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Transcript abundance of bovine NRAMP1 and iNOS genes among *Brucella* sero-reactive cattle and buffalo

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Brucella abortus **and** ***Brucella melitensis*** **are the causative agents associated with brucellosis among cattle and buffaloes, respectively. NRAMP1 and iNOS are the best characterized genes conferring resistance against several intracellular organisms. The present study was undertaken to analyze transcript abundance of NRAMP1 and iNOS genes among** ***Brucella*** **sero-reactive cattle and buffalo. Our studies revealed that seropositive animals have significantly (P<0.05) higher mRNA transcript expression of both genes as compared to seronegative animals. Therefore, the present study shows that both** ***NRAMP1*** **and** ***iNOS*** **genes are important candidates for the host response to** ***Brucella*** **among cattle and buffalo.**

Key words: Bovine brucellosis, NRAMP1, iNOS.

INTRODUCTION

Brucellosis is an infectious bacterial disease caused by members of genus *Brucella* of which *Brucella abortus* and *Brucella melitensis* affect cattle and buffaloes (Ahmed et al., 2010; Ali et al., 2014). Apart from these two species, *Brucella suis* has also been reported in bovines (Tae et al., 2012). *Brucella* is a Gram negative intracellular pathogen that can survive in a variety of host cells, particularly within mononuclear phagocytic cells or macrophages (Smith and Ficht, 1990). Brucellosis is widespread in most countries of the world. Brucellosis causes abortion, milk production reduction and increases the intercalving period (Carvalho et al., 2010). It also causes retention of placenta, acute metritis and interferes with the breeding program through involuntary culling. In males, it causes orchitis and epididymitis leading to production of infected semen and even permanent sterility.

Conventional methods of control (e.g., use of antibacterial drugs and vaccination) have significantly reduced the incidence of infectious diseases, including brucellosis, yet the latter is causing havoc to livestock industries. Furthermore indiscriminate use of antimicrobials agents are making microbes resistant. Therefore, there is a need to develop alternative strategies to combat infectious diseases. One of the alternatives could be to improve the overall genetic resistance of animals to the infectious pathogens. Accordingly, the present breeding goals for high productivity must be balanced with the improved functional traits including health and disease resistance traits. Natural resistance to brucellosis has been reported in the past in swine (Cameron et al., 1943) and cattle

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(Templeton et al., 1990). Specific genes controlling resistance to brucellosis have not been identified. However, the macrophages from resistant or susceptible animals exhibited differential ability to control the intracellular replication of bacteria (Price et al., 1990). The Ity/Lsh/Bcg gene is one of the best characterized genes conferring resistance/susceptibility against several different intracellular pathogens (Plant et al., 1982). These genes are now designated as NRAMP1 (natural resistance associated macrophage protein 1) and are recently renamed as solute carrier protein (slc11a1). NRAMP1 affects the intraphagosomal microbial replication by modulating divalent cations in the phagosome (Gruenheid and Gros, 2000). Strong association of NRAMP1 genotypes has been observed in a tuberculosis outbreak in a community of aboriginal Canadians (Greenwood et al., 2000) and Gambian population in West Africa (Bellamy et al., 1998). For leprosy, a role of NRAMP1 in susceptibility to leprosy has been suggestive in a large familial study in South Vietnam (Abel et al., 1998). (GT)n microsatellite polymorphism at the 3' UTR in NRAMP1 of cattle was found to be associated with resistance/susceptibility to *B. abortus* in cattle (Barthel et al., 2001; Paixao et al., 2006; Paixao et al., 2007) and buffalo (Capparelli et al., 2007; Ganguly et al., 2008). Nevertheless, several other studies indicated that NRAMP1 may not be the only major gene conferring resistance/susceptibility against brucellosis (Medina and North, 1996; Bellamy, 1999; Guilloteau et al., 2003). Kumar et al. (2005) also demonstrated lack of association between resistance/susceptibility to brucellosis and the 3' UTR polymorphism of the NRAMP1 gene in crossbred cattle.

Nitric oxide (NO) is one inorganic gas that serves as a biological messenger in a wide variety of physiological processes (Moncada et al., 1991). NO is important in many biological functions that include vascular homeostasis, neurotransmission and defense against infectious agents (Nathan, 1992). NO is produced after the 5-electron oxidation of L-arginine by a family of nitric oxide synthase (NOS) enzymes, forming the free radical, NO and the byproduct, citrulline (Korth et al., 1994). Three district isoforms of the NOS enzyme have been isolated. One NOS is inducible (iNOS, type II NOS, NOS2) which is not typically expressed in resting cells and is induced by various substances including endotoxin, cytokines and microbial products (Moncada et al., 1997).

