CANDIDATE GENE POLYMORPHISM AND ITS ASSOCIATION WITH RESISTANCE / SUSCEPTIBILITY TO TUBERCULOSIS IN CATTLE

THESIS

Submitted to the
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Roll No. 5028

IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF

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DIVISION OF ANIMAL GENETICS
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Dated: 01-06-**2013**

Certificate

This is to be certified that the research work embodied in this thesis entitled **"Candidate gene polymorphism and its association with resistance/ susceptibility to tuberculosis in cattle"** *submitted by* **Dr. Mohd Baqir, Roll No. 5028,** *for the award of* **Master of Veterinary Science** *Degree in* **Animal Genetics and Breeding** *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.*

It is further certified that **Dr. Mohd Baqir, Roll No. 5028,** *has worked for more than 21 months in the Institute and has put in more than 150 days attendance under me from the date of registration for the* **Master of Veterinary Science** *Degree in this Deemed University, as required under the relevant ordinance.*

(DEEPAK SHARMA)
Chairman
Advisory Committee

Certificate

We the undersigned members of Advisory Committee of Dr. Mohd Baqir ,Roll No. 5028 a candidate for the degree of **Master of Veterinary** *Science with the major discipline* ***Animal Genetics and Breeding,*** *agree that the thesis entitled "**Candidate gene polymorphism and its association with resistance/susceptibility to tuberculosis in cattle**" may be submitted in partial fulfillment of the requirement for the degree.*

We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented for the award of ***Master of Veterinary Science*** *degree of this Institute.*

It is further certified that the candidate has completed all the prescribed requirements governing the award of ***Master of Veterinary Science*** *degree of the Deemed University, Indian Veterinary Research Institute.*

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Place: **Izatnagar, Bareilly** **(Mohd Baqir)**

Dated: 1-6-2013

%	:	Percent
/	:	Per
@	:	At the rate of
µg	:	Microgram
µl	:	Microlitre
µM	:	Micro molar
A	:	Adenine
bp	:	Base pair
^{0}C	:	Degree Celsius
Cm	:	Centimeter
C	:	Cytosine
D	:	Day
dATP	:	Deoxy adenosine triphosphate
dCTP	:	Deoxy cytosine triphosphate
dGTP	:	Deoxy guanosine triphosphate
dNTP	:	Deoxy nucleoside triphosphate
DNA	:	Deoxy ribose nucleic acid
dTTP	:	Deoxy thymine triphosphate
F	:	Forward primer
Fig	:	Figure
EDTA	:	Ethylenediamine tetra acetic acid
g	:	Gram
G	:	Guanine
hr	:	Hour
IU	:	International unit
mgcl2	:	Meganesium chloride
mg	:	Milligram
ml	:	Milliliter
mm	:	Millimeter
Min	:	Minute
MW	:	Molecular weight
M	:	Molar
ng	:	Nanogram
NFW	:	Nuclease free water
N	:	Number
OD	:	Optical density
OR	:	Odds ratio
PIC	:	Polymorphism information content
PBS	:	Phosphate buffer saline
PCR	:	Polymerase chain reaction
RBC	:	Red blood cell
rpm	:	Rotation per minute
Sec	:	Second

NaOH	:	Sodium hydroxide
T	:	Thymine
TBE	:	Tris borate EDTA
TE	:	Tris EDTA
U	:	Units
UV	:	Ultravolit
V	:	Volts
V/cm	:	Volts per centimeter
w/v	:	weight/ volume

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1 Introduction

The genus Mycobacterium comprises over 100 species. The majority of these are found in the environment and are not normally associated with disease in humans or animals. However a small number are pathogenic. Within this pathogenic sub-group is the so-called *M. tuberculosis* complex which comprises *M. tuberculosis, M. africanum, M. canettii, M. pinnipedii, M. microti, M. bovis* and *M. bovis* subspecies *caprae*. These are generally regarded as host adapted but with the ability to infect other species. The slowly growing non-photochromogenic bacilli members of the *Mycobacterium tuberculosis* complex: *M. bovis* and *M. caprae* species are causative agents for infectious bacterial disease - Bovine tuberculosis (BTB). However, *M. bovis* is the most universal pathogen among mycobacteria and affects many vertebrate animals of all age groups. Although, cattle, goats and pigs are found to be most susceptible, while sheep and horses are showing a high natural resistance (Radostits *et al*., 2000; Thoen *et al*., 2006). Bovine tuberculosis (BTB) is a chronic infectious disease of animals characterised by the formation of granulomas in tissues and organs, more significantly in the lungs, lymph nodes, intestine and kidney including others. BTB has been a cause of great economic loss constraining international trade of animals and their products. In developed countries, BTB in animals is a rarity with occasional severe occurrences in small groups of herds. In developing countries, however, such as in 46% of African, 44% of Asian and 35% of the South American and the Caribbean countries, sporadic occurrences and enzootic occurrences of BTB have been reported (Cosivi *et al.,* 1998).

BTB has significant public health importance, apart from being the most important disease with serious effects on animal production (O'Reilly and Daborn, 1995). However, zoonotic BTB is present in most developing countries where surveillance and control activities are often inadequate or unavailable. Studies revealed that among humans, the disease incidence is higher in farmers, abattoir workers and others who worked with cattle. In India, however there is little information available on the transmission of bovine tuberculosis and its impact on human health.

Generally treatment for BTB is not recommended in animals since there is no cost effective treatment. The socio-economic condition of people in India, social customs prevailing which prevent the slaughtering of affected cows and the wild life reservoirs of the bacterium make BTB much difficult to eradicate. Therefore, there is an urgent need to develop some alternative strategies to combat infectious diseases.

An ideal approach to the control of zoonotic infectious diseases in animals is the development of genetic resistance. The basic principle is the identification of already resistant animals and further use of its superior genotype for the production of new population which could resist the disease causing organism. Phenotypic measurement of disease resistant traits, however, is often difficult because experimental exposure to infections are neither feasible due to high cost involvement nor permissible due to ethical reasons. Nevertheless, indirect selection of disease resistance traits using suitable markers can serve as one of the alternatives. Molecular markers have provided enormous scope to unravel the genetic variations at DNA sequence level. These markers can be utilized for selection of disease resistance either using linkage analysis or candidate gene approach. Availability of a large population, correct pedigree information and a reference family are the prerequisites for linkage analysis. Thus under present circumstances, it may not be a feasible approach for studying the genetic basis of disease resistance. Alternatively, candidate gene approach, in which genes that influence the progression of the disease are identified, can serve as a useful tool in delineating the underlying genetic mechanism against disease resistance. In recent years, significant progress on the identification and characterization of candidate genes, microsatellite markers and comparative gene mapping has been made. Identification of individual candidate genes which control natural resistance and the actions of these genes will greatly expand the knowledge of genetic resistance to bacterial diseases and the possibility for practical application.

There may well be other benefits to this strategy of breeding for TB resistance. Several other obligate intracellular bovine pathogens may interact with their host using similar mechanisms to those likely to be discovered for M. bovis. These organisms include *Brucella abortus*, *Salmonella enterica* and *Mycobacterium avium paratuberculosis*. It is conceivable that by selecting animals to be more resistant to BTB, one could also serendipitously select for increased resistance to these other

pathogens. In addition, there is evidence (Brotherstone *et al*. 2010) that there is no genetic association between milk yield and the genetic susceptibility to BTB, indicating that there is little evidence that selection for increased milk yield has increased susceptibility to BTB or that selecting for resistance will reduce productivity.

Considering these aspects the study is undertaken with the following objectives:

Objectives

- To detect the polymorphism in candidate genes for susceptibility/resistance to tuberculosis
- To explore the association of polymorphism to susceptibility and resistance against T.B infection in cattle.

Despite considerable evidence of a genetic component to TB resistance, modest effort has been directed toward identifying bovine genetic susceptibility loci. Indeed, it is only recently that effort has been directed toward quantifying the host genetic influence (Bermingham *et al*. 2009; Brotherstone *et al.* 2010). Also, European B. taurus cattle appear to be more susceptible to M. bovis infection than B. indicus cattle as Ameni *et al.* (2007) presented data from a cohort of 2500 zebus, 1900 crossed (zebu–Holstein) and 900 Holstein cattle. Not only was prevalence of BTB higher in the Holstein population compared with the other two, but severity of pathology in skin test-positive animals was also significantly greater. Disease risks were also estimated, with Holstein cattle 2.32 times more likely to be diseased than zebu cattle.

2.1 NRAMP 1/SLC11A1(Natural resistance-associated macrophage protein 1/ Solute carrier)

Genetic studies in mice have demonstrated that a gene called natural resistance-associated macrophage protein 1 *(NRAMP1*, formerly the *Lsh/lty/Bcg* gene) controls innate resistance and susceptibility for *Mycobacterium bovis* (Gros *et al.,* 1981), *Mycobacterium intracellular* (Goto *et al.,* 1984), *Leishmania donovani* (Crocker *et al.,* 1984), and several other mycobacteria. The *NRAMP1* gene mediates activity of macrophages against intracellular parasites during the early stages of infection (Blackwell *et al.,* 1994). *NRAMP1* is a member of the solute carrier *{SLC11A1)* family of ion transporter (Horin and Matiasovic, 2000), which is an integral trans-membrane protein and expresses particularly on phagosome of macrophage (Gruenheid *et al.,* 1997). *NRAMP1* affects the intraphagosomal microbial replication primarily by eliminating Mn^2 (Supek *et al.,* 1996) or other divalent cations (Fe^2, Mn^2, Co^2 etc.) from phagosomal interior (Gruenheid and Gros , 2000; Forbes and Gross. 2003), which serve as essential cofactors for their survival by helping in many microbial metabolic processes. Beside this, other

pleiotropic effects of *NRAMP1* namely production of reactive oxygen and nitrogen products (Denis *et al.,* 1988; Barton *et al.,* 1995; Anas *et al.,* 1997), higher level of antigen processing and MHC class II molecules expression (Lang *et al.,* 1997) and enhanced phagolysosomal fusion further augment the antimicrobial activity. A point mutation of G169D in murine *Nrampl* causes differential resistance/susceptibility towards a number of antigenically different intracellular microorganisms (Vidal *et al.*. 1993). Such candidate gene approaches in BTB resistance research have concentrated mainly on the bovine natural resistance-associated macrophage protein (NRAMP1) gene owing to the ubiquity with which it has been identified as a candidate in mouse and human studies. A microsatellite in the 3' untranslated region (UTR) of the bovine NRAMP gene has been found to be associated with natural resistance to brucellosis infection in cattle (Adams & Templeton 1998) In a study, comprising 33 cattle with positive results to the tuberculin test, of which nine were assigned a resistant phenotype and 24 a susceptible phenotype, no association was observed between the bovine 3' UTR microsatellite and resistance to M. bovis infection (Barthel *et al.* 2000). Kadarmideen *et al.,* (2011) genotyped a total of 211 Chadian cattle for a microsatellite within the SLC11A1 candidate gene and study revealed statistically significant effects of SLC11A1 alleles ($P < 0.001$) on most BTB traits. Polymorphisms (alleles 211, 215 and 217) are significantly related to lower incidence of BTB traits in cattle. Selvaraj *et al* 2002 found *NRAMP1 gene polymorphisms 823 C/T (exon* 8) associated with resistance to tuberculosis (TB). In one study in human The G > C mutation of intron 4 of NRAMP1 gene might be a susceptible factor to PTB. (Qu *et al.,* 2006). Studies at 469+14 G/C (INT4), 1465-85 G/A, and C274T polymorphisms of NRAMP1 in ethnic Russians with and without tuberculosis (N =58 and 127 respectively) none of the polymorphisms was associated with TB. (Puzyrev et al.,2002).

2.2 SP110/Ipr1 *(nuclear body protein/ Intracellular pathogen resistance 1)*

Recently, the mouse gene Ipr1 (intracellular pathogen resistance-1) on the locus sst1 (super-susceptibility to tuberculosis 1) on chromosome1 has been shown to essentially contribute to innate immunity in a murine model of Mycobacterium tuberculosis infection. (Pan *et al*., 2005). Ipr1 is encoded within the sst1 locus, and it is expressed in tuberculosis lung lesions and macrophages of sst1-resistant mice but not

sst1-susceptible mice. Upregulation of Ipr1 after infection occurs in macrophages from sst1 resistant mice but not in macrophages from susceptible mice, and expression of an Ipr1 transgene construct in macrophages from susceptible animals resulted in the control of mycobacterial replication and the eventual induced death of infected cells. Thus, Ipr1 might play an important role in preventing tuberculosis by mediating control of M tuberculosis within its prime target cell, the macrophage(Kramnik, 2008). Polymorphisms of the human *SP110 nuclear body protein* (*SP110*)gene, orthologous to murine *Intracellular pathogen resistance 1* (*Ipr1*), have been reported to be associated with tuberculosis. Tosh *et al*., (2006) analysed 20 SNPs in human families from Gambia (West Africa) and reported 3 SNPs (rs2114592,sp110int10 and rs3948464) associated with genetic susceptibility to tuberculosis. For these well established reasons the SP110 (Speckled 110) gene is considered as one of promising candidates bovine tuberculosis susceptibility gene. Larrañaga *et al.,(*2010) in an association study of 14 SNPs in two independent populations repoted the SNP c.587A>G to be significantly associated with MAP (*Mycobacterium avium* subspecies *paratuberculosis)* infection. Larrañaga *et al., (*2011) studied association between SNP c.587A>G in *SP110* gene and susceptibility to *Mycobacterium bovis* infection in cattle. A total of 158 cows (50 infected and 108 non-infected) from a multibreed sample were included in the study. Neither SNP c.587A>G alleles nor genotypes showed significant association with susceptibility to *Mycobacterium bovis* infection in the analyzed cattle population. In an association study in Ghana (West Africa), for twenty-one SP110 gene variants studied, no associations of human pulmonary tuberculosis with Sp110 variants were found (Thye *et al.,* 2006). Twenty SNPs were detected in Holstein-Friesian cattle and the application of the SNP polymorphisms to the case-control samples revealed no association with MAP (*Mycobacterium avium* subspecies *paratuberculosis)* (Ruiz *et al.,* 2009). Lei et al.,2012 found no significant association for five SNPs (rs1135791, rs9061, rs11556887, rs3948464, rs1346311) in human Chinese population for *M. tuberculosis* infection. Genetic association study suggests a role for *SP110* variants in lymph node tuberculosis but not pulmonary tuberculosis in north Indians(Abhimanyu *et al* (2011).

2.3 IL12RB1

Interleukin 12 receptor, beta 1 is a subunit of the interleukin 12 receptor. IL12RB1 is also known as CD212 (cluster of differentiation 212).The protein encoded

by this gene is a type I transmembrane protein. This protein binds to interleukine 12 (IL12) with a low affinity, and is thought to be a part of IL12 receptor complex. The coexpression of this and IL12RB2 proteins was shown to lead to the formation of high affinity IL12 binding sites and reconstitution of IL12 dependent signaling. The lack of expression of this gene was found to result in the immunodeficiency of patients with severe mycobacterial and Salmonella infections. Thus host cytokine machinery play an important role in protecting against mycobacterial infection. The host cytokine response is an important target in this regard as it plays a major role in regulating the defense against mycobacterial species (Coussens 2004). After Mycobacterium tuberculosis infection, (mRNA) il12rb1 is spliced by dendritic cells (DCs) to form an alternative (mRNA) il12rb1Deltatm that encodes the protein IL-12Rbeta1DeltaTM. Compared with IL-12Rbeta1, IL-12Rbeta1DeltaTM contains an altered C-terminal sequence and lacks a transmembrane domain. M. tuberculosis-exposed DCs express IL-12Rbeta1DeltaTM to enhance IL-12Rbeta1-dependent migration and promote M. tuberculosis-specific T cell activation. IL-12Rbeta1DeltaTM thus represents a novel positive-regulator of IL12Rbeta1-dependent DC function and of the immune response to M. tuberculosis (Robinson *et al.*,2010). In humans there is reported association of IL12RB1 polymorphisms with pulmonary tuberculosis (Remus *et al.*, 2004; Kusuhara *et al.*, 2007). Pant *et al.,* 2011 found one SNP (c.81 T>C)in IL12RB1 associated with the MAP infection status in resource population of 446 dairy Holsteins.

