Does the Natural resistance associated macrophage protein (Nramp) gene confer resistance/susceptibility in bovines against mycobacterial infection?

G. S. Naveen Kumar†, M. G. Govindiah†, C. S. Nagaraj†, A. Bindu* and T. J. Rasool*†

*Rajiv Gandhi Centre for Biotechnology, Jigarthanda, Thiruvananthapuram 695 014, India
†Department of Animal Genetics and Breeding, University of Agricultural Sciences, Hebbal, Bangalore 560 024, India

The progression of mycobacterial infection in mice is known to be controlled by the 'Bcg' locus which is known to harbour the Natural resistance associated macrophage protein (Nramp) gene. In mice, specific point mutations at this locus have been shown to segregate with resistance and susceptibility to tuberculosis (TB). In order to verify whether a similar situation exists in the bovine genome, bovine Nramp genomic sequences from tuberculosis-infected and uninfected animals were analysed by PCR-RFLP. Alu I and Taq I appear to be the enzymes of choice for detecting haplotypes as they could detect three and two allelic patterns, respectively. The frequency of the alleles between the infected and random groups did not show any variation leaving a doubt on the role of a specific Nramp sequence on TB resistance/susceptibility in bovines.

BOVINE tuberculosis is a serious threat not only to the livestock industry but also to public health. In spite of its economic and zoonotic importance, the disease is rampant in our bovine population where stamping out policy and treatment of the bacterial infection with suitable antibiotics is not feasible due to practical reasons. Hence the only viable proposition to control the disease in livestock is by developing cattle breed genetically resistant to Mycobacterium bovis.

The role of heredity in resisting tuberculosis (TB) infection was known prior to the discovery of Mycobacterium bacilli itself. Some ethnic groups of men and some strains of mice are naturally resistant to TB.1,2 In humans, the contribution of genetic factors to resistance and susceptibility to TB is also documented by racial differences3. Studies of twins also proved greater concordance of TB among monozygotic twins than dizygotic twins4. Donovick et al.3 showed that survival time of mice strains after intravenous injection with mycobacterium varied considerably. In cattle, genetic basis of TB is supported by the fact that a fraction of the herd does not pick up infection in spite of staying in the infected herd for many years.

1For correspondence. (e-mail: rgcbt@md2.vsnl.net.in)

Natural resistance to infection by mycobacterium and other intracellular parasites in mice is controlled by a single locus 'Bcg' (refs 6, 7), the gene of which is expressed in the macrophages. A candidate gene for the 'Bcg' locus was isolated by positional cloning and was designated as Natural resistance associated macrophage protein, Nramp, which codes for an integral membrane protein of macrophage8. The role of Nramp in resistance to intracellular pathogens has also been confirmed by in vivo gene disruption studies9. Cloning and sequencing of the Nramp sequence from 27 mouse strains revealed 5 nucleotide sequence variations. One of the variations, which resulted in the replacement of glycine with aspartic acid at the 169th amino acid position, clearly distinguished the resistant and susceptible inbred strains of mice8,10.

If such variations exist in the bovine population, they should offer a simple means to identify the resistant and susceptible varieties of cattle strains, thereby enabling the development of TB-resistant cattle. In order to verify this, genomic DNA samples from a TB-infected cattle herd were tested by PCR-RFLP technique to identify the resistant and susceptible bovine Nramp alleles, if any.

Ten animals each of crossbred cattle that were proved to be TB-positive and TB-negative by single intradermal tuberculin test were used for the study. The level of taurus inheritance in these animals varied from 12.5 to 75%.

Animals which were positive for the last five years based on single intradermal test (SID) and which had an intradermal response of greater than 15 mm diameter were grouped as tuberculin positive. Further, M. bovis was also isolated from milk samples of 8 of the 10 animals grouped as positive.

Genomic DNA was isolated from 10 ml of venous blood by the salting out procedure. DNA was dissolved in 500 μl of TE and the concentration and purity was checked by the standard spectrophotometric method.

Two pairs of primers were designed by PC gene package based on the homologous sequence published for human NRAMP 1 gene. The first set of primers was designed to amplify exon 5 and 6 of human NRAMP 1 gene. The second set of primers as designed from the most conserved regions of exon 5 to exon 7 of the human cDNA sequence.

The primer sequences are

P1 5' CCCTCCCCTAAATGAGGATC 3' 
P2 5' CCCACCA CTC CCC TAT GAG GTG 3' 
P3 5' TCCGATGCAGGAAGTATC 3' 
P4 5' GCC AAA GGT CAA GGC CAT AAT GG 3' 

About 300 ng of the genomic DNA was amplified in a reaction mixture of 50 μl, using 50 pmol of either P1, P2 or P3, P4 primer combination as per standard
sion at 72°C for 90 s. A final extension for 7 min resulted in uniform sized products. The second primer was used at a higher annealing temperature of 60°C. The authenticity of the bovine PCR product was confirmed by hybridizing with a labelled 1.8 kb mouse cDNA probe.

