GENOTYPING OF 3' UTR OF THE *