2.4 IL12RB2

The beta 2 subunit of the interleukin (IL)-12 receptor (IL-12R beta 2) has been shown to play an essential role in differentiation of T helper 1 (Th1) cells in the murine and human system, and antibodies raised against IL-12R beta 2 recognized this molecule on human Th1 but not Th2 cells. However, while the cytokines secreted by clones of murine cells allowed the definition of distinct T helper cell subsets, bovine clones with polarized Th1 and Th2 cytokine profiles were rarely found. cloned bovine IL-12R beta2 (boIL-12R beta 2) DNA complementary to RNA (cDNA) from the start codon to the 3' end of the mRNA. Comparison of boIL-12R beta 2 cDNA with human and murine IL-12R beta 2 cDNA sequences revealed homologies of 85 and 78%, respectively. The deduced protein showed the hallmark motifs of the cytokine receptor

superfamily including the four conserved cysteine residues, the WSXWS motif and fibronectin domains in the extracellular part as well as a STAT4 binding site in the intracellular part of the molecule. Using real-time reverse transcription-polymerase chain reaction, upregulation of mRNA expression of this molecule could be demonstrated in cultured bovine lymph node cells stimulated with phytohemagglutinin (Waldvogel *et al*.,2002). One SNP in IL12RB2 (c.-511A>G) associated with the MAP infection status in resource population of 446 dairy Holsteins found by Pant *et al*., 2011

2.5 DC-SIGN

Dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) C-type lectin is almost exclusively expressed at the cell surface of DC. In addition to its normal function facilitating contact of DC with T cells, DC-SIGN has been shown to bind a variety of pathogens, including *Mycobacterium bovis*. In particular, C-type lectins detect pathogens by their characteristic carbohydrate structures and internalise them for further antigen processing and presentation (McGreal *et al.,* 2005). DC-SIGN can initiate innate immunity by modulating toll-like receptors though the detailed mechanism is not yet known. (Dunnen *et al*.,2008) DC-SIGN together with other C-type lectins is involved in recognition of tumors by dendritic cells.Polymorphism of the promoter of DC-SIGN gene in Caucasians was studied at positions -336A/G and -871/G in 134 patients with pulmonary tuberculosis and 112 healthy individuals, who lived in the Irkutsk Region, Eastern Siberia. Comparison of DC-SIGN gene polymorphism in patients with tuberculosis and healthy controls revealed no significant differences in loci -336A/ G and -871A/G (Ogarkov *et al.,*2007). Barreiro *et al.,* 2005 tested whether polymorphisms in CD209, the gene encoding DC-SIGN, are associated with susceptibility to tuberculosis through sequencing and genotyping analyses in a South African cohort and observed an association between two CD209 promoter variants (-871G and -336A) and decreased risk of developing tuberculosis. To directly analyze the role of human DC-SIGN during mycobacterial infection, Schaefer *et al.,* 2008 generated conventional transgenic (tg) mice (termed "hSIGN") using CD209 cDNA under the control of the murine CD11c promoter. After high dose aerosol infection with the strain *M. tuberculosis* H37Rv, hSIGN mice showed massive accumulation of DC-SIGN cells in infected lungs, reduced tissue damage and prolonged survival. Human DC-SIGN,

protection by limiting tuberculosis-induced pathology. DC-SIGN is the major *Mycobacterium tuberculosis* receptor on Human Dendritic Cells (Tailleux *et al*., 2002). The bovine orthologue of human DC-specific ICAM-3 grabbing non-integrin C-type lectin (DC-SIGN) gene has recently been identified and functionally characterized. DC-SIGN receptor interacts and binds to M. bovis BCG It is worth speculating that in cattle, as in humans, polymorphic variation in the DC-SIGN gene may be associated with TB resistance(Yamakawa *et al.,* 2008).

2.6 IL23R

Interleukin 23 receptor is a type I cytokine receptor. The protein encoded by this gene is a subunit of the receptor for IL23A/IL23. This protein pairs with the receptor molecule IL12RB1/IL12Rbeta1, and both are required for IL23A signaling. This protein associates constitutively with Janus kinase 2 (JAK2), and also binds to transcription activator STAT3 in a ligand-dependent manner. Investigating the association between a functional single nucleotide polymorphism (SNP) in the interleukin-23 receptor gene (IL23R; rs11209026, 1142 G(wild type) → A(reduced function), Arg381Gln) and disease severity outcome in pulmonary tuberculosis (TB), Ben-Selma *et al*.,(2012) reported the reduced-function polymorphism 1142G → A encoded by IL23R influences the outcome of disease severity of active pulmonary TB in Tunisian patients. Focusing on all SNPs within 1 Mb of IL23R,Settles *et al.,(*2009) found no loci in the vicinity of this genes to be associated with any Map infection phenotype. Pant *et al.,* (2011) found one SNP in IL23R c.1417 A>C associated with the MAP infection status in resource population of 446 dairy Holsteins.

3.1. Resource panel

The present study was conducted on animals of mixed cattle population (containing Kosi, Sahiwal and crossbred animals), maintained at Shri Mataji Goshala, Barsana, Mathura. The population included male and females of various age groups. The animals were maintained under similar feeding and management conditions. A total of 245 animals were screened for bovine tuberculosis (bTB) by performing single intradermal comparative cervical tuberculin test (SICCT) keeping in view of prevalence rate of tuberculosis. On the basis of the result of SICCT two groups were formed i.e. tuberculin test positive animals affected with tuberculosis and the second group were negative for tuberculin test and supposed to be free of tuberculosis. Subsequently, the data on the selected animals based on SICCT will also be recorded and 35 tuberculin positive and 45 tuberculin negative animals were included in our investigation for genotyping.

3.2.Tuberculin test procedure (SICCT)

Preliminary screening for TB was carried out using the purified protein derivative (PPD) tuberculin skin test (SICCT). Prior to inoculation with PPD the skin thickness at site of injection was measured. Approximately, 0.1 ml PPD was injected into the mid-neck, and the skin-fold thickness of each injection site was measured after 72 h. The reactions were then categorized as positive (>4 mm thickness) and negative (<2 mm thickness).

3.3. Collection and storage of blood

About 6 ml of venous blood was collected from jugular vein of each of the selected animal in a sterile polypropylene tube containing 0.5 ml of 2.7% EDTA as an anticoagulant. The tubes containing blood was then kept on ice until it was transferred to the laboratory. For the isolation of serum about 10 ml of blood was collected in a

sterile vacutainer tube with anti coagulant and was kept in slanting position for 1 hour for the separation of serum. It was then centrifuged at 2000 rpm for 15 minutes for further separation of the serum. The serum thus separated will be transferred to aliquot kept under refrigeration (-20^{o} C).

3.4. Isolation of DNA

DNA from the whole blood collected from the experimental animals was isolated using Promega Wizard® Genomic DNA extraction Kit, using following procedures.

1. Added 900µl of Cell Lysis Solution to a sterile 1.5ml micro centrifuge tube.
2. Gently rocked the tube of blood until thoroughly mixed; then transferred 300 µl of blood to the tube containing the Cell Lysis Solution. Inverted the tubes 5–6 times to mix properly.
3. Incubated the mixture for 10 minutes at room temperature (invert 2–3 times once during the incubation) to lyse the red blood cells. Centrifuge at 13,000–16,000 rpm for 20 seconds at room temperature.
4. Removed and discarded as much supernatant as possible without disturbing the visible white pellet. Approximately 10–20µl of residual liquid would be remained in the tube had been frozen, repeat Steps 1–4 until pellet was white.
5. Vortex the tube vigorously until the white blood cells was resuspended for 10–15 seconds.

Completely resuspended the white blood cells to obtain efficient cell lysis.

6. Added Nuclei Lysis Solution at the rate of 300µl for 300µl sample volume the tube containing the resuspended cells. Pipetted the solution 5–6 times to lyse the white blood cells. The solution should become very viscous. If clumps of cells were visible after mixing, incubate the solution at 37°C until the clumps were disrupted. If the clumps were still visible after 1 hour, added 100µl of additional Nuclei Lysis Solution.
7. Added 1.5 µl of RNase Solution to the nuclear lysate, and mixed the sample by inverting the tube 2–5 times. The mixture was incubated at 37°C for 15 minutes, and then cooled to room temperature.
8. Added 100µl of Protein Precipitation Solution to the nuclear lysate, and vortexed vigorously for 10–20 seconds. Small protein clumps might be visible after vortexing.
9. Centrifuged at 13,000 rpm for 3 minutes at room temperature. A dark brown protein pellet should be visible at this stage.

10. Transferred the supernatant to another clean 1.5ml micro centrifuge tube containing 300µl of room-temperature isopropanol. Left this residual liquid in the tube to avoid contaminating the DNA solution with the precipitated protein.
11. Gently mixed the solution by inversion until the white thread-like strands of DNA form a visible mass.
12. Centrifuged at 13,000 rpm for 1 minute at room temperature. The DNA would be visible as a small white pellet.
13. Decanted the supernatant, and added 300µl of room temperature 70% ethanol to the DNA. Gently inverted the tube several times to wash the DNA pellet and the sides of the micro centrifuge tube.
14. Centrifuged at 13,000 rpm for 1 minute at room temperature. The DNA would be visible as a small white pellet.
15. Carefully aspirated the ethanol using a sequencing pipette tip. The DNA pellet was very loose at this point and care was taken to avoid aspirating the pellet into the pipette. Inverted the tube on clean absorbent paper and air-dried the pellet for 10–15 minutes.
16. Added 100 µl of DNA Rehydration Solution to the tube and rehydrated the DNA by incubating at 65°C for 1 hour. Periodically mixed the solution by gently tapping the tube.
17. The DNA was then stored at -20^{o} c.

3.4.1. Quantification of DNA

DNA quantification was done using Qubit® 2.0 Fluorometer (Invitrogen,USA). The procedure was one using 1-2 µL of DNA solution. The procedure was carried out as follows

1. Set up two Assay Tubes for the standards and one tube for each user sample.
2. Prepared the Qubit™ Working Solution by diluting the Qubit™ reagent 1:200 in Qubit™ buffer. Prepared 200 µL of Working Solution for each standard and sample.
3. Prepared the Assay Tubes according to the table below.

	Standard	Sample
Volume of Working So-	190 µL	198-199 µL
Volume of Standard	10 µL	—
Volume of User Sample	—	1–2 µL
Total Volume in each Assay	200 µL	200 µL

4. Vortexed all tubes for 2–3 seconds.
5. Incubated the tubes for 2 minutes at room temperature
6. Insert the tubes in the Qubit® 2.0 Fluorometer and take readings.
7. Using the Dilution Calculator feature of the Qubit® 2.0 Fluorometer, the stock concentration of the original sample was determined.

Those DNA samples having a minimum concentration of 50 µg/ml were used for further study.

3.4.2. Quality checking of DNA

The Quality of extracted genomic DNA will be assessed through 1 % horizontal submarine agarose gel electrophoresis as below-

1. The gel casting plate was sealed with adhesive tape and placed on a leveled table surface.
2. Agarose (1 % w/v) was boiled in 1X TBE (Refer Annexure for composition) buffer. After boiling it was cooled to 55ºC and then ethidium bromide (0.5µg/ ml) was added at the rate of 3 µl per 10 ml of agarose solution. The gel was gently poured into the casting tray avoiding bubble formation and was allowed to solidify at room temperature.
3. After solidification, the comb and adhesive tape were removed.
4. The gel casting tray was submerged in gel tank of electrophoresis unit having 1 X TBE buffer.

5. DNA samples for loading were prepared by mixing 2µl of genomic DNA, 8 µl of 1 X TBE and 2µl of 1X loading dye. Samples were carefully loaded in the wells.

Electrophoresis was performed at 2-5 volts/cm for one hour and then gel was visualized and photographed under UV transilluminator. Uncut Lambda (Bangalore Genei, India) DNA (500ng) will be used as reference DNA. After electrophoresis gel was examined on safe image viewer for any sheared DNA. Photographs of gels were taken using Digital camera and were documented and prints were taken as necessary. The good quality DNA samples having intact DNA bands without any smearing were selected for further analysis. The genomic DNA from each animal was diluted into the working solution using nuclease free water (NFW) so as to get a final concentration of 50µg/ml.

3.5. PCR Assay

The Polymerase Chain Reaction (PCR) is a powerful technique which results in rapid production of DNA, sometimes referred to as "molecular photocopying" was invented by Kary Mullis in 1980s. It is an in vitro method for enzymatically synthesizing defined sequence of DNA. The reaction used two oligonucleotide primers that hybridize to apposite strands and flank the target DNA sequence to be amplified. A heat stables DNA polymerase such as Taq polymerase catalyses the elongation of the primers. Template denaturation at 94°C, primer annealing generally between 52-64 °C (depending on primer annealing temperature), and extension at 72°C of the annealed primers by the polymerase results in exponential accumulation of a specific DNA fragment. The primer extension products synthesized in a given cycle can serve as the template in the next cycle. The number of target DNA copies approximately doubles every cycle.

3.5.1. Template DNA

The DNA concentrations of the isolated DNA samples were determined and their working solutions were prepared by diluting up to the final concentration of 50 ng/ µl with NFW.

3.5.2. Primers

The success of a PCR experiment is almost wholly dependent upon the oligonucleotide primers. The primers need to be designed such that one recognizes the

sense strand of the DNA to be replicated (i.e. is the same sequence as the antisense strand) while the other recognizes the antisense strand of the target DNA (i.e. is the same sequence as the sense strand). Typically, primers will have the following characteristics. They will be between 17 and 30 nucleotides in length – sufficient to allow unique annealing to a single sequence within a genome. They will have a GC content of approximately 50 per cent.

The primers for the current study was designed using software oligoanalyser for the SNPs present in the concerned candidate genes from NCBI(www.ncbi.nlm.nih.gov). The list of primers and respective SNPs amplified is given in the Table.1(a) & 1(b).

3.5.3. PCR reaction setup

Initially varying various parameters like annealing temperature and $Mgcl_2$ concentration, the amplification conditions were standardized. Finally these standardized conditions were used.

PCR amplification was carried out in a final volume of 25 µl reaction mixture, in 0.2 ml thin PCR tubes. Each PCR tubes containing 50 ng (Approx.) genomic DNA, 1.5 mM $MgCl_2$, 1X reaction mixture, 200 µM of each dNTP(dATP,dGTP,dCTP and dTTP), 1 U of *taq* DNA polymerase enzyme and 5 pmol of each forward and reverse primer. In tabular form it is given below.

Sl. No.	Reaction Component	Volume		Final Concentration
1	5X Taq buffer with	5 µl		
2	$MgCl_2$	1.5 µl		1.5mM
3	dNTP mix (2.5 mM)	2 µl		200µM
4	W Forward primer (1µg/µl)	2.5 µl		1µM
5	W Reverse primer (1µg/µl)	2.5 µl		1µM
6	Taq DNA polymerase (3U/ µl)	0.33 µl		1U
7	Autoclaved distilled water	Add to 24 µl		-
8	Template DNA (50 ng/ µl)	1.0 µl		50 ng

3.5.4. PCR amplification conditions

The amplification for all the primers was carried out in a DNA Engine Thermal Cycler Machine (M/s BioRad, USA) in the below mentioned PCR program.

1. Initial denaturation at 94^0C for 4 minutes, followed by 25 cycles of PCR, each

cycle consists of:

i. Denaturation at 94^0C for 30 seconds.

ii. Annealing at Tm ^{0}C for 30 seconds.

iii. Extension at 72^0C for 30 seconds.

2. Final extension at 72^0C for 5 minutes.

3. Finally 4^0C forever.

3.5.5 Checking of amplified product

The amplified PCR products checked by submarine horizontal gel electrophoresis with agarose gel of 2.4 % w/v 1X TBE for the conformation of amplification. The amplified products visualized as a single compact band of expected size under UV light and will be documented by gel documentation system. The Figure1 (a, b & c) shows the PCR products of all 15 SNPs.