8 µl of the PCR product was directly digested in a reaction volume of 25 µl using 5 different restriction enzymes, *Hae*III, *Rsa*I, *Taq*I, *Alu*I and *Sau*3A according to the manufacturer's recommended conditions. The digested DNA samples were size separated using 6% non-denaturing poly-acrylamide gel and run for 2 h at 60 V. The digested products were visualized by ethidium bromide staining and photographed immediately. The alleles were scored in comparison with the standard DNA marker.

DNA samples from chronic TB-infected cattle and apparently healthy and TB-negative cattle were used for amplifying *Nramp*-specific gene using human-specific primers. Whereas the first set of primers did not amplify a product from the bovine genome in spite of using a low annealing temperature, the second set of primers amplified a product of 890 bp both from the human and cattle genome. In order to confirm the authenticity of the PCR product to be *Nramp*-specific, the product was hybridized with random primer labelled mouse *Nramp* cDNA probe. The hybridization experiment confirmed that the PCR product is indeed *Nramp*-specific (Figure 1). The amplified product should cover the homologous region to human exon 5, 6 and 7 and intron 5 and 6 as per the design of the primer.

The internal sequence variation among the experimental animal groups was analysed by PCR-RFLP method. Five different tetracutter enzymes were used for the purpose. *Hae*III enzyme resolved a monomorphic pattern comprising four restriction sites resulting in five fragments of 300 bp, 200 bp, 215 bp, 75 bp and 70 bp (Figure 2). *Rsa*I and *Sau*3A enzymes also revealed a monomorphic pattern with a single enzyme site each. *Taq*I revealed a dimorphism of the sequence giving two alleles. The two genotypes were characterized by fragments of 515 bp and 375 bp, and 590 bp, 515 bp, 375 bp and 300 bp (Figure 3). The pattern clearly reveals that the second genotype is a heterozygote and the dimorphism is due to shifting of the restriction site in the two genotypes.

Among the enzymes tested, *Alu*I was the most informative as it could resolve three different genotypic patterns within the group (Figure 4). The first pattern, comprising two sites and three fragments of 540 bp, 270 bp and 80 bp had a frequency of 0.263 in the group. The second allelic pattern had four sites and also exhibited the same frequency. The third pattern appeared to be an heterozygote as it displayed all the fragments of pattern I and pattern II (lane 10, Figure 4).

Figure 2. Polyacrylamide gel analysis of bovine *Nramp* PCR product after digestion with *Hae*III restriction enzyme. a. Tuberculin-negative group. Lane M, molecular weight marker (pBR 322 *Hae*III digest); lane U, uncut PCR product of 890 bp; lanes 1–8, *Hae*III digested PCR product of different animals from tuberculin-negative group showing fragments of 300 bp, 230 bp, 215 bp, 75 bp and 70 bp. b. Tuberculin-positive group. Lane M, molecular weight marker (pBR 322 *Hae*III digest); lane U, uncut PCR product of 890 bp; lanes 1–6, *Hae*III digested PCR product of different animals from tuberculin-negative group showing fragments of 300 bp, 230 bp, 215 bp, 75 bp and 70 bp.
Table 1. Summary of allelic pattern obtained with the five restriction enzymes

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Band pattern</th>
<th>Allelic forms</th>
<th>Genotype frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>HaeIII</td>
<td>300 bp, 230 bp, 215 bp, 75 bp and 70 bp</td>
<td>Homozygotic</td>
<td>1</td>
</tr>
<tr>
<td>RsaI</td>
<td>550 bp and 340 bp</td>
<td>Homozygotic</td>
<td>1</td>
</tr>
<tr>
<td>TaqI</td>
<td>515 bp and 375 bp, 590 bp, 515 bp, 375 bp and 300 bp</td>
<td>Homozygotic</td>
<td>0.9</td>
</tr>
<tr>
<td>AluI</td>
<td>540 bp, 270 bp and 80 bp, 290 bp, 270 bp, 130 bp, 120 bp and 80 bp</td>
<td>Homozygotic</td>
<td>0.263</td>
</tr>
<tr>
<td>Sau3A</td>
<td>500 bp and 390 bp</td>
<td>Homozygotic</td>
<td>1</td>
</tr>
</tbody>
</table>

Infectious diseases are controlled by genetic parameters. The concept of developing TB-resistant cattle is a promising area in this direction as the genetic basis of TB-infection in mammals is well documented. Linkage analysis, in vivo gene disruption experiments, transfection experiments, etc. confirm the role of murine N ramp gene in controlling intracellular parasitic diseases. Cloning and sequencing of N ramp alleles from different inbred strains of mice has revealed that a single point mutation at the 975th nucleotide position which resulted in an amino acid substitution at the 169th position segregated well between susceptible and resistant strains. If this situation exists in bovines also, then it offers a simple method for marker-assisted selection to develop TB-resistant cattle.

