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Molecular characterization of the NRAMP1 gene in buffalo

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ABSTRACT - NRAMP1 (natural-resistance-associated macrophage protein) gene influences the initial phase of bacterial cellular infections, regulating macrophage activation. Recent literature on buffalo has attempted to associate the genotypes at the polymorphic microsatellite, that is located in the 3'-UTR of the gene, with either susceptibility to brucellosis or improved macrophage function. However, contradictory results were reported. In the present work, we have sequenced the whole coding region, as well as part of the introns and UTRs, of the NRAMP1 gene in 49 Mediterranean buffaloes, including both serologically positive and negative animals to Brucella abortus test. We have detected 12 mutations. Nineteen haplotypes were built from the detected variant alleles, so demonstrating the high variability of this gene in buffalo, but no significant differences in haplotype frequencies were found between serologically positive/negative animals.

Key words: NRAMP1 gene, Buffalo, Mutation detection, Haplotypes.

Introduction - NRAMP1 (natural-resistance-associated macrophage protein) gene influences the initial phase of bacterial cellular infections, regulating macrophage activation (Blackwell et al., 2000). NRAMP1 belongs to a well-conserved group of genes in various mammalian species (Vidal et al., 1993). A Gly to Asp substitution at position 169 of the mouse NRAMP1 protein is invariably associated with the resistant/susceptible phenotypes to Mycobacterium bovis, Salmonella typhimurium, and Leishmania donovani (Ables et al., 2002). The transcript of the gene was sequenced by Feng et al. (1996) in cattle (Acc. no. U12862) and in water buffalo (Acc. no. U27105); by Matthews and Crawford (1998) in sheep and deer (Acc. no. AF005380 and AF005379). A (GT)ₙ microsatellite repeat was located in the 3'-UTR, 60 nucleotides downstream from the TGA stop codon, in all four species. Ables et al. (2002) have sequenced the NRAMP1 gene in various breeds of cattle and buffalo but did not find any polymorphism at the position which corresponds to the same amino acid of the murine NRAMP1-resistant phenotype. Ganguly et al. (2008) evaluated the association of the NRAMP1 polymorphisms of the 3'-UTR with the macrophage function, demonstrating that the (GT)₁₃ allelic variant is significantly associated with the improved macrophage function in buffalo. Capparelli et al. (2007) demonstrated that the (GT)₁⁶ carrier buffaloes are inherently resistant to brucellosis. On the contrary, Kumar et al. (2005) found that, in the Indian zebu and crossbred cattle, the microsatellite genotype is not associated with resistance to brucellosis. In the present work, we sequenced the whole coding region, as well as part of the introns and UTRs, of the NRAMP1 gene in 49 Mediterranean buffaloes with the purpose of detecting mutations.

Material and methods - The serological tests for brucellosis were carried out by rapid serum agglutination and complement fixation tests (Alton et al., 1975). DNA from two groups of buffaloes of the same farm (23 serologically positive and 26 negative to Brucella abortus tests) was extracted from fresh blood samples and was amplified with primers designed on cattle and buffalo sequences
(DQ848779.1, DQ493965, U27105) in order to produce 7 amplicons; the PCR primers, \( T_{\text{annealing}} \) and locations on buffalo gene are reported in Table 1. The amplification was performed in a final volume of 20 µl, containing 100 ng genomic DNA, 0.2 mM of each dNTP, 20 pmol of each primer, and 1 U GoTaq® DNA Polymerase (Promega).

The detection of sequence variations was performed on a ABI Prism 310 DNA sequencer by using the ABI Prism BigDye Terminator Cycle Sequencing, Ready Reaction Kits (version 1.1 - Applied Biosystems). Haplotype analysis was performed by using the Arlequin 3.0 software (Excoffier et al., 2005).

### Table 1. Primers, amplicons size and location.

| Forward primer | Reverse primer | T.a. | Size (bp) | Covered region in *Bubalus*
|----------------|----------------|------|-----------|-----------------------------|
| gggagaggtgcagcaacctca | tcctaccctgcatagga | 60°C | 315 | Promoter, partial exon 1 |
| cagccacgctcagcagag | aatcttaagcgccacagct | 58°C | 914 | Partial exon 1 to partial intron 2 |
| cttgccagggctcatctcag | tttctgtgccattgaacctt | 58°C | 290 | Partial intron 2 to partial intron 3 |
| tcctccctacagtgaagtc | gtcacctcagggttcaagtaa | 62°C | 443 | Partial intron 3 to partial intron 4 |
| ggcctcggcgaactgagac | gctgaaggctctcctg | 61°C | 387 | Partial intron 6 to partial intron 7 |
| acatgtgatgctcagagtaaa | gctggtcttgaagtaagga | 58°C | 578 | Partial intron 9 to partial intron 11 |
| gaaaatgcggcaggggtctac | tgtctcagcggcactagct | 60°C | 572 | Partial intron 13, to 3'UTR |
| gctgtgatgatgtaggctgtagtag | ttggccttacctcagaaactt | 60°C | 110 | 3'UTR |