3.6 PCR-RFLP Assay

Mass genotyping of all Case: Control resource population was done by using PCR-RFLP for all the 15 SNPs. For this the restriction enzyme digestion reaction were setup as follows:

PCR product	20 ul
Restriction enzyme	1 U
RE buffer	2.5 ul
NDW	2.5 ul
Total	25 ul

The restriction enzymes used and the condition of digestion are given in Table.2

The restriction digested products were resolved in 3 % to 5 % agarose gel and visulaized under UV light after staining with ethidium bromide and genotyping was done based on the restriction digested products size.

3.7 Allele frequencies and their distribution

Gene and genotype frequencies of various identified alleles/genotypes were calculated.

Genotype frequency= Number of individuals of a particular genotype/Total Number of individuals of all genotypes.

Gene frequency = (2D+H)/2N

Where, D=Number of homozygote animals of a particular genotype

H= Number of heterozygote animals having both alleles

N= Total number of individuals

Genotype of every animal was recorded manually from the autoradiograph. Genotyping involved the recording of the homozygous or heterozygous state of the animal, as well as the size of the respective alleles in base pairs. On population basis, the number of alleles, their size and frequencies for different markers was recorded for each breed.

3.8 Polymorphism information content (PIC)

Expected value of PIC for each locus will be calculated as per (Botstein et al., 1980):

$$PIC = 1 - \sum_{i=1}^{n} pi2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2\, pi2\, pj2$$

3.9 Statistical analysis

The association between various allelic variants with Bovine tolerance/ susceptibility was worked out by suitable statistical techniques using different procedures of SAS 9.3. The missing data was considered as most frequently available genotype of SNP. The PROC ALLELE procedure of the SAS 9.3 used for the estimation of PIC, HWE and heterozygosity. The PROC LOGISTIC procedure of SAS 9.3 was used to find association of allelic and genotypic frequencies with bTB. The ODDs ratio of genotypes was calculated in affected population versus their contemporary genotypes.

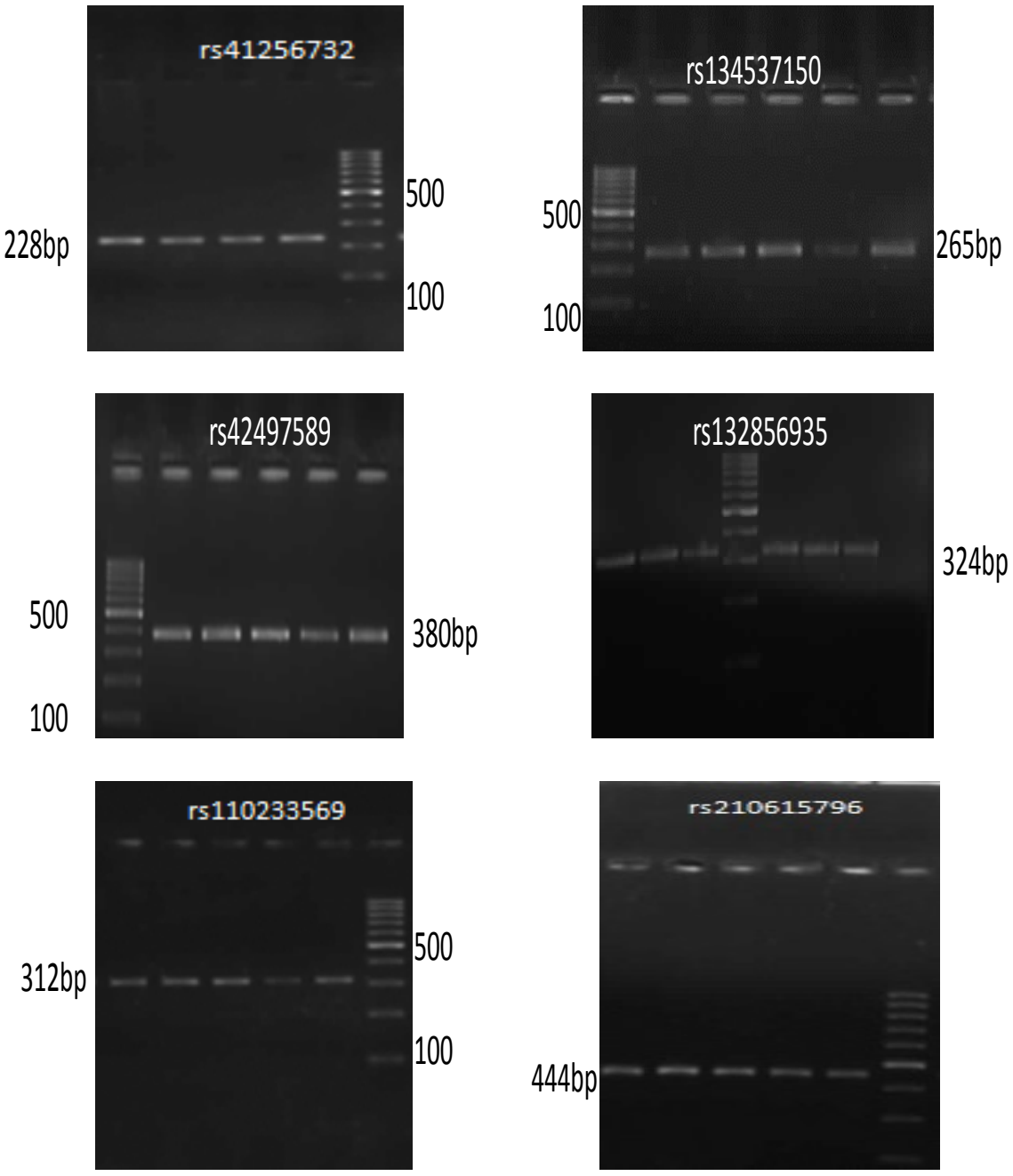

Fig.1(a) PCR amplifications under optimised conditions

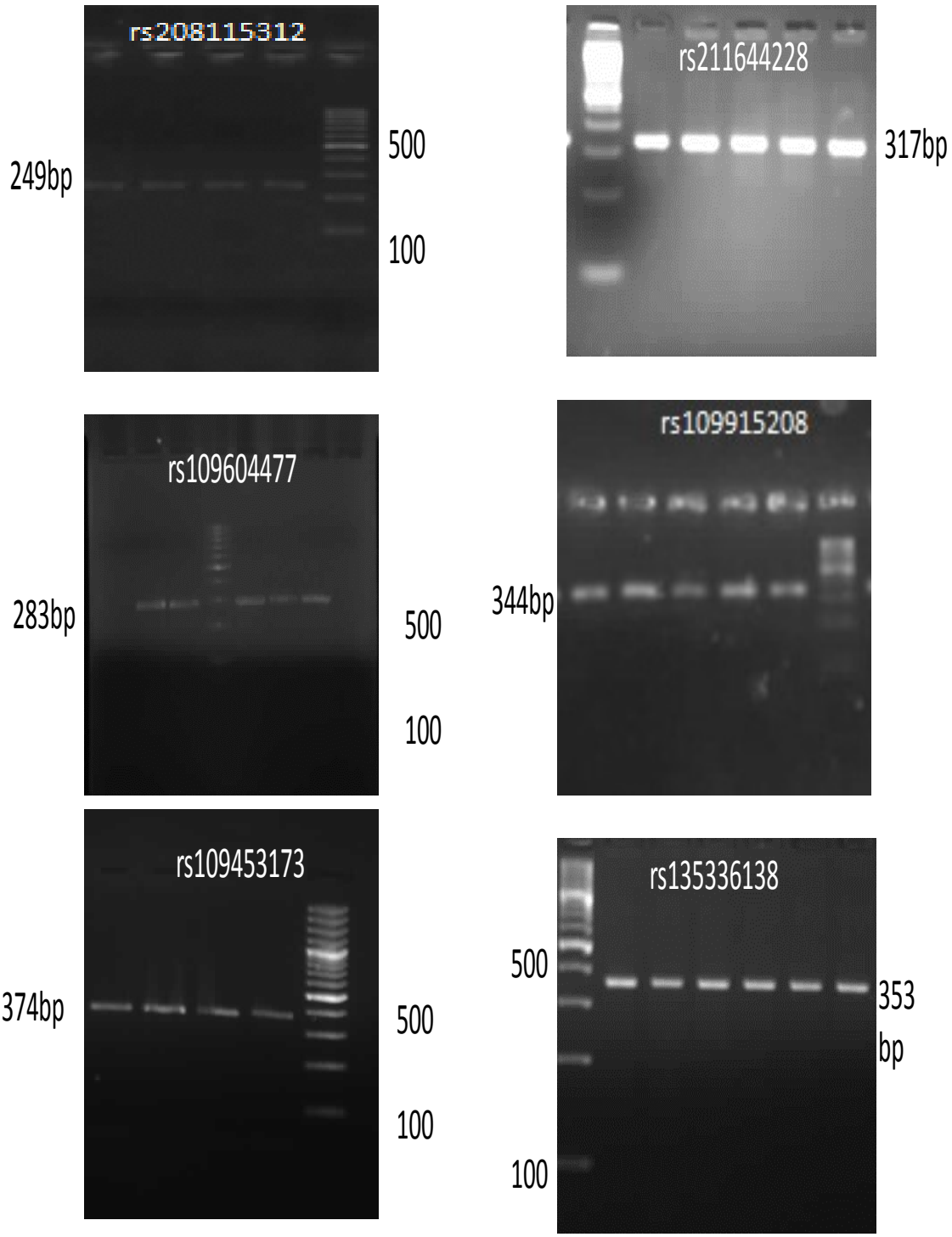

Fig.1(b) PCR amplifications under optimised conditions

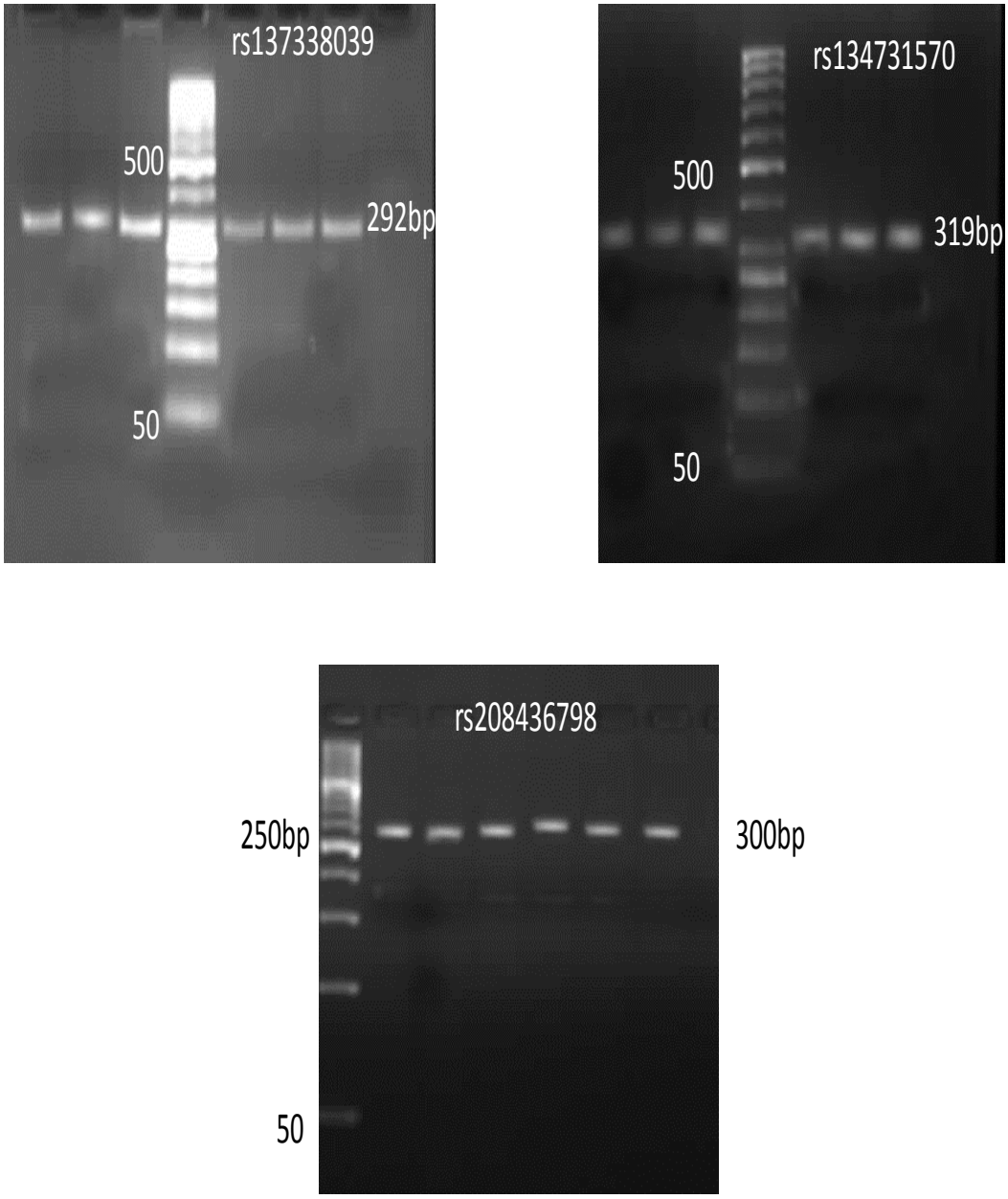

Fig.1(c) PCR amplifications under optimised conditions

SNP ID	Gene	Chromosome no.	SNP	Primers	Annealing temp. (°C).	Restriction enzyme	Fragment sizes	Amino acid change
rs41256732	SP110	**2**	**C/T**	TTCTGACTGCATCTGCCAAG TCCAGGAATCTGAGGTTTGG	**57**	**HpaII**	228,190, 38	Leu- Gln
rs134537150	SP110	**2**	**A/G**	GAATGTGAACGGTGGTGAGG CAACAGCTTCCAGGCTCATC	**59**	**EarI**	265, 193, 72	3'UTR
rs42497589	IL23R	**3**	**C/T**	CCCTCTTCAGATTGCAAGGC ACTTGGGAAACAATGCCAGG	**59**	**HpaII**	380,246, 134	Pro-pro
rs132856935	IL12RB1	**7**	**C/T**	GGAGCCCTTCCTATTGATCC TCTGCTTCCTGTCCTTCAAG	**57**	**HpyCH4III**	324,200, 124	Met- Thr
rs110233569	IL12RB1	**7**	**C/G**	TAGAGTTTGCCGAGAGAGTG TGGTAATGGAGGATGCAGG	**57**	**AluI**	312, 201, 111	Gln-His
rs210615796	IL12RB1	**7**	**A/G**	GGTTAGAGTTTGCCGAGAGAG CAGACTTGTGCGAGTAGGAG	**57**	**SmaI**	444, 275, 169	Arg-Glu
rs208115312	IL12RB2	**3**	**C/T**	ATCTGGGCGGTGCTACTAAG	**59**	**NlaIII**	249,150, 99	Val-Met

Table.1 (a) : **List of SNPs, primers & restriction enzymes used.**

rs135336138	IL12RB2	**3**	**G/T**	TCCTGCCTGAAGTTTC CTTG TTTGCTGGGTTGGGT AGTTG	**58**	**HaeIII**	353,205, 148	Thr-Pro
rs211644228	IL12RB2	**3**	**A/G**	TGGAACCCTGAACAA GTTGG CTTGTCCTGTCTCTGC TGTG	**58**	**PstI**	317, 199, 118	Arg-Cys
rs109604477	SLC11A1	**2**	**C/T**	CTTCCCCTTCTTATCT CCCG GCCAATGACTTCCTG CATG	**57**	**BsmI**	283,219, 64	Ile-Thr
rs109915208	SLC11A1	**2**	**C/T**	TGGACTGGAGGGTAA GAACG AGGGAGGAATGCAG GTAGATG	**59**	**Bpu10I**	344,215, 129	Ala-Val
rs109453173	SLC11A1	2	**C/G**	ATCTCCTTCCTACTGC CCG CACAAACTGTCCCGC GTAG	58	**PstI**	374, 293, 81	Pro-Ala
rs137338039	DC-SIGN	7	**C/T**	AGGCTTAGAGAGTGA CTTGC ACCTTGAAAGCAGAT TTGGC	**57**	**BsmAI**	292,197, 95	5’
rs134731570	DC-SIGN	7	**C/T**	ACCCCCAGAAATTCC TGAAC TAAATGCAGATTCCT GGGCC	**58**	**BtsCI**	319,217, 102	Gly-Glu
rs208436798	DC-SIGN	7	**C/T**	CTTCTGGAAAAAAGG GGAGC TACTAAGGGGATGGG CATTC	**57**	**HpaII**	300, 201,99	3’UTR

Table.1 (b) : **List of SNPs, primers & restriction enzymes used.**

S.No.	ENZYME	COMPANY	Con./µl)	USED Units /sample	BUFFER	REACTION TEMPERATURE
1.	HpaII	Fermentas	10	1	Buf.Tango (10X)	37
2.	HaeIII	Fermentas	10	1	Buf.R	37
3.	PstI	Thermo	10	1	Buf"O"	37
4.	EarI	Thermo	10	1	Buf.Tango (10X)	37
5.	HpyCH4III	Thermo	10	1	Buf.Tango (10X)	65
6.	AluI	Fermentas	10	1	Buf.Tango (10X)	37
7.	SmaI	Thermo	10	1	Buf.Tango (10X)	30
8.	NlaIII	NEB	10	1	NEBuffer 4 (10X)	37
9.	BsmI	Fermentas	10	1	Buf"R"	37
10.	Bpu10I	Thermo	5	1	Buffer Bpu10I (10X)	37
11.	BsmAI	Fermentas	10	1	Buf.Tango (10X)	37
12.	BtsCI	Thermo	10	1	Buf.Tango (10X)	55

Table 2 : Conditions of restriction enzyme digestion.

