNFa) gene belonging to a potential regulatory sequence, confers susceptibility to bTB (Papaianni *et al*., 2017) indicating that polymorphism in cytokine may have an important role as resistance/ susceptible gene in bTB. A further SNPs in the 3'UTR region of Interferon gamma (IFNg) which is a part of the target sequence recognized by microRNA 125b also increases susceptibility to bTB (Iannaccone *et al.*, 2018a) showing that microRNAs have a double role beside the ones as biomarker (Iannaccone *et al*., 2018b) Thus, because polymorphisms in cytokines may have an important role in bTB, we decided to investigate the genetic variability in IL-10 of water buffalo related to resistance/susceptibility to bTB.

## MATERIAL AND METHODS

### Samples collection

Speciment samples were collected from 184 animals reared in different herds located in Campania region (south Italy) and grouped in cases (positive to hypersensitivity and microbiological test, 59 samples) and controls (negative to multiple hypersensitivity test, 125 samples) according to previous work (Alfano *et al*., 2014). DNA was extracted using QIAamp DNA mini kit according to manufacturer's procedure and purity was analyzed using Nanodro specfotometer. Only samples with ratio A260/280 higher than 1.8 were used for further analysis.

### IL-10 amplification and polymorphism discovery

Using primers designed (forward: 5'-TTCATCTCCCAATGCAAGCAAGCTA-3'; reverse:5'-ATCGGATTTCAGAGGTCTTCC GTTTAT-3') on water buffalo genome sequence (GeneBank NW_005783511, 356114. 360857), we amplified and sequenced a genomic DNA region of 300 nucleotides spanning the coding region of the exon 5 in 10 cases and 10 controls. Amplification thermal conditions were the following: 5 minutes at 95°C and then 30 seconds at 95°C, 30 seconds at 55°C, and 40 seconds at 72°C (40 cycles), with a final extension for 5 minutes at 72°C and sequencing was performed by external company (Microgem, S.r.l., Italy) Genotyping for the g.4002C>T polymorphism was carried out using the following primer: C-allele primer: 5'-TACATAGAAACCTACGTGACAAC-3'; T-allele primer: 5'-TACATAGAAACCTACGTGA CAAT-3'; common reverse: 5'-ATCGGATTT CAGAGGTCTTCCGTTTAT-3'.

### Bioinformatic and statistical analysis.

Sequence alignments was performed using and results were aligned using by use of Chromas software (Technelysium, Queensland, Australia). Secondary protein structure analysis was carried out using https://zhanglab.ccmb.med. umich.edu/I-TASSER/ (Roy *et al*., 2010). ORs and 95% confidence intervals were calculated by Fisher's exact test using the statistical package GraphPad Prism version 5 (GraphPad, La Jolla, CA, USA). Hardy–Weinberg equilibrium by the Hardy–Weinberg calculator (http://www.oege.org/

software/hwe-mr-calc.shtml).

## RESULTS AND DISCUSSION

A genomic DNA sequence spanning the exon 5 of the IL-10 gene in water buffalo was amplified and sequence alignments show a point transversion g.4002C>T. which is responsible of an amino acid substitution p.(Thr175Met) in the primary protein sequence. Using a free web tool for protein secondary structure prediction (https://zhanglab.ccmb.med.umich.edu/I-TASSER/), we found that the substitution p.(Thr175Met) lengthens the a-helix compared to the wild type sequence (Figure 1).

Thus, to test the possibility that g.4002C>T polymorphism is a bTB, we genotyped using an allele-specific PCR all remaining samples and results are summarized in Table 1.

Homozygous subjects TT are more represented in the cases group (22 out of 59; frequency: 0.37) compared to controls groups (24 out of 125; frequency: 0.19); conversely, the genotype CC is more common in the controls group (52 out of 125; frequency: 0.42) compared to the cases group (14 out of 59; frequency: 0.27). The comparison of the CC *vs* TT ratio between case and control subjects by Fisher's exact test, showed an odds ratio (OR) of 2.97; with the 95% confidence interval (CI) ranging between 1.231 and 6.667; P-value (two sided) = 0.0088. This result strongly suggests a predisposition of TT subjects to bTB because the OR is in the range between 2.1 and 5.7 (Ioannidis et al, 2001). Moreover, as expected in case of association (Anderson *et al*., 2005), genotypic frequencies at the *IL-10* locus were in equilibrium among controls and in disequilibrium among cases (Table 2).