The present study was undertaken to characterize and study the expression profile of NRAMP1 and iNOS genes among Indian native buffalo (Murrah) and cattle (Tharparker) breeds.

MATERIALS AND METHODS

Experimental animals

A total of 75 Tharparkar cattle and 125 Murrah buffaloes bred and maintained at the Livestock Production and Management Farm of IVRI were used in the present study. All the animals were in the dry/lactation/early pregnant stages and apparently healthy. Records pertaining to their date of birth, parity, calving, abortion and vaccination were also collected.

Collection of samples

Blood (1.5 ml) was aseptically collected from the jugular vein of each animal into a sterile tube containing EDTA (0.5 M, pH 8.0) (@ 50 µl/ml of blood) as anticoagulant and mixed gently to prevent clotting for DNA isolation. Another 10 ml of blood was collected from the same animal into a sterile glass tube containing no anticoagulant for separation of serum for the serological tests. The blood samples for serum separation were kept in slanting position for better yield. The next day, serum was harvested by centrifugation of the tubes at approximately 3000 rpm for 30 min. The straw colored serum was aliquoted into 1.5 ml micro centrifuge tubes and stored at -20°C without addition of preservatives.

Serological testing

Three different serological tests viz., Rose Bengal plate test (RBPT), standard tube agglutination test (STAT) and indirect enzyme linked immunosorbent assay (iELISA) were carried out. Animals that were negative and positive for all the three tests were included in the study as control or infected animals.

Rose Bengal plate test (RBPT)

The RBPT was performed on glass plates according to the method described by Alton et al. (1988). 30 µl serum was mixed with 30 µl RBPT antigen on a glass plate using a glass rod and the plate was gently rotated. Any degree of agglutination within three minutes was considered as positive. A positive control was maintained in which 30 µl of *Brucella* positive serum was used. The RBPT antigen and the *Brucella* positive serum were obtained from the Division of Biological products, IVRI, Izatnagar, India.

Standard tube agglutination test (STAT)

The procedure described by Alton et al. (1988) was followed. The antigen, a pure smooth culture of *Brucella abortus* strain 99 in phenol saline was obtained from the Division of Biological products, IVRI, Izatnagar. Two-fold dilutions of the sera were performed. In each tube 0.5 ml of the *Brucella* plain antigen was mixed and incubated for 20 h at 37°C. The tubes were examined for agglutination and compared with standards containing different levels of antigen in 0.5% phenol saline. Serum dilution which matches in opacity with tube containing 0.5 ml antigen in 1.5 ml of phenol saline represents 50% agglutination and was considered as the end point. A titer of 80 I.U. and above was considered positive. However, samples showing a titer of 40 I.U. were classified as doubtful reactors and titer below 40 I.U. were considered as negative.

Indirect enzyme linked immunosorbent assay (indirect ELISA)

The indirect ELISA was performed according to the method described by Nielsen et al. (1984). The optimum antigen concentration for coating the plates was determined by following the checkerboard titration method. Each reaction was set up in duplicate. Briefly, 100 ng of protein antigen (*Brucella* LPS) in 100 µl

Table 1. Primers used for the present study.

Gene	Primers sequence (5' to 3')	Reference	Length of amplicons
NRAMP1	F: GGGCGAGGTCTGCCATCT R: GATGGTCAGCCAGAGGAGAATG	Self designed	61 bp
iNOS	F: TCTGCAGACACGTGCGTTATG R: GCCGTACTTGGGCTTCCA	Self designed	79 bp
β-actin	F: AGCTCGCCATGGATGATGA R: TGCCGGAGCCGTTGT	Self designed	53 bp

of carbonate-bicarbonate buffer (pH 9.6) was coated on a 96 well flat bottom ELISA plates (Nunc, Germany) and left overnight at 4°C. The antigen solution was discarded and plates were then washed three times with PBS (pH 7.2) containing 0.05% Tween 20 (PBS-T). After washing with PBS-T, 100 µl of test serum (diluted 1:200 in PBS-T) was placed in the wells of the microtitre plates and incubated at 37°C for 1 h. After incubation, the plates were washed three times with PBS-T and 100 µl of rabbit anti-bovine HRPO conjugate (Sigma, USA) diluted 1:20000 in PBS-T was added to each well and the plates were incubated at 37°C for 1 h. The plates were finally washed three times with PBS-T. 100 µl of Orthophenylene Diamine (OPD, Sigma, USA) (0.6 mg/ml) in substrate buffer containing H_2O_2 (0.025%) was added to each well. Plates were incubated for 10-15 min in the dark, and the reaction was stopped by adding 100 µl of 2 N H_2SO_4. The optical density was measured at 492 nm by a microplate reader. Results were standardized on each plate by inclusion of 1:200 dilution of a positive control. A negative control serum (1:200) and reagent blank were also included on each plate. Samples having O.D. value 2.5 times those of negative control serum were considered positive for brucellosis.