SP110 C912T (rs41256732)

This SNP lies in the exonic region of SP110 and is non-synonymous , resulting a change of amino acid Leu-Gln. The mass PCR-RFLP revealed polymorphism within as well as between the case and control population. The estimates for polymorphic Information content (PIC), heterozygosity and allelic diversity were 0.3749 , 0.5238 and 0.4997 respectively. The chi square test revealed that the population was not in HWE(Table.3). At this SNP site, two alleles i.e. C and T and three genotypes i.e. CC, CT and TT were observed. While the CC genotype showed the fragments of 190 bp, and 38 bp, TT genotype showed the restriction fragment of 228 bp and the CT genotype showed the restriction fragments of 288 bp, 190 bp, 38 bp(Fig.2a). While the frequency of T allele was 0.4857 in case and 0.4898 in control, C allele had the frequency of 0.5143 and 0.5102 in case and control population respectively. Similarly the frequency of genotype TT, CT and CC were 0.2000, 0.5714, and 0.2286 in case and 0.2449, 0.4898, and 0.2653 in control, respectively. The probability values showed that the genotype (P = 0.75) as well as allele (p =0.95) had nonsignificant effect on occurrence of bovine tuberculosis. The ODDs ratio of C verses T was 1.01 (0.55-1.87; 95% CI), where as ODDs ratio of CC verses TT and CT verses TT were 1.05(0.29-3.80; 95% CI) ; 1.42(0.47-4.31; 95% CI) respectively (Table.4 & 5a).

SP110 A1415G (rs134537150)

This SNP lies in the 3'UTR of SP110. The mass PCR-RFLP revealed polymorphism within as well as between the case and control population. The estimates for polymorphic Information content (PIC), heterozygosity and allelic diversity were 0.3646 , 0.4881 and 0.4795 respectively. The chi square test revealed that the population was in HWE(Table.3). At this SNP site, two alleles i.e. A and G and three genotypes i.e. AA, AG and GG were observed. While the GG genotype showed the fragments of 193 bp, and 72 bp, AA genotype showed the restriction fragment of 268

bp and the AG genotype showed the restriction fragments of 265 bp, 193 bp, 72 bp (Fig.2b). While the frequency of G allele was 0.3286 in case and 0.4490 in control, A allele had the frequency of 0.6714 and 0.5510 in case and control population respectively. Similarly the frequency of genotype AA, AG and GG were 0.4000, 0.5429, and 0.0571 in case and 0.3265, 0.4490, and 0.2245 in control, respectively. The probability values showed that the genotype (P = 0.08) as well as allele (p = 0.11) had nonsignificant effect on occurrence of bovine tuberculosis. The ODDs ratio of A verses G was 1.66(0.88-3.15; 95% CI), where as ODDs ratio of AA verses GG and AG verses GG were 4.81(0.90-25.52; 95% CI) ; 4.74(0.93-24.16; 95% CI) respectively.(Table.4 & 5a).

IL23R C1683T (rs42497589)

This SNP lies in the exonic region of IL23R and is synonymous. The mass PCR-RFLP revealed polymorphism within as well as between the case and control population. The estimates for polymorphic Information content (PIC), heterozygosity and allelic diversity were 0.0454 , 0.0238 and 0.0465 respectively. The chi square test revealed that the population was not in HWE(Table.3). At this SNP site, two alleles i.e. C and T and three genotypes i.e. CC, CT and TT were observed. While the TT genotype showed the fragment of 380 bp, CC genotype showed the restriction fragments of 245 bp and 135 bp and the CT genotype showed the restriction fragments of 380 bp, 245 bp, 135 bp (Fig.3a). While the frequency of T allele was 0.0286 in case and 0.0204 in control, C allele had the frequency of 0.9714 and 0.9796 in case and control population respectively. Similarly the frequency of genotype TT, CT and CC were 0, 0.0571, and 0.9429 in case and 0.0204, 0, and 0.9796 in control, respectively. The probability values showed that the genotype (P = 0.09) as well as allele (p =0.73) had nonsignificant effect on occurrence of bovine tuberculosis. The ODDs ratio of C verses T was 0.70(0.09-5.15; 95% CI), where as ODDs ratio of CC verses TT and CT verses TT were >999.999 (<0.001-999.999; 95% CI) ; >999.999(<0.001-999.999; 95% CI) respectively.(Table.4 & 5a).

IL12RB1 C1946T (rs132856935)

This SNP lies in the exonic region of IL12RB1 and is non-synonymous, resulting a change of amino acid Met-Thr. The mass PCR-RFLP revealed polymorphism within as

well as between the case and control population. The estimates for polymorphic Information content (PIC), heterozygosity and allelic diversity were 0.0665, 0.0714 and 0.0689 respectively. The chi square test revealed that the population was not in HWE (Table.3). At this SNP site, two alleles i.e. C and T and two genotypes i.e. CT and TT were observed. While the CC genotype showed the fragments of 200 bp, and 124 bp, TT genotype showed the restriction fragment of 324 bp and the CT genotype showed the restriction fragments of 324 bp, 200 bp, 124 bp (Fig.3b). While the frequency of T allele was 0.9714 in case and 0.9592 in control, C allele had the frequency of 0.0286 and 0.0408 in case and control population respectively. Similarly the frequency of genotype TT, CT and CC were 0.9429, 0.0571, and 0 in case and 0.9184, 0.0816, and 0 in control, respectively. The probability values showed that the genotype (P =0.66) as well as allele (p =0.66) had nonsignificant effect on occurrence of bovine tuberculosis. The ODDs ratio of C verses T was 0.69(0.12-3.88; 95% CI), where as ODDs ratio of CT verses TT was 0.68(0.11-3.94; 95% CI) . (Table.4 & 5a).

IL12RB1 C1194G (rs110233569)

This SNP lies in the exonic region of IL12RB1 and is non-synonymous , resulting a change of amino acid Gln-His. The mass PCR-RFLP revealed polymorphism within as well as between the case and control population. The estimates for polymorphic Information content (PIC), heterozygosity and allelic diversity were 0.3153, 0.3929 and 0.3922 respectively. The chi square test revealed that the population was in HWE (Table.3). At this SNP site, two alleles i.e. C and G and three genotypes i.e. CC, CG and GG were observed. While the CC genotype showed the fragments of 197 bp, and 115 bp, GG genotype showed the restriction fragment of 312 bp and the CG genotype showed the restriction fragments of 312 bp, 197 bp, 115 bp(Fig.4a). While the frequency of G allele was 0.6857 in case and 0.7653 in control, C allele had the frequency of 0.3143 and 0.2347 in case and control population respectively. Similarly the frequency of genotype GG, CG and CC were 0.4571, 0.4571, and 0.0857 in case and 0.5918, 0.3469, and 0.0612 in control, respectively. The probability values showed that the genotype (P = 0.47) as well as allele (p =0.25) had nonsignificant effect on occurrence of bovine tuberculosis. The ODDs ratio of C verses G was 1.49(0.75-2.97; 95% CI), where as ODDs ratio of CC verses GG and CG verses GG were 1.81(0.32-10.04; 95% CI) ; 1.70(0.68-4.26; 95% CI) respectively(Table.4 & 5a).

IL12RB1 A1160G (rs210615796)

This SNP lies in the exonic region of IL12RB1 and is non-synonymous , resulting a change of amino acid Arg-Glu. The mass PCR-RFLP revealed monomorphism within as well as between the case and control population(Fig.9b).

IL12RB2 C721T (rs208115312)

This SNP lies in the exonic region of IL12RB2 and is non-synonymous , resulting a change of amino acid Val-Met. The mass PCR-RFLP revealed monomorphism within as well as between the case and control population(Fig.4b).

IL12RB2 G1330T (rs135336138)

This SNP lies in the exonic region of IL12RB2 and is non-synonymous , resulting a change of amino acid Thr-Pro. The mass PCR-RFLP revealed monomorphism within as well as between the case and control population(Fig.5a).

IL12RB2 A2176G (rs211644228)

This SNP lies in the exonic region of IL12RB2 and is non-synonymous , resulting a change of amino acid Arg-Cys. The mass PCR-RFLP revealed polymorphism within as well as between the case and control population. The estimates for polymorphic Information content (PIC), heterozygosity and allelic diversity were 0.0232, 0.0238 and 0.0235 respectively. The chi square test revealed that the population was in HWE (Table.3). At this SNP site, two alleles i.e. A and G and two genotypes i.e. AG and GG were observed. While the AA genotype showed the fragments of 203 bp, and 114 bp, GG genotype showed the restriction fragment of 317 bp and the AG genotype showed the restriction fragments of 317 bp,203 bp, 114 bp(Fig.5b). While the frequency of G allele was 0.9857 in case and 0.9898 in control, A allele had the frequency of 0.0143 and 0.0102 in case and control population respectively. Similarly the frequency of genotype GG, AG and AA were 0.9714, 0.0286, and 0 in case and 0.9796, 0.0204, and 0 in control, respectively. The probability values showed that the genotype (P =0.81) as well as allele (p =0.81) had nonsignificant effect on occurrence of bovine tuberculosis. The ODDs ratio of A verses G was 1.40(0.08-22.86; 95% CI), where as ODDs ratio of AG verses GG was 1.41(0.08-23.36; 95% CI)Table.4 & 5a.

SLC11A1 C468T (rs109604477)

This SNP lies in the exonic region of SLC11A1 and is non-synonymous , resulting a change of amino acid Ile-Thr. The mass PCR-RFLP revealed polymorphism within as well as between the case and control population. The estimates for polymorphic Information content (PIC), heterozygosity and allelic diversity were 0.3737 , 0.9286 and 0.4974 respectively. The chi square test revealed that the population was not in HWE(Table.3). At this SNP site, two alleles i.e. C and T and two genotypes i.e. CT and TT were observed. While the CC genotype showed the fragment of 283 bp, TT genotype showed the restriction fragments of 219 bp and 64bp and the CT genotype showed the restriction fragments of 283 bp, 219 bp, 64 bp(Fig.6a). While the frequency of T allele was 0.5857 in case and 0.5000 in control, C allele had the frequency of 0.4143 and 0.5000 in case and control population respectively. Similarly the frequency of genotype TT, CT and CC were 0.1714, 0.8286, and 0 in case and 0, 0.1000, and 0 in control, respectively. The probability values showed that the genotype (P = 0.008) as well as allele (p =0.27) had significant effect on occurrence of bovine tuberculosis. The ODDs ratio of C verses T was 0.70(0.38-1.31; 95% CI), where as ODDs ratio of CT verses TT was <0.001(<0.001 to >999.9>; 95% CI) Table.4 & 5b.

SLC11A1 C723T (rs109915208)

This SNP lies in the exonic region of SLC11A1 and is non-synonymous , resulting a change of amino acid Ala-Val. The mass PCR-RFLP revealed polymorphism within as well as between the case and control population. The estimates for polymorphic Information content (PIC), heterozygosity and allelic diversity were 0.0454, 0.0476 and 0.0465 respectively. The chi square test revealed that the population was in HWE (Table.3). At this SNP site, two alleles i.e. C and T and two genotypes i.e. CC and CT were observed. While the CC genotype showed the fragments of 215 bp, and 129 bp, TT genotype showed the restriction fragment of 344 bp and the CT genotype showed the restriction fragments of 344 bp, 215 bp, 129 bp(Fig.6b). While the frequency of T allele was 0 in case and 0.0408 in control, C allele had the frequency of 0.1000 and 0.9592 in case and control population respectively. Similarly the frequency of genotype TT, CT and CC were 0, 0, and 0.1000 in case and 0, 0.0816, and 0.9184 in control, respectively. The probability values showed that the genotype (P =0.03) as well as allele (p = 0.03) had significant effect on occurrence of bovine tuberculosis. The ODDs ratio of C verses

T was >999.9(<0.001->999.999; 95% CI), where as ODDs ratio of CC verses CT was 999.9(<0.001->999.999; 95% CI).(Table.4 & 5a).

SLC11A1 C1139G (rs109453173)

This SNP lies in the exonic region of SLc11A1 and is non-synonymous , resulting a change of amino acid Pro-Ala. The mass PCR-RFLP revealed polymorphism within as well as between the case and control population. The estimates for polymorphic Information content (PIC), heterozygosity and allelic diversity were 0.3519, 0.4881 and 0.4557 respectively. The chi square test revealed that the population was not in HWE (Table.3). At this SNP site, two alleles i.e. C and T and three genotypes i.e. CC, CG and GG were observed. While the CC genotype showed the fragments of 274 bp, and 100 bp, GG genotype showed the restriction fragment of 374 bp and the CG genotype showed the restriction fragments of 374 bp, 274 bp, 100 bp(Fig.7). While the frequency of G allele was 0.7143 in case and 0.6020 in control, C allele had the frequency of 0.2857 and 0.3980 in case and control population respectively. Similarly the frequency of genotype GG, CG and CC were 0.5143, 0.4000, and 0.0857 in case and 0.3265, 0.5510, and 0.1224 in control, respectively. The probability values showed that the genotype (P = 0.22) as well as allele (p = 0.13) had nonsignificant effect on occurrence of bovine tuberculosis. The ODDs ratio of C verses T was 0.60(0.31-1.16; 95% CI), where as ODDs ratio of CC verses GG and CG verses GG were 0.44(0.09-2.07; 95% CI) ; 0.46(0.18-1.17; 95% CI) respectively.(Table.4 & 5b).

DC-SIGN C292T (rs137338039)

This SNP lies in the promoter region of DC-SIGN. The mass PCR-RFLP revealed polymorphism within as well as between the case and control population. The estimates for polymorphic Information content (PIC), heterozygosity and allelic diversity were 0.2608, 0.2857 and 0.3084 respectively. The chi square test revealed that the population was not in HWE(Table.3). At this SNP site, two alleles i.e. C and T and three genotypes i.e. CC, CT and TT were observed. While the CC genotype showed the fragments of 197 bp, and 95 bp, TT genotype showed the restriction fragment of 292 bp and the CT genotype showed the restriction fragments of 292 bp, 197 bp, 95 bp(Fig.8a). While the frequency of T allele was 0.7286 in case and 0.8674 in control, C allele had the frequency of 0.2714 and 0.1326 in case and control population respectively. Similarly

the frequency of genotype TT, CT and CC were 0.5429, 0.3714, and 0.0857 in case and 0.7551, 0.2245, and 0.0204 in control, respectively. The probability values showed that the genotype (P = 0.09) as well as allele (p = 0.02) had nonsignificant effect on occurrence of bovine tuberculosis. The ODDs ratio of C verses T was 2.43(1.11-5.34; 95% CI), where as ODDs ratio of CC verses TT and CT verses TT were 5.84(0.56-60.03; 95% CI) ; 2.30(0.86-6.10; 95% CI) respectively.(Table.4 & 5b).