### Table 2. Detected haplotypes in the sampled buffaloes.

| Pos. | g86a | g906a | g973a | c275t | g6t | g31a | g422a | a201t | c319g | del 154^55 | t132c | g7(n) |
|------|------|-------|-------|-------|-----|------|-------|-------|-------|------------|------|-------|
| 1    | G    | G     | T     | T     | G   | A    | A     | G     | -     | T          | GT(12) |
| 2    | G    | G     | G     | T     | G   | G    | A     | A     | G     | -          | T     | GT(12) |
| 3    | G    | G     | G     | C     | G   | G    | G     | A     | C     | CC         | T     | GT(16) |
| 4    | G    | G     | G     | G     | G   | G    | A     | C     | CC    | T          | GT(12) |
| 5    | G    | G     | A     | C     | G   | A    | A     | T     | C     | CC         | C     | GT(9)  |
| 6    | A    | A     | G     | T     | G   | G    | A     | A     | G     | -          | T     | GT(12) |
| 7    | G    | G     | G     | C     | G   | G    | A     | C     | -     | T          | GT(12) |
| 8    | G    | G     | G     | T     | G   | G    | A     | A     | G     | CC         | T     | GT(12) |
| 9    | G    | G     | A     | C     | G   | A    | A     | T     | C     | CC         | T     | GT(16) |
| 10   | A    | G     | G     | T     | T   | G    | A     | A     | G     | -          | T     | GT(12) |
| 11   | A    | A     | G     | T     | G   | G    | A     | A     | G     | -          | T     | GT(12) |
| 12   | G    | G     | A     | C     | G   | A    | A     | A     | C     | CC         | C     | GT(9)  |
| 13   | G    | G     | A     | C     | G   | A    | A     | A     | G     | CC         | C     | GT(9)  |
| 14   | G    | G     | G     | T     | G   | G    | A     | T     | C     | -          | T     | GT(12) |
| 15   | G    | G     | G     | C     | G   | G    | A     | G     | CC    | T          | GT(16) |
| 16   | G    | G     | G     | T     | G   | G    | A     | A     | C     | -          | T     | GT(12) |
| 17   | G    | G     | G     | T     | G   | G    | G     | A     | C     | CC         | T     | GT(16) |
| 18   | A    | A     | G     | T     | G   | G    | A     | A     | C     | CC         | T     | GT(12) |
| 19   | G    | G     | G     | T     | G   | G    | A     | T     | C     | CC         | C     | GT(9)  |
The significance of differences in haplotype frequency between serologically positive/negative animals was calculated with Chi-square test.

Results and conclusions - The buffalo sequences not previously described were deposited into NCBI with Acc. no. DQ390205, DQ441408, DQ390206. A total of 12 allelic variants were identified and the SNP positions, relative to the available sequences, are reported in Table 2. Using these polymorphisms, a total of 19 haplotypes were inferred occurring at variable frequency (from 1% to 35%) (Table 2).

Three allelic variants (respectively, GT\textsubscript{(9)}, GT\textsubscript{(12)}, and GT\textsubscript{(16)}) at the polymorphic microsatellite in the 3'-UTR were identified, with GT\textsubscript{(12)} allele occurring most frequently (67%), followed by GT\textsubscript{(16)} allele (18%) and GT\textsubscript{(9)} (14%). No significant differences in the frequency of GT\textsubscript{(n)} variant between serologically positive/negative animals were detected.

In the analysed sample, only six haplotypes showed a frequency higher than 6% and for them we reported the differences in haplotype frequency between serologically positive/negative animals (Table 3). The remaining 13 rare haplotypes occurred in only 2.2% of serologically positive animals but in 32.9% of the negative ones.

Chi-square analysis did not show significant differences in haplotype frequencies between serologically positive/negative animals.

In this work we showed, for the first time, the complexity of NRAMP1 gene, evident from the high number of haplotypes, despite the low sample size of the analysed animals. In order to assess the influence of the different genotypes on disease resistance, it is therefore necessary to perform deeper investigation with wider buffalo populations.

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| Table 3. Haplotype frequency in serologically positive/negative animals. |
|-----------------|--------|---|--------|---|
| Haplotype | Positive | Negative | |
| n. | freq. % | n. | freq. % |
| 1 | 3 | 6.5 | 3 | 5.8 |
| 2 | 21 | 45.6 | 13 | 25.0 |
| 3 | 8 | 17.4 | 5 | 9.6 |
| 5 | 7 | 15.2 | 3 | 5.8 |
| 6 | 5 | 10.9 | 6 | 11.3 |
| 8 | 1 | 2.2 | 5 | 9.6 |

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