Grouping of animals

On the basis of serotyping, animals were classified into seropositive and seronegative groups. A total of 32 animals (8 in each group) were studied for the expression profile of NRAMP1 and iNOS genes.

Total RNA isolation by leukocytes separated from blood

A 15 ml sterile centrifuge tube containing 4 ml of Hisep, lymphocyte separation medium (HiMedia, India) and 8 ml of diluted blood (1:1 dilution with sterile PBS) was layered gently onto the separation medium in a laminar flow hood with the two layers remaining immiscible. The tube was centrifuged at 2100 rpm for 30 min at room temperature (RT). The opaque interface layer between the plasma and the separation medium was aspirated and transferred to a new centrifuge tube. The volume of the contents was made up to 10 ml using isotonic PBS. The tube was centrifuged at 1500 rpm for 15 min at RT. The pellet obtained was retained in the centrifuge tube while the supernatant was discarded. The pellet was again washed with 10 ml of isotonic PBS. The tube was again centrifuged at 1500 rpm for 15 min at RT. The supernatant was gently discarded and the pellet was dissolved in TRI-Reagent (Sigma, India) for direct isolation of RNA following the manufacturer's instruction. An aliquot of the total RNA was subjected to denaturing agarose gel (1%) electrophoresis to assess the quality and integrity of the RNA. The purity of total RNA was checked by using the Nanodrop Spectrophotometer reading at OD_{260} and OD_{280} taken against 2 µl nuclease free water as blank. The concentration of total RNA was estimated as follows: RNA concentration (µg/µl) = OD_{260} x (dilution factor) x 40/1000.

Expression profiling of NRAMP1 and iNOS using quantitative real-time PCR

Real-time qPCR (Mode 7500 Applied Biosystems Real-Time PCR System) was used to elucidate the differential expression of NRAMP1 and iNOS genes with respect to seroractivity. Reverse transcription of total RNA was carried out using a ProtoScript first strand cDNA synthesis kit (New England Biolabs, Beverly, MA, USA) as per the manufacturer's recommendations employing the M-MuLV reverse transcriptase and random primers. The cDNA product was stored at -20°C. A diluted 1:10 solution of the cDNA was used to perform the downstream PCR amplification and real-time qPCR. Real-time fluorescence detection method (PCR) was used to quantify the RNA expression of the candidate gene. Gene specific primers were designed using Primer Express 3.0 software on the basis of respective cDNA sequence (Table 1). β-Actin was used as a house keeping gene (endogenous control) for the analysis of data.

All PCR reactions were performed in triplicate. Amplification was carried out in 20 µl volume reaction containing 10 µl 1X SYBR Green PCR master mix (Applied Biosystems, USA), 1 µl (5 pmols) of each gene-specific forward and reverse primer, 4 µl of cDNA template and 4 µl nucleus free water. For PCR, samples were activated at 95°C for 10 min. Amplification was performed for 40 cycles at 95°C for 15 s and 60°C for 60 s. Negative and positive controls were included for the real-time qPCR assay. In negative controls, cDNA was not added. The efficiency of the selected primer pairs was calculated as follows: a serial dilution of the template was run with each primer pair and the log of the dilution was plotted against the C_T value of each dilution. The slope of the resulting regression equation was used to calculate the efficiency of each reaction using $E= -1+10^{(-1/slope)}$ equation. For each sample, a dissociation curve was generated after completion of amplification and analyzed in comparison with the negative and positive controls, to determine the specificity of the PCR reaction. The mRNA expression of candidate genes was calculated by Relative Quantitation (RQ) using the Comparative C_T Method (Livak et al., 2001).

Statistical analysis

Association between the genotypes and gene expression of the NRAMP1 related to *Brucella* were tested with a General Linear Model (ANOVA, SPSS 14). Significant differences were determined by one-way ANOVA using the SPSS program.