DC-SIGN C319T (rs134731570)

This SNP lies in the exonic region of DC-SIGN and is non-synonymous , resulting a change of amino acid Gly-Glu. The mass PCR-RFLP revealed monomorphism within as well as between the case and control population(Fig.9a).

DC-SIGN C300T (rs208436798)

This SNP lies in the 3'UTR of DC-SIGN. The mass PCR-RFLP revealed polymorphism within as well as between the case and control population. The estimates for polymorphic Information content (PIC), heterozygosity and allelic diversity were 0.1494, 0.0357and 0.1626 respectively. The chi square test revealed that the population was not in HWE(Table.3). At this SNP site, two alleles i.e. C and T and three genotypes i.e. CC, CT and TT were observed. While the CC genotype showed the fragments of 201 bp, and 99 bp, TT genotype showed the restriction fragment of 300 bp and the CT genotype showed the restriction fragments of 300 bp, 201 bp, 99 bp(Fig.8b). While the frequency of T allele was 0.1286 in case and 0.0612 in control, C allele had the frequency of 0.8714 and 0.9388 in case and control population respectively. Similarly the frequency of genotype TT, CT and CC were 0.0857, 0.0857, and 0.8286 in case and 0.0612, 0, and 0.9389 in control, respectively. The probability values showed that the genotype (P = 0.05) as well as allele (p = 0.13) had nonsignificant effect on occurrence of bovine tuberculosis. The ODDs ratio of C verses T was 0.44(0.15-1.30; 95% CI), where as ODDs ratio of CC verses TT and CT verses TT were 0.63(0.11-3.33; 95% CI) and >999.999(<0.001->999.999; 95% CI) respectively.(Table.4 & 5b).

Linkage disequilibrium analysis

The linkage disequilibrium analysis revealed that the significantly linked loci/SNPs were rs41256732 with rs134537150 and rs132856935 ; rs134537150 with rs132856935 and rs208436798 ; rs42497589 with rs137338039 ; rs132856935 with rs211644228 and rs137338039 ; rs137338039 with rs208436798. All other loci/ SNPs of investigation were having non-significant LD suggested that there was no linkage between alleles of these loci.

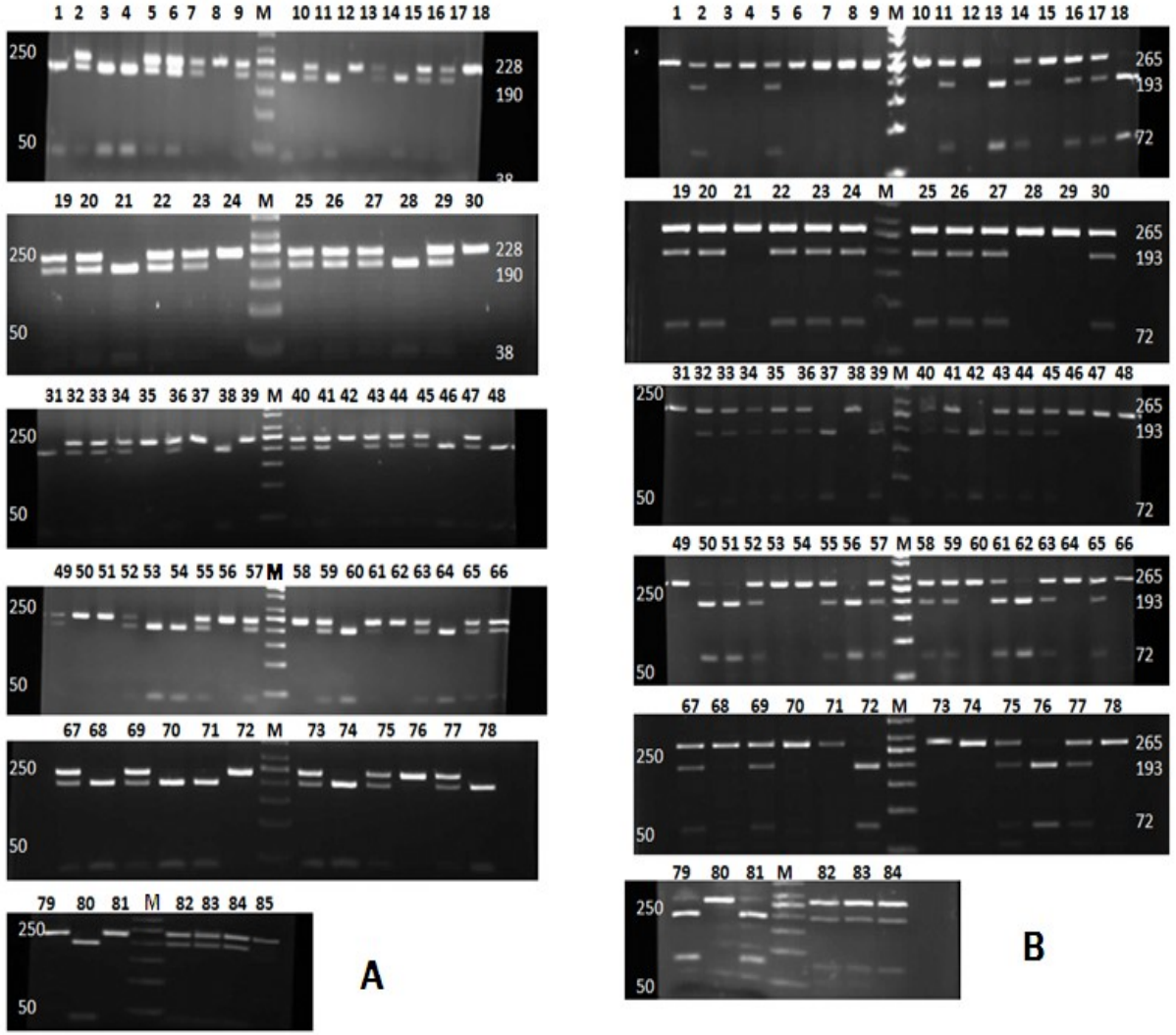

Fig . 2 . PCR RFLP profile for SNP rs41256732 (A) and rs134537150 (B) resolved at 5 and 4 % agarose gel, respectively. Lane 1 to 35: Case; Lane M : 50 bp ladder, Fermentas ; Lane 36 to 84: control

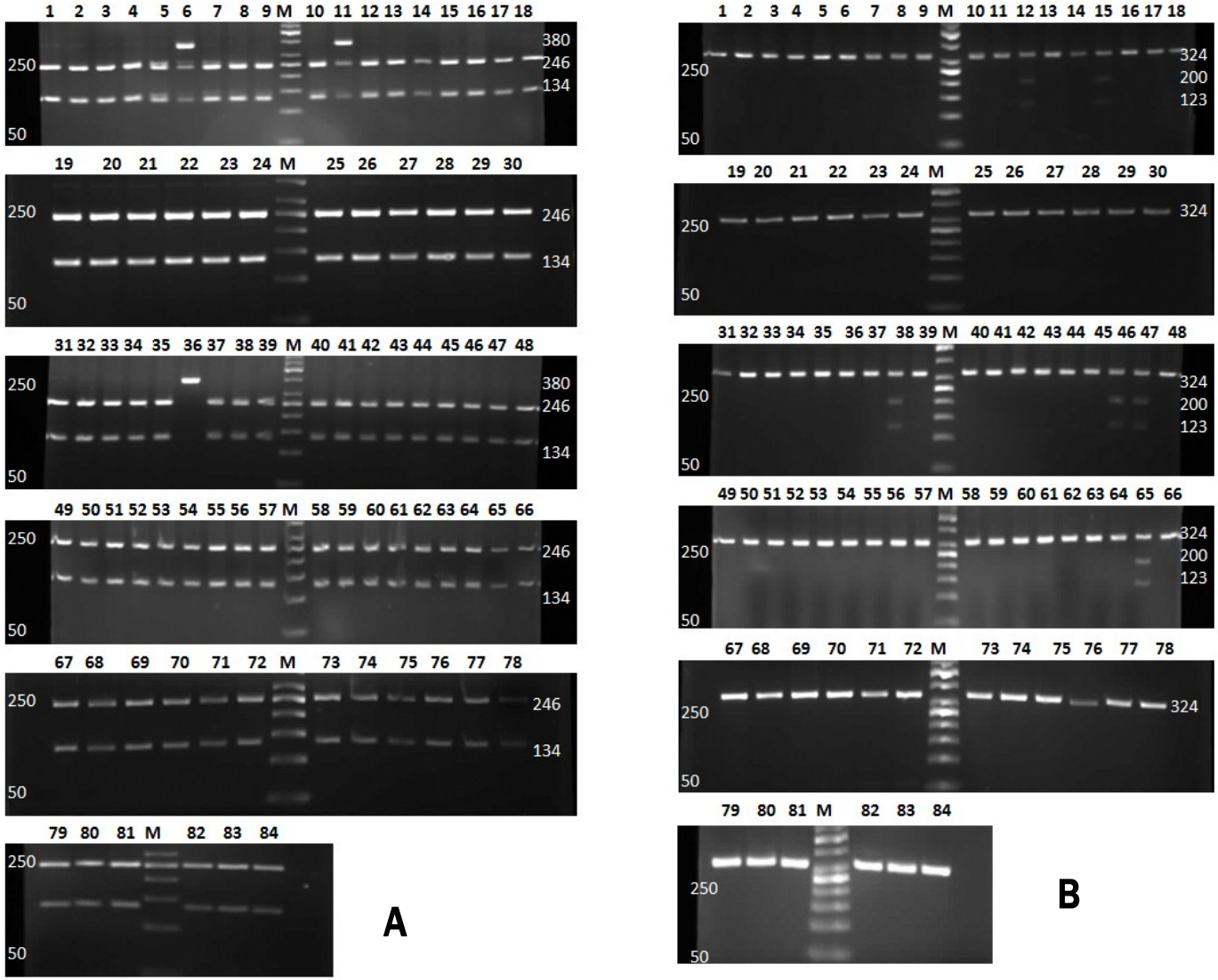

Fig 3 . PCR RFLP profile for SNP rs42497589 (A) and rs132856935 (B) resolved at 3 and 3 %, respectively agarose gel. Lane 1 to 35: Case; Lane M : 50 bp ladder, Fermentas ; Lane 36 to 84: control

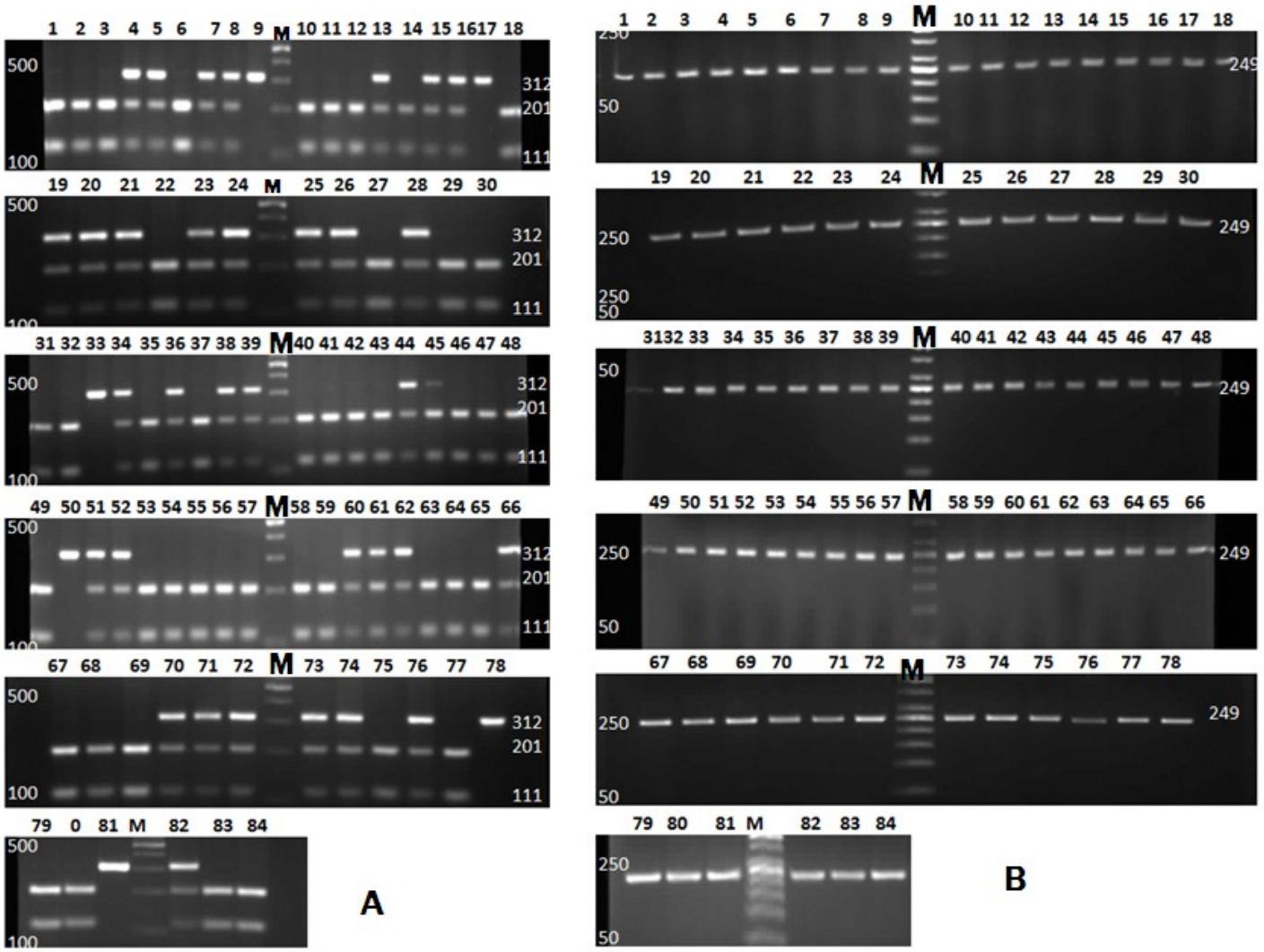

Fig 4 . PCR RFLP profile for SNP rs110233569 (A) and rs208115312 (B) resolved at 4 and 5 % agarose gel, respectively. Lane 1 to 35: Case; Lane M : 50 bp ladder, Fermentas ; Lane 36 to 84: control