Animals staying together in an organized farm, both of which were infected with TB and which were healthy in spite of staying with infected animals were scored for variation at the N ramp 1 homologous sequences. The 890 bp PCR product amplified with human-specific primer was digested with 5 restriction enzymes for the internal restriction enzyme site variation. Table 1 gives the summary of the allelic pattern obtained with the 5 restriction enzymes. Only AluI and TaqI could detect variation within the 890 bp region.

The detection of very little polymorphism at the bovine N ramp 1 locus is not surprising in spite of using 5 tetracutter enzymes, as N ramp belongs to a family of conserved genes. However, comparison of the allelic pattern and their frequency among TB-infected and non-infected groups did not show any difference between the groups, reducing the significance of N ramp polymorphism detected in the animals. It also raises a doubt whether N ramp alone is responsible for conferring resistance against M. bovis in bovines, or any other gene.

Development of disease-resistant varieties is one area which has received scanty attention from animal scientists in spite of the fact that the prognosis of many
Spectrin exhibits chaperone-like activity

Abhijit Chakrabarti* and Shekhar Bhattacharya
Biophysics Division, Saha Institute of Nuclear Physics, 37 Belgachia Road, Calcutta 700 037, India.

Erythroid spectrin, a cytoskeletal protein, inhibits refolding of denatured heme enzyme, horseradish peroxidase (HRP). The denatured enzyme after 30 min refolding in the absence and presence of spectrin showed significant differences in the enzyme activity. The enzymic activity of HRP decreased in the presence of spectrin when compared with the activity in absence of spectrin. This inhibitory effect of spectrin is abolished when it was preincubated with magnesium-ATP indicating that spectrin exhibits chaperone-like activity.

SPECTRIN is the major structural protein of the erythrocyte membrane skeleton. It is an elongated αβ-heterodimer with large molecular weight of approximately 460,000 Dalton.1-3 Spectrin isoforms have been identified in a wide variety of nonerythroid cells where they are also localized predominantly along cellular plasma membranes.5 Spectrin binds to a wide range of ligands starting from metal ions like calcium and molybdenum, fatty acids and phospholipids to proteins like actin, ankyrin and calmodulin.6-7 The major function of spectrin is presumed to establish the cytoskeletal network that provides mechanical strength to cells. However, its capability to interact with a wide range of different ligands and its abundance also in nonerythroid tissues suggests that spectrin might have a role to play in other functions as well. Like some of the heat-shock proteins,3,4 and the eye lens structural protein α-crystallin7, spectrin interacts with phospholipid membranes.6-7 Spectrin binds to fatty acids and many other hydrophobic ligands8-10 which is also seen in chaperone proteins that bind hydrophobic fluorescent probes like ANS and bis-ANS (ref. 11). The heat-shock proteins and a few structural proteins, e.g. eye lens α-crystallin have been shown to exhibit chaperone-like activity by interacting with the denatured proteins12-14. Also, recently the cytoskeletal protein, tubulin, has been shown to exhibit chaperone-like activity in preventing the aggregation of other proteins15. In view of these properties of the erythroid spectrin, we tried to investigate if spectrin can act as molecular chaperone by interacting with the unfolded proteins. We chose a heme enzyme, horseradish peroxidase (HRP) for such a study because of (i) the abundance of haemoglobin in erythrocytes that was earlier known to interact with spectrin16 and (ii) a monomeric protein whose folding pathway has been worked out17-18 and its activity can be readily determined in a continuous assay.

The enzyme activity of HRP (Type VI, Sigma) was measured according to the ABTS assay method18 by following its change in absorbance at 405 nm in 50 mM phosphate-citrate buffer, pH 5.0. Spectrin dimers were purified from ovine erythrocyte ghost membranes following published protocols19,20. HRP (100 μg/ml) was incubated with 6 M guanidinium chloride (GdmCl, Sigma) for 2.5 h at 25°C for denaturation. Refolding was initiated by 40-fold dilution of the denatured HRP in 50 mM Tris-HCl, pH 7.8 with or without dimERIC spectrin. After 30 min of refolding at 25°C, the enzyme activity was measured by ABTS assay method. The extent of reactivation of the denatured HRP

Received 20 April 1999; revised accepted 15 June 1999