RESULTS AND DISCUSSION

The present study examined the differential expression profile of bovine NRAMP1 and iNOS genes among *Brucella* seropositive and seronegative animals. Consi-

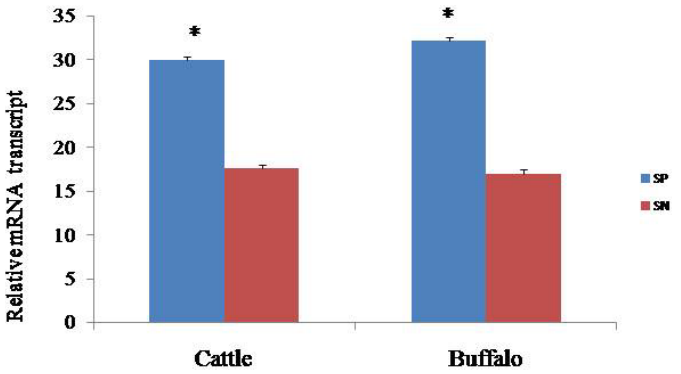

Figure 1. Relative expression of NRAMP1 transcript in leukocyte samples of sero-reactive animals using beta actin to normalize and compare the target gene expression. *Significant differences at P < 0.05. SP, Seropositive; SN, seronegative.

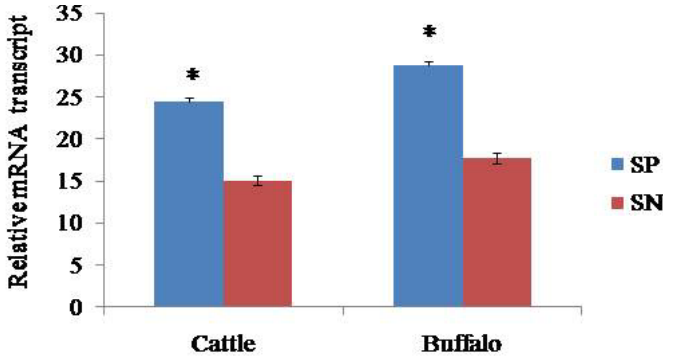

Figure 2. Relative expression of iNOS transcript in leukocyte samples of sero-reactive animals using beta actin to normalize and compare the target gene expression. *Significant differences at P < 0.05. SP, Seropositive; SN, seronegative.

dering the reality that no single serological test renders absolutely accurate result, the animals were screened using three different serological tests viz., Rose Bengal plate test (RBPT), Standard tube agglutination test (STAT) and indirect enzyme linked immunosorbent assay (iELISA). Among the three serological tests in 75 cattle and 125 buffaloes, RBPT showed 31 (41%), iELISA showed 23 (31%) and STAT showed 28 (37%) seropositive animals in cattle, whereas RBPT showed 29 (23%), ELISA showed 22 (18%) and STAT showed 38 (30%) seropositive in murrah buffalo.

Our findings gave an opportunity to identify the serological test that selected the most positive animals among the three tests. From the present findings, the ELISA detected more cattle with brucellosis as compared to STAT and RBPT. ELISA has been reported to be a more sensitive test as compared to other conventional tests (Al Dahouk et al., 2003).

ELISA provides a similar or better sensitivity than both RBPT and CFT, but like classical tests, ELISA is unable to differentiate infected animals from recently vaccinated animals (Jimenez et al., 1992; Blasco et al., 1994) or animals infected with cross-reacting bacteria (Garin-Bastuji et al., 2006).

After the post-serological tests, we screened the transcript profiling of NRAMP1 and iNOS genes among the identified seropositive and seronegative animals using quantitative PCR. Our preliminary findings showed that, *Brucella* seropositive (30.23±0.675 and 31.86±0.553) animals had significantly (P<0.05) higher NRAMP1 mRNA transcript levels than seronegative (16.67±0.334 and 15.48±0.319) groups for both cattle and buffalo, respectively (Figure 1). Similarly, our findings also highlight the mRNA transcript expression of the iNOS gene which was significantly (P<0.05) higher among seropositive (24.86±0.454 and 28.84±0.498) animals than seronegative (14.87±0.312 and 18.92±0.287) groups (Figure 2). Earlier, Korou et al. (2010) reported NRAMP1 mRNA expression increased in macrophages infected with *Mycobacterium avium* subspecies *paratuberculosis*. Similarly, Thacker et al. (2007) evaluated iNOS gene expression in response to *Mycobacterium bovis* infection.

In summary, the expression profile of antibacterially related gene NRAMP1 as well as iNOS may play important roles in conferring genetic susceptibility/-resistant to cattle infected with brucellosis. In the present study, we do not know whether the expression of transcripts of the candidate genes reflect only messenger RNA or also protein expression, since post-transcriptional regulation was not evaluated. In the future, proteomic studies would validate our present findings.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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