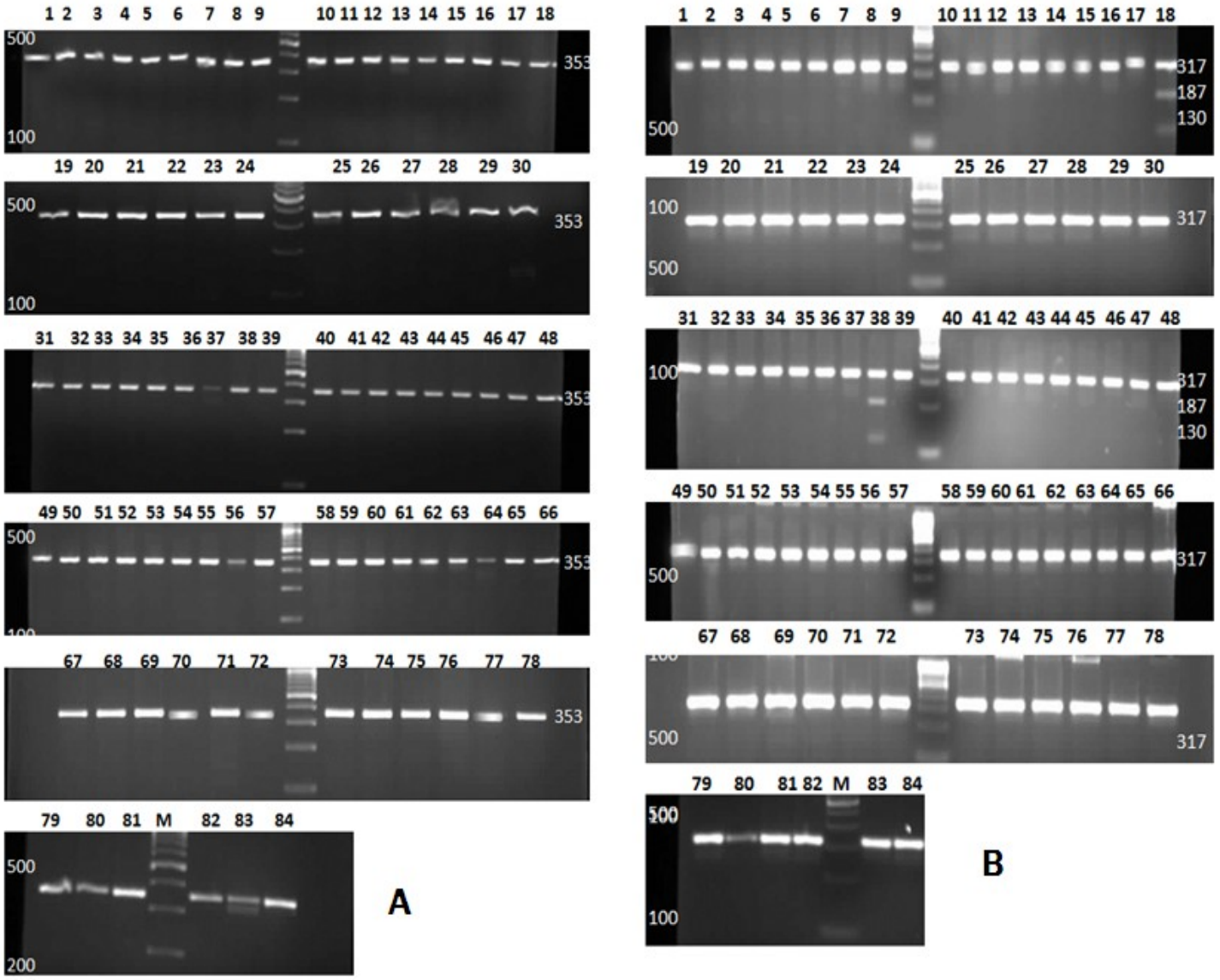

Fig 5 . PCR RFLP profile for SNP rs135336138 (A) and rs211644228 (B) resolved at 3 and 3 %, respectively agarose gel. Lane 1 to 35: Case; Lane M : 50 bp ladder, Fermentas ; Lane 36 to 84: control

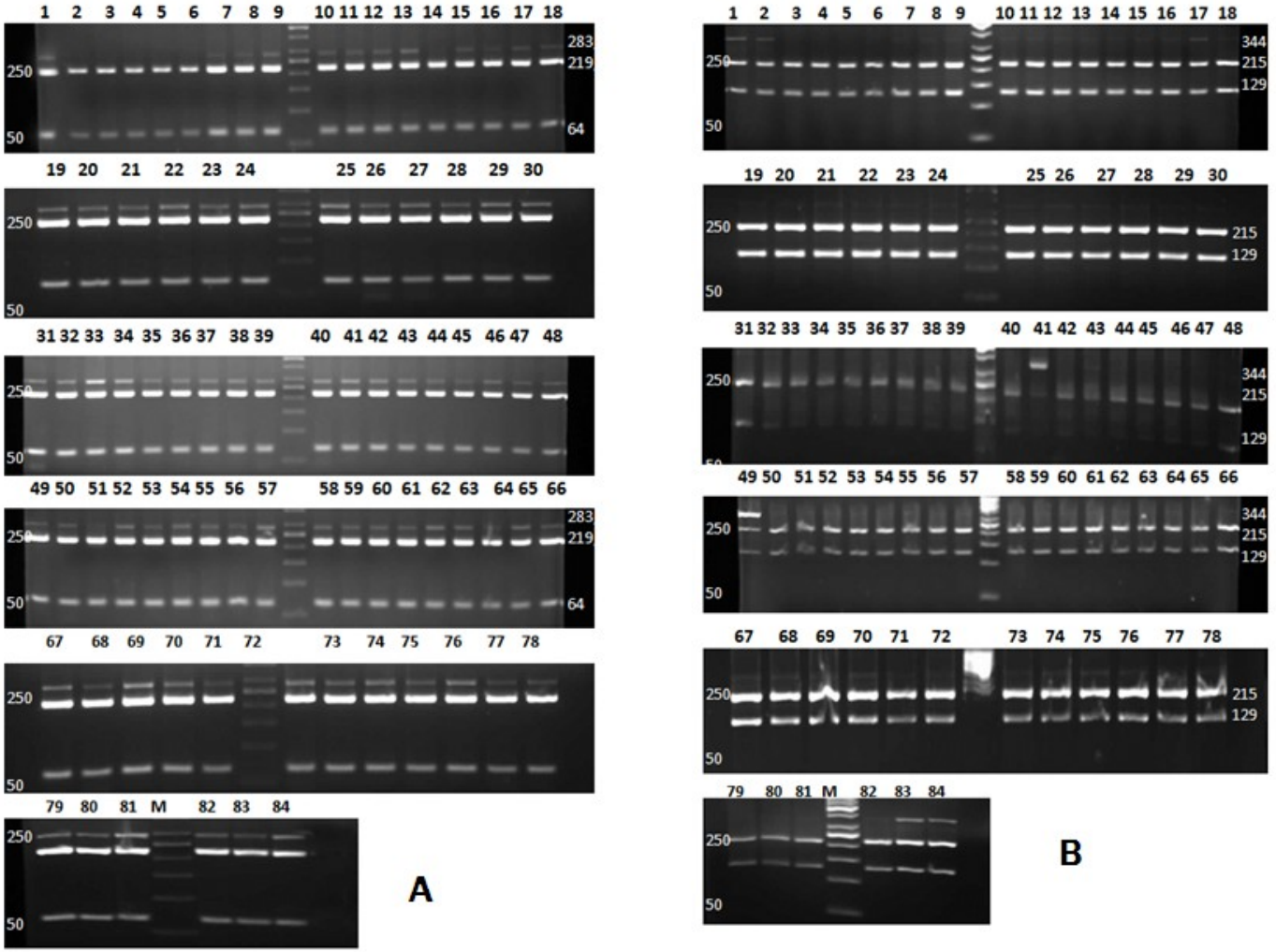

Fig 6 . PCR RFLP profile for SNP rs109604477 (A) and rs109915208 (B) resolved at 5 and 3 % agarose gel, respectively. Lane 1 to 35: Case; Lane M : 50 bp ladder, Fermentas ; Lane 36 to 84: control

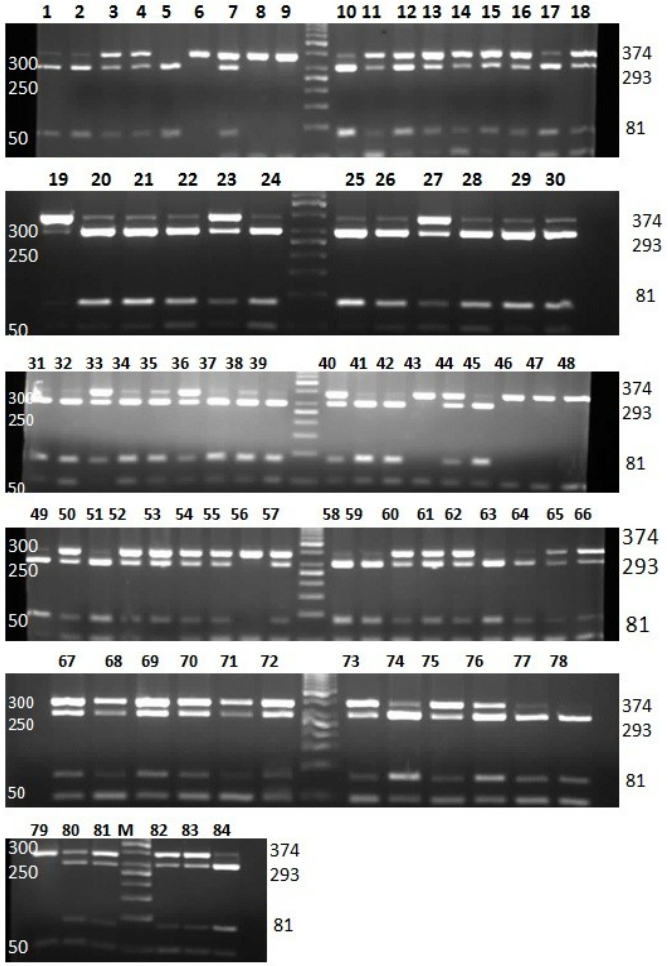

Fig 7 . PCR RFLP profile for SNP rs109453173 resolved at 4 % agarose gel. Lane 1 to 35: Case; Lane M : 50 bp ladder, Fermentas ; Lane 36 to 84: control

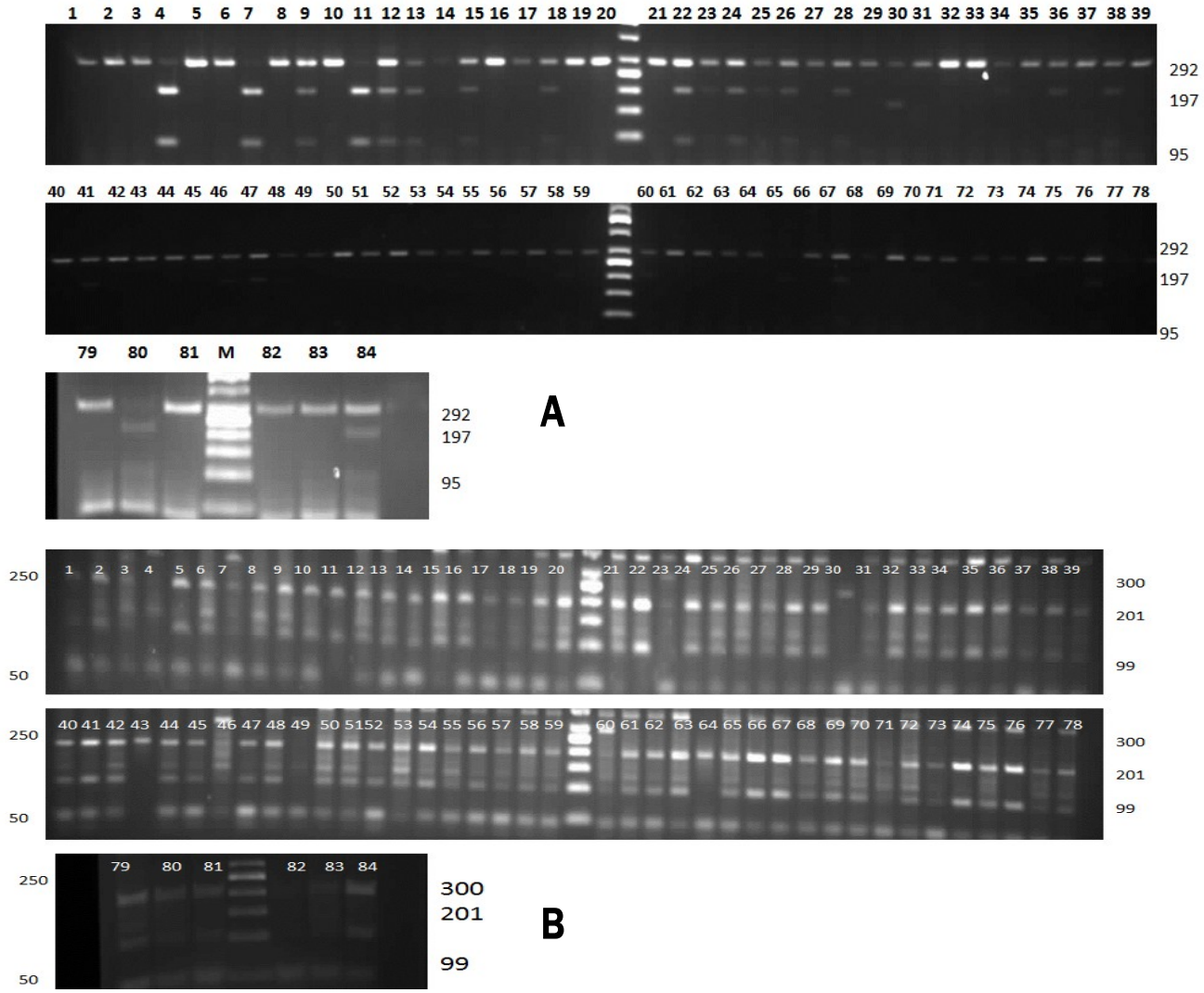

Fig 8 . PCR RFLP profile for SNP rs137338039 (A) and rs208436798 (B) resolved at 4 and 4 % respectively agarose gel. Lane 1 to 35: Case; Lane M : 50 bp ladder, Fermentas ; Lane 36 to 84: control

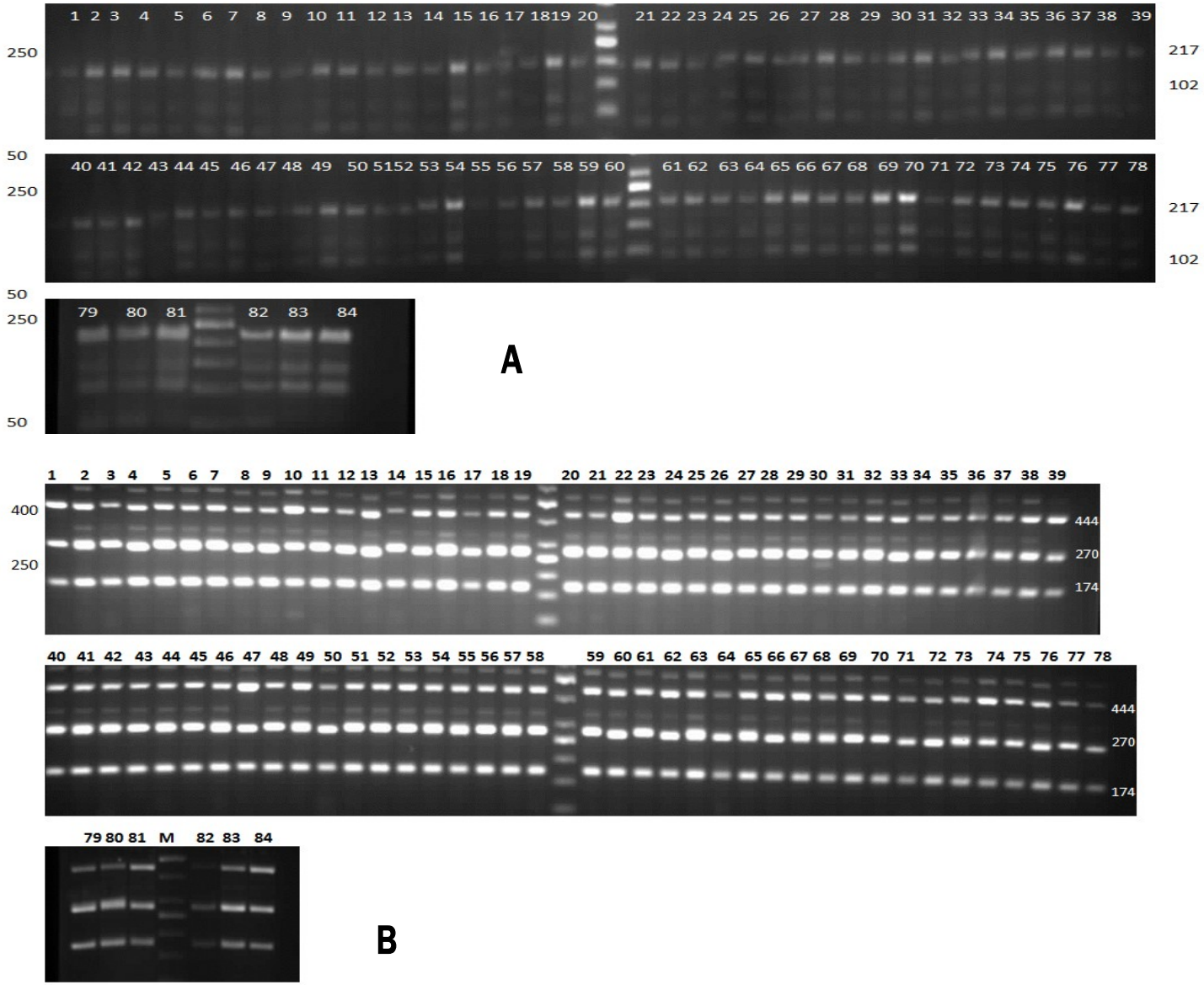

Fig 9 . PCR RFLP profile for SNP rs134731570 (A) and rs210615796 (B) resolved at 4 and 3 % agarose gel, respectively. Lane 1 to 35: Case; Lane M : 50 bp ladder, Fermentas ; Lane 36 to 84: control