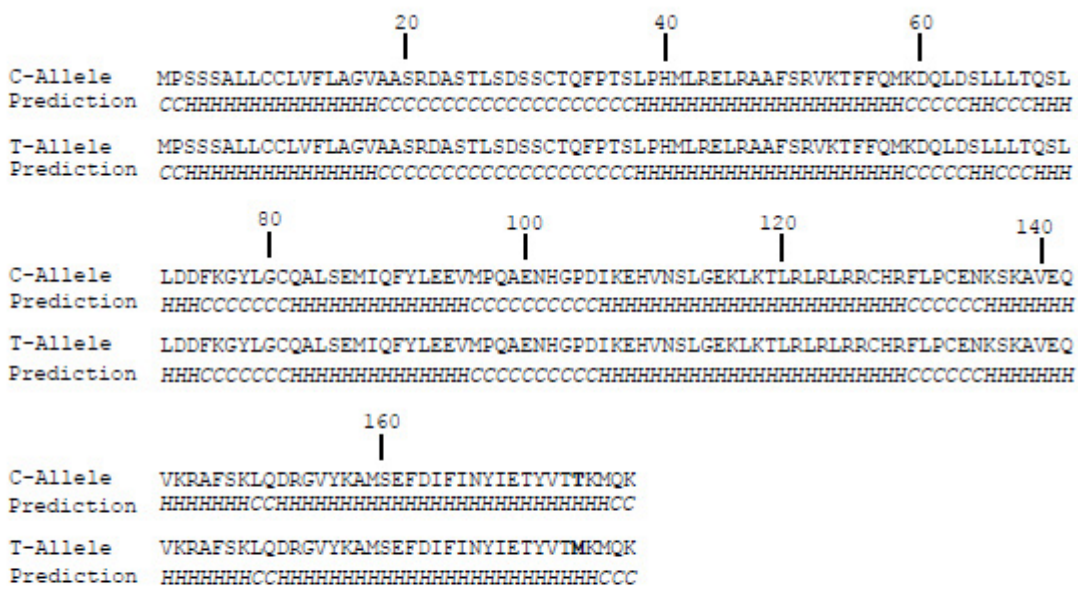


Figure 1. Predictive comparative analysis of IL-10 proteins translated from C-allele at the site g.4002. Sequences are compared based on secondary structure generate by I-TASSER software (http://zhanglab.ccmb.med.umich.edu/L-TASSER). C: coil H: helix.

Table 1. Association between susceptibility to *M. bovis* infection and the g.4002C>T polymorphism in water buffalo.

| Genotypes | Controls | Cases | OR (95% CI) | P-value[aa] |
|---|---|---|---|---|
| CC | 52 | 14 | - | - |
| CT | 49 | 23 | 2.14 (0.988 to 4.630) | 0.0764 |
| TT | 24 | 22 | 2.97 (1.231 to 6.667) | 0.0088 |

[aa]Fisher's exact test.

Table 2. Hardy-Weinberg equilibrium based on g.4002G>A in water buffalo infected and not infected with *M. bovis*.

| Status | GG | AG | AA | $\chi^2$ |
|---|---|---|---|---|
| Cases | 14 | 23 | 22 | 4.65 |
| Controls | 52 | 49 | 24 | 3.81 |

## CONCLUSION

In conclusion, our data demonstrated that susceptibility to bovine tuberculosis in water buffalo is influenced by the polymorphism g.4002C>T in *IL-10* gene probably consequent to a change in the protein secondary structure. This polymorphism might represent a valid tool for marker assisted selection against bovine tuberculosis in water buffalo.

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