Marker Summary								
Locus	Number of Indiv	Number of Alleles	Polymorph Info Content	Heterozygosity	Allelic Diversity	Test for HWE		
						Chi-Square	DF	Pr > ChiSq
rs41256732	84	2	0.3749	0.5238	0.4997	0.1953	1	0.6586
rs134537150	84	2	0.3646	0.4881	0.4795	0.0269	1	0.8698
rs42497589	84	2	0.0454	0.0238	0.0465	19.9881	1	<.0001
rs132856935	84	2	0.0665	0.0714	0.0689	0.1152	1	0.7343
rs110233569	84	2	0.3153	0.3929	0.3922	0.0002	1	0.9881
rs211644228	84	2	0.0232	0.0238	0.0235	0.0122	1	0.9121
rs109604477	84	2	0.3737	0.9286	0.4974	63.0933	1	<.0001
rs109915208	84	2	0.0454	0.0476	0.0465	0.0500	1	0.8231
rs109453173	84	2	0.3519	0.4881	0.4557	0.4242	1	0.5149
rs137338039	84	2	0.2608	0.2857	0.3084	0.4542	1	0.5004
rs208436798	84	2	0.1494	0.0357	0.1626	51.1570	1	<.0001

Table 3 : Polymorphism at different SNPs and Chi-square test for HWE

SNP	Allele	Allele frequency		p-Value	Odds ratio (95% CI)
		Case	Control		
rs41256732	T	34(48.57)	48(48.98)	0.95	1
	C	36(51.43)	50(51.02)		1.01 (0.55-1.87)
rs134537150	G	23(32.86)	44(44.90)	0.11	1.00
	A	47(67.14)	54(55.10)		1.66 (0.88 to 3.15)
rs42497589	T	2(2.86)	2(2.04)	0.73	1.00
	C	68(97.14)	96(97.96)		0.70 (0.09 to 5.15)
rs132856935	T	68(97.14)	94(95.92)	0.66	1.00
	C	2(2.86)	4(4.08)		0.69 (0.12 to 3.88)
rs110233569	G	48(68.57)	75 (76.53	0.25	1.00
	C	22(31.43)	23 (23.47)		1.49 (0.75 to 2.97)
rs211644228	G	69(98.57)	97(98.98)	0.81	1.00
	A	1(1.43)	1(1.02)		1.40 (0.08 to 22.86)
rs109604477	T	41(58.57)	49(50.00)	0.27	1.00
	C	29(41.43)	49(50.00)		0.70 (0.38 to 1.31)
rs109915208	T		4(4.08)	0.03	1.00
	C	70(1.000)	94(95.92)		>999.9 (<0.001 to >999.999)
rs109453173	G	50(71.43)	59(60.20)	0.13	1.00
	C	20(28.57)	39(39.80)		0.60 (0.31 to 1.16)
rs137338039	T	51(72.86)	85(86.74)	0.12	1.00
	C	19(27.14)	13(13.26)		2.43(1.11 to 5.34)
rs208436798	T	9(12.86)	6(6.12)	0.13	1.00
	C	61(87.14)	92(93.88)		0.44 (0.15 to 1.30)

Table 4 : Allele frequencies and their association with susceptibility to bTB in Case: Control population

SNP	Genotype	Genotype frequency		p-Value	Odds ratio (95% CI)
		Case	Control		
rs41256732	TT	7 (20.00)	12 (24.49)	0.75	1.0
	CC	8 (22.86)	13 (26.53)		1.05 (0.29 to 3.80)
	CT	20 (57.14)	24 (48.98)		1.42 (0.47 to 4.31)
rs134537150	GG	2(5.71)	11(22.45)	0.08	1.00
	AA	14(40.00)	16(32.65)		4.81 (0.90 to 25.52)
	AG	19(54.29)	22(44.90)		4.74 (0.93 to 24.16)
rs42497589	TT		1(2.04)	0.09	1.00
	CC	33(94.29)	48(97.96)		>999.999(<0.001->999.999)
	CT	2(5.71)			>999.999(<0.001->999.999)
rs132856935	TT	33(94.29)	45(91.84)	0.66	1.00
	CT	2(5.71)	4(8.16)		0.68 (0.11 to 3.94)
rs110233569	GG	16(45.71)	29(59.18)	0.47	1.00
	CC	3(8.57)	3(6.12)		1.81 (0.32 to 10.04)
	CG	16(45.71)	17(34.69)		1.70 (0.68 to 4.26)
rs211644228	GG	34(97.14)	48(97.96)	0.81	1.00
	AG	1(2.86)	1(2.04)		1.41 (0.08 to 23.36)

Table 5(a) : Genotypic frequencies and their association with susceptibility to bTB in Case : Control population

Cont..

SNP	Genotype	Genotype frequency		p-Value	Odds ratio (95% CI)
		Case	Control		
rs109604477	TT	6(17.14)		0.008	1.00
	CT	29(82.86)	49(1.000)		<0.001 (<0.001 to >999.9)
rs10915208	CC	35(1.000)	45(91.84)	0.03	>999.9 (<0.001 to >999.9)
	CT		4(8.16)		1.00
rs109453173	GG	18(51.43)	16(32.65)	0.22	1.00
	CC	3(8.57)	6(12.24)		0.44 (0.09 to 2.07)
	CG	14(40.00)	27(55.10)		0.46 (0.18 to 1.17)
rs137338039	TT	19(54.29)	37(75.51)	0.09	1.00
	CC	3(8.57)	1(2.04)		5.84 (0.56 to 60.03)
	CT	13(37.14)	11(22.45)		2.30 (0.86 to 6.10)
rs208436798	TT	3(8.57)	3(6.12)	0.06	1.00
	CC	29(82.86)	46(93.89)		0.63 (0.11 to 3.33)
	CT	3(8.57)			>999.999 (<0.001 to >999.999)

Table 5(b) : Genotypic frequencies and their association with susceptibility to bTB in Case : Control population

Out of the 15 SNPs genotyped given in Table , four SNPs were monomorphic (rs210615796 in IL12RB1; rs208115312 in IL12RB2; rs135336138 in IL12RB2; rs134731570 in CD-SIGN) and thus removed prior to analysis.

SP110 gene

The SNP (**rs41256732**) locus in SP110 gene revealed that the ODDs ratio of C verses T was 1.01(0.55-1.87; 95% CI)and non-significantly differing in case animals as compared to control animals where as ODDs ratio of CC verses TT and CT verses TT were 1.05(0.29-3.80; 95% CI) ; 1.42(0.47-4.31; 95% CI) respectively and all the three genotypes were not differing significantly (P≤0.05) in case-control animals.

The SNP (**rs134537150)** locus in SP110 revealed that the ODDs ratio of A verses G was 1.66(0.88-3.15; 95% CI) and non-significantly differing in case animals as compared to control animals where as ODDs ratio of AA verses GG and AG verses GG were 4.81(0.90-25.52; 95% CI) ; 4.74(0.93-24.16; 95% CI) respectively and all the three genotypes were not differing significantly (P≤0.05) in case-control animals.

Polymorphisms of the *SP110 nuclear body protein* (*SP110*)gene have been reported to be associated with tuberculosis (Tosh *et al*., 2006). Larrañaga *et al.,*2011 studied association between SNP c.587A>G in *SP110* gene and susceptibility to *Mycobacterium bovis* infection in cattle and reported that neither SNP c.587A>G alleles nor genotypes showed significant association with susceptibility to *Mycobacterium bovis* infection.

IL23R

The SNP (**rs42497589)** locus in IL23R revealed that the ODDs ratio of C verses T was 0.70(0.09-5.15; 95% CI) and non-significantly differing in case animals as compared to control animals where as ODDs ratio of CC verses TT and CT verses TT were >999.999(<0.001-999.999; 95% CI); >999.999(<0.001-999.999; 95% CI) respectively and all the three genotypes were not differing significantly (P≤0.05) in

case-control animals.

Ben-Selma et al.,(2012) reported the reduced-function polymorphism 1142G → A encoded by IL23R influences the outcome of disease severity of active pulmonary TB in Tunisian patients. Pant et al., (2011) found one SNP in IL23R c.1417 A>C associated with the MAP infection status in resource population of 446 dairy Holsteins.

IL12RB1

The SNP (**rs132856935)** locus in IL12RB1 revealed that the ODDs ratio of C verses T was 0.69(0.12-3.88; 95% CI) and non-significantly differing in case animals as compared to control animals where as ODDs ratio of CT verses TT was 0.68(0.11-3.94; 95% CI) and the two genotypes were not differing significantly (P≤0.05) in case-control animals.

The SNP (**rs110233569)** locus in IL12RB1 revealed the ODDs ratio of C verses G was 1.49(0.75-2.97; 95% CI) and non-significantly differing in case animals as compared to control animals where as ODDs ratio of CC verses GG and CG verses GG were 1.81 (0.32-10.04; 95% CI) ; 1.70(0.68-4.26; 95% CI) respectively and all the three genotypes were not differing significantly (P≤0.05) in case-control animals.

In humans there is reported association of IL12RB1 polymorphisms with pulmonary tuberculosis (Remus *et al.*, 2004; Kusuhara *et al*., 2007). Pant *et al.,* 2011 found one SNP (c.81 T>C)in IL12RB1 associated with the MAP infection status in resource population of 446 dairy Holsteins.

IL12RB2

The SNP (**rs211644228)** locus in IL12RB2 revealed the ODDs ratio of A verses G was 1.40(0.08-22.86; 95% CI) and non-significantly differing in case animals as compared to control animals where as ODDs ratio of AG verses GG was 1.41(0.08-23.36; 95% CI) and the two genotypes were not differing significantly (P≤0.05) in case-control animals.

Pant *et al*., (2011) reported one SNP in IL12RB2 (c.-511A>G) associated with the MAP infection status in resource population of 446 dairy Holsteins.

SLC11A1

The SNP (**rs109604477)** locus in SLC11A1 revealed the ODDs ratio of C verses T was 0.70(0.38-1.31; 95% CI), where as ODDs ratio of CT verses TT was <0.001(<0.001 to

>999.9>; 95% CI) . The genotype frequencies observed at rs109604477 were significant ($p \leq 0.01$). The odds of CT genotype verses TT genotype was about zero because the TT genotype was exclusively present in Bovine Tuberculosis positive animals (6 out of 35 animals). It suggested that selection against TT homozygote at this locus will reduce the Bovine Tuberculosis cases in cattle

The SNP (**rs109915208)** locus in SLC11A1 revealed the ODDs ratio of C verses T was >999.9(<0.001->999.999; 95% CI), where as ODDs ratio of CC verses CT was 999.9 (<0.001->999.999; 95% CI). The allelic frequency of rs109915208 was significantly affecting the Bovine Tuberculosis. The odds of 'C' allele verses 'T' allele was towards infinity because all 'T' alleles were exclusively present in control population. The odds of CT genotype verses CC genotype was towards infinity because the CT genotype were exclusively present in TB positive animals which suggested that selection for heterozygotes will confer resistance against TB cases in cattle.

The SNP (**rs109453173)** locus in SLC11A1 revealed the ODDs ratio of C verses T was 0.60(0.31-1.16; 95% CI) and non-significantly differing in case animals as compared to control animals where as ODDs ratio of CC verses GG and CG verses GG were 0.44 (0.09-2.07; 95% CI) ; 0.46(0.18-1.17; 95% CI) respectively and all the three genotypes were not differing significantly ($P \leq 0.05$) in case-control animals.

Selvaraj et al.,2002 reported NRAMP1 gene polymorphisms at c.823 C/T (exon 8) associated with resistance to tuberculosis (TB). In a study in human the G > C mutation of intron 4 of NRAMP1 gene as a susceptible factor to PTB. (Qu et al., 2006). Studies at 469+14 G/C (INT4), 1465-85 G/A, and C274T polymorphisms of NRAMP1 in ethnic Russians with and without tuberculosis(N =58 and 127 respectively)none of the polymorphisms was associated with TB. (Puzyrev et al.,2002).

DC-SIGN

The SNP (**rs137338039)** locus in DC-SIGN revealed the ODDs ratio of C verses T was 2.43(1.11-5.34; 95% CI) and non-significantly differing in case animals as compared to control animals where as ODDs ratio of CC verses TT and CT verses TT were 5.84(0.56 -60.03; 95% CI) ; 2.30(0.86-6.10; 95% CI) respectively and all the three genotypes were not differing significantly ($P \leq 0.05$) in case-control animals.

The SNP (**rs208436798)** locus in DC-SIGN revealed the ODDs ratio of C verses T was 0.44(0.15-1.30; 95% CI) and non-significantly differing in case animals as compared to

control animals where as ODDs ratio of CC verses TT and CT verses TT were 0.63(0.11 -3.33; 95% CI) and >999.999(<0.001->999.999; 95% CI) respectively and all the three genotypes were not differing significantly ($P \leq 0.05$) in case-control animals.

Comparison of DC-SIGN gene polymorphism in patients with tuberculosis and healthy controls revealed no significant differences in loci -336A/ G and -871A/G (Ogarkov *et al.,*2007). Barreiro *et al.,* 2005 tested whether polymorphisms in CD209, the gene encoding DC-SIGN, are associated with susceptibility to tuberculosis through sequencing and genotyping analyses in a South African cohort and observed an association between two CD209 promoter variants (-871G and -336A) and decreased risk of developing tuberculosis.

The linkage disequilibrium analysis revealed that the significantly linked loci/SNPs were rs41256732 with rs134537150 and rs132856935 ; rs134537150 with rs132856935 and rs208436798 ; rs42497589 with rs137338039 ; rs132856935 with rs211644228 and rs137338039 ; rs137338039 with rs208436798. All other loci/ SNPs of investigation were having non-significant LD suggested that there was no linkage between alleles of these loci. But in our investigation only two loci/SNPs i.e rs109604477 and rs109915208 in SLC11A1 were significantly associated with Bovine Tuberculosis and were not having significant LD. It suggested that the alleles at these loci were segregating independently with each other and chances of getting haplotypes was rare.

6 Summary

The genus Mycobacterium comprises M. *tuberculosis*, M. *africanum*, M. *canettii*, M. *pinnipedii*, M. *microti*, M. *bovis* and M. *bovis* subspecies *caprae*. However, M. *bovis* is the most universal pathogen among mycobacteria and affects many vertebrate animals of all age groups. In developing countries, however, such as in 46% of African, 44% of Asian and 35% of the South American and the Caribbean countries, sporadic occurrences and enzootic occurrences of bTB have been reported. The bTB has significant public health importance, apart from being the most important disease with serious effects on animal production. Generally treatment for bTB is not recommended in animals since there is no cost effective treatment. The socioeconomic condition of people in India and prevailing religious customs prevent the slaughtering of affected cows make bTB much difficult to eradicate. Therefore, there is an urgent need to develop some alternative strategies to combat infectious diseases. An ideal approach to the control of the infectious diseases in animals is the development of genetic resistance. Candidate gene approach can serve as a useful tool in delineating the underlying genetic mechanism against disease resistance. Some of the candidate genes having role in resistance /susceptibility to infectious diseases are SLC11A1, IL12RB1, IL12RB2, IL23R, DC-SIGN and SP110. Solute like carrier family 11 A1 (SLC11A1) also known as NRAMP1 (Natural resistance-associated macrophage protein 1). The NRAMP1 gene mediates activity of macrophages against intracellular parasites during the early stages of infection and affects the intraphagosomal microbial replication. Interleukin 12 receptor, beta 1 is a subunit of the interleukin 12 receptor. The coexpression of this and IL12RB2 proteins lead to the formation of high-affinity IL12 binding sites and reconstitution of IL12 dependent signaling. The lack of expression of this gene was found to result in the immunodeficiency of patients with severe mycobacterial and Salmonella infections. The beta 2 subunit of the interleukin (IL)-12 receptor (IL-12R

beta 2) play an essential role in differentiation of T helper 1 (Th1) cells in the murine and human system. Interleukin 23 receptor is a type I cytokine receptor and required for IL23A signaling. Dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) C-type lectin is almost exclusively expressed at the cell surface of DC. In addition to its normal function facilitating contact of DC with T cells, DC-SIGN bind a variety of pathogens, including *Mycobacterium bovis*. SP110 play an important role in preventing tuberculosis by mediating control of M tuberculosis within its prime target cell, the macrophage. There are reported association of SNPs in SLC11A1, IL12RB1, IL12RB2, IL23R, DC-SIGN and SP110 to resistance /susceptibilty to tuberculosis in humans as well as in animals. More than 245 animals from Shree Mata Gosala, Barsana, Cattle Farm, Atta, Jalon and Akha village, Bareilly were screened for presence to bovine tuberculosis. Single Intradermal Comparative Cervical Tuberculin (SICCT) test done and increase in thickness of skin after 72 h of intradermal injection of tuberculin antigen noted to develop Case and Control resource panel as per standard protocol. Then 35 positive and 45 Negative animals were selected as case-control panel and 6ml blood collected from jugular vein in tubes containing EDTA were stored at -20 C. Promega wizard kit used to Isolate genomic DNA. The concentration was determined using Qubit Flurometer. 1 ul of genomic DNA was resolved on 1 % agarose gel stained with ethidium bromide or SYBRR Safe DNA gel stain and quantification was made by comparing the intensity of the band with the intensity of a known quantity of Lambda DNA. Only thick and without smearing were chosen for further processing.

Primers for the total 15 SNPs in these genes were designed using Oligoanalyser for amplification of the loci. Concerned amplicons were amplified under optimized PCR condition. The PCR product are resolved in 1.5% agarose gel and visualized under UV light after staining with ethidium bromide. The Restriction enzyme digestion was made at the optimized conditions and the restriction digested products were resolved in 3 % to 5 % agarose gel and visulaized under UV light after staining with ethidium bromide. Mass genotyping of all case: control resource population was done by using PCR-RFLP. The PROC ALLELE procedure of the SAS 9.3 used for the estimation of PIC, HWE and heterozygosity. The PROC LOGISTIC procedure of SAS 9.3 was used to find association of allelic and genotypic frequencies with bTB. The case-control population was genotyped by using PCR-RFLP for all the fifteen SNPs. Out of the 15 SNPs genotyped , four SNPs were monomorphic (rs210615796 in IL12RB1; rs208115312 in

IL12RB2; rs135336138 in IL12RB2; rs134731570 in CD-SIGN) and thus removed prior to analysis. For eleven SNPs, polymorphism was found suggesting the presence of these SNPs in our resource population.

At the SNPs rs41256732, rs134537150, rs42497589, rs132856935, rs110233569, rs211644228, rs109453173, rs137338039, rs208436798 the respective gene & genotype frequencies were not differing significantly in case & control population. But the gene & genotype frequencies were differing significantly in case & control population at rs109604477 and rs109915208. At rs109604477, two genotypes TT (219bp and 64bp) and CT (283bp, 219bp and 64bp) were observed. Also at rs109915208 locus, two genotypes, CC (215bp and 129bp) and CT (344bp, 215bp and 129bp) were found, while three genotypes GG (293bp and 81bp), CC (374bp) and CG (374bp, 293bp and 81bp) were observed at rs109453173 locus. A very high heterozygocity of 0.928 was observed at rs109604477 but it was moderate at rs109453173 (0.488) and very low (0.047) at rs109915208. Except the very low PIC of 0.0454 at rs109915208, moderate PIC (0.352 to 0.374) was estimated at other two SNP loci. Similar trend was seen for allelic diversity. Except for rs10960477, the population was in HW equilibrium (HWE) for the SNPs pertaining to SLC11A1 gene. The chi-square probabilities revealed that out of three SNPs from SLC11A1 gene, two had the significant association with the susceptibility to tuberculosis. At rs109604477, while the frequencies of TT and CT genotypes were 0.171 and 0.828, respectively in case population, only one genotype (CT) was observed in control population. The genotypic frequencies between case and control population differed significantly (P=0.008). The frequencies of C and T allele did not differed significantly (P=0.27). At rs109915208, while the frequencies of CC and CT genotypes were 0.918 and 0.082 respectively, in control population, only one genotype (CC) was present in case population. The differences for the genotypic frequencies between case and control population were found to be statistically significant (P=0.027). While the T and C allele had the frequency of 0.041 and 0.959 in control population and in SICCT positive animals only C allele was observed. The difference in allelic frequencies between case and control population was found significant (P=0.03). The genotypes observed at rs109604477 and rs109915208 loci showed significant association with the susceptibility to bovine tuberculosis in cattle. At rs109604477, the odds ratio (OR) of CT verses TT genotype

was close to zero suggesting that animals having TT genotype were more susceptible for bTB infection as compared to CT genotype. Similarly at rs109604477 locus OR of C allele verses T allele was 0.70 (0.38-1.31; 95 % CI), however chi-square probability revealed non-significant (P=0.27) effect of allele on susceptibility to tuberculosis. At rs109915208, the OR of CC verses CT genotype was approaching towards infinity suggesting that animals having CT genotype were less susceptible for bTB infection as compared to CC genotype. The chi-square probability revealed significant effect (P=0.03) of allele at rs109915208 locus on susceptibility to tuberculosis. The OR of C allele verses T allele was approaching towards infinity, suggesting the association of T allele with resistance to bTB as compared to C allele. Both of the SNPs were non-synonymous causing an amino acid change. While the mutation of C to T causes change of Isoleucine to Threonine at rs109604477. These changes of amino acids may have some role in susceptibility/resistance of animals for bovine tuberculosis.

Taken altogether results it could be concluded that SNPs of SLC11A1 gene had significant role for susceptibility to bovine tuberculosis but other genes can't be excluded as having no role for susceptibility to bovine tuberculosis. These findings need further validation of markers on larger population by including few more confirmatory diagnostic tests.

7 Mini Abstract

In present study polymorphisms at total fifteen SNPs in six genes namely SLC11A1, IL12RB1, IL12RB2, IL23R, DC-SIGN and SP110 were investigated by PCR-RFLP method for finding their association with susceptibility to bovine tuberculosis (bTB) in cattle. Total 245 animals were tested with single intradermal comparative cervical tuberculin test (SICCT) for finding positive animals for tuberculosis. The statistical analysis revealed two SNPs (rs109915208 and rs109604477 in SLC11A1) were significantly associated to tuberculosis. At rs109915208 locus very low PIC of 0.0454 was observed whereas, moderate PIC (0.352 to 0.374) was estimated at rs109604477. At rs109915208 locus the genotypic as well as allelic frequencies were differing significantly ($P<0.05$) in case-control animals where the OR of CC *verses* CT genotype and OR of C *verses* T allele was approaching towards infinity suggesting that animals having CT genotype and T allele were less susceptible for bTB as compared to their contemporary genotype/allele. But, at rs109604477 locus animals with TT genotype were found to be significantly ($P=0.008$) more susceptible for bTB infection than the animals with CT genotypes. Both of the significantly associated SNPs were non-synonymous causing an amino acid change.

अर्थात् छह जीन SLC11A1, IL12RB1, IL12RB2, IL23R, DC-SIGN और SP110 में कुल पंद्रह SNPs पर वर्तमान अध्ययन बहुरूपताओं में, पीसीआर RFLP विधि से जांच की गई. कुल 245 पशुओं तपेदिक के लिए सकारात्मक जानवरों को खोजने के लिए एक त्वचा के अंदर तुलनात्मक ग्रीवा टुबरकुलीन परीक्षण (SICCT) के साथ परीक्षण किया गया. सांख्यिकीय विश्लेषण थे पता चला दो SNPs (SLC11A1 में rs109915208 और rs109604477) काफी तपेदिक तक जुड़े. rs109915208 ठिकाना पर PIC बहुत कम (.0454) मध्यम PIC (0.352-0.374) rs109604477 में अनुमान लगाया गया था. rs109915208 ठिकाना पर genotypic के साथ ही allelic आवृत्तियों में काफी (पी <0.05) भिन्न थे मामला नियंत्रण पशुओं में जबकि OR सीसी तुलना सीटी जीनोटाइप की और OR सी तुलना टी एलील अनंत की ओर आ रहा था उनके समकालीन की तुलना में सुझाव है कि सीटी जीनोटाइप और टी एलील होने जानवरों बीटीबी के लिए कम अतिसंवेदनशील थे. लेकिन, rs109604477 ठिकाना पर सीटी जीनोटाइप के साथ जानवरों के तुलना टीटी जीनोटाइप के साथ (पी = 0.008) काफी बीटीबी के लिए अतिसंवेदनशील पाए गए. दोनों काफी जुड़े SNPs एमिनो एसिड परिवर्तन हो रहा था.

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APPENDIX-I

Chemicals/Equipments/Lab wares/ miscellaneous items

Chemicals	**Source**
Agarose (low EEO)	Himedia
Boric acid	SRL, Qualigens
Bromophenol blue	Hi-media
dNTPs (dATP, dGTP, dTTP, dCTP)	Promega
EDTA	Analytical Rasayam, SRL
Ethanol	Bengal Chemicals
Ethidium bromide	Sigma, SRL
Isopropanol	Qualigens, SRL
Magnesium chloride	
Proteinase-K	Promega
Potassium bicarbonate	Merck, Himedia
Tris base	Promega, Amresco, Qualigens
Taq DNA polymerase	Promega
Taq DNA polymerase buffer	Promega
Tris buffer	Promega
Ladder DNA marker-100 bp Ladder DNA marker-50 bp Ladder DNA marker-20 bp Lambda DNA ladder	Promega Promega Promega
Oligonucleotide primers	IDT
Xylene cyanol	Hi-media
Equipments	**Sources**
Autoclave	Universal
Centrifuge R8C	REMI, Jouan

Refridgerated centrifuge	REMI
Deep Freezer (-20°C)	Vestfrost
Distillation plant	Scientronic instruments
Gel documentation system	Syngene
Horizontal gel electrophoresis	Bangalore genei
Ice box	Torson
Ice flaking machine	Scotsman
Incubator	S. P. Scientronic instruments
Magnetic stirrer	Corning
Micro-centrifuge	Microspin FV-2400
Micropiptte (all ranges)	Eppendorf
pH meter	Tunco
Power pack	Bangalore genei, Tarson
Refrigerator	Godrej, LG
Spectrophotometer	Beckman
Thermal cycler	Bio Rad, DNA engine
UV transiluminator	Syngene
Vortexer	Yorco
Water bath	York Scientific
Weighing balance (Digital)	Sartorious
Microwave Oven	LG
Lab wares	
3.1 Glassware	
Beakers Conical flasks Measuring cylinders Pasteur pipettes 10 ml pipettes Reagent bottles	Borosil

3.2 Plastic wares	
Polypropylene centrifuge tube (15 ml and 50 ml)	Tarson
Eppendorf tube (1.5 ml and 0.5 ml)	Axygen USA
PCR tube (0.2 ml)	Axyxen USA
Micro tips (All ranges)	Axygen USA

(4) Miscellaneous items

Micellaneous items	
Adhesive tapes	Needles
Aluminum foils	Para film
Forceps	Marker pens
Disposable gloves	Papers
Cotton	Threads
Scissors	Cello-tape
Blotting paper	Thermometers
Disposable Syringe	Razor blades

Wizard® Genomic DNA Purification Kit (Promega) 500 isolations

Each system contains sufficient reagents for 500 isolations of genomic DNA from **300µl** of whole blood samples. Includes:

- 500ml Cell Lysis Solution
- 250ml Nuclei Lysis Solution
- 125ml Protein Precipitation Solution
- 100ml DNA Rehydration Solution
- 1.25ml RNase Solution

1. 0.5M EDTA solution (pH 8.0)

EDTA disodium salt	18.61 gm
Double distilled water (up to)	100 ml

Dissolve EDTA in about 80 ml of autoclaved distilled water by keeping it on magnetic stirrer for one hour, and then adjust the pH to 8.0 by NaOH pellets before making to final volume. Autoclave and store at room temperature.

2. RBC Lysis Buffer

Ammonium chloride (NH_4Cl)	8.3 gm
Potassium bicarbonate ($KHCO_3$)	1.0 gm
0.5 M EDTA (pH 8.0)	299ml
Autoclaved distilled water (up to)	1000 ml

Stored at room temperature

3. 70% ethanol

Ethanol	70 ml

Distilled water	30 ml

Mix thoroughly and store in amber colored bottle at 4^0C.

4. 10X TBE

Tris base	108 gm
Boric acid	55 gm
0.5M EDTA (pH 8.0)	40.0 ml
Distilled water (up to)	1000 ml

Autoclaved and stored at room temperature.

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5. 20,000X Ethidium bromide (10 mg/ml)

Ethidium bromide	10 mg
Distilled water	1 ml

Wrap in aluminum foil and store in dark place at room temperature.

1. Gel loading Dye (6X)

a) Type I

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Sucrose in water	40 % (w/v)

Mix and store at 4°C.

b) Type IV

Bromophenol blue	0.25%
Sucrose in water	40% (w/v)

Mixed and stored at 4^0C.

Enzymes and Biological

1. Proteinase-K (20 mg/ml)

Proteinase-K	20 mg
Autoclaved distilled water	1 ml

Store at -20^0C. Proteinase-K is highly active protease of the subtillisin type that is purified from the mold *Tritirachum album* Limber, which has the function of digesting the proteins.

2. *Taq* DNA polymerase enzyme

Taq DNA polymerase	5 U/µl

Store at -20^0C.

3. Primers Primer
Working solution concentration 10 ng/ml
Store at –20°C.

Stock primers are supplied in lyophilized condition. The stock primers are dissolved in nuclease free water to give the concentration of100pM/µl in the stock solution. The working concentration of primers required for amplification is 10 pM/µl. The working solution of primers is prepared by taking 10 µl of stock solution and 90 µl of nuclease free water.

4. 100 bp Ladder DNA

It contains 10 bands of double stranded DNA fragments ranging from 100 to 1000 bp.

5. 50bp Ladder DNA

It contains 17 bands of dsDNA fragments ranging from 50 to 1350bp.

6. Lambda DNA

7. 10 X *Taq* DNA buffer

Tris HCl (pH 8.8)	100 mM
KCl	500 mM
$MgCl_2$	15 mM
Triton X-100	1%

Store at -20^0C.

8. dNTPs mix solution

The mixture contains all the dNTPs (dATP, dGTP, dTTP, dCTP) each in the concentration of 100 mM.

9. Wizard® Genomic DNA Purification Kit (Promega) 500 isolations

Each system contains sufficient reagents for 500 isolations of genomic DNA from 300µl of whole blood samples. Includes:

- 500ml Cell Lysis Solution
- 250ml Nuclei Lysis Solution
- 125ml Protein Precipitation Solution
- 100ml DNA Rehydration Solution
- 1.25ml RNase Solution

Curriculum Vitae

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- Graduated (B.V.Sc & A.H) from College of veterinary Science & Animal Husbandry, SKUAST-K .
- Recipient of ICAR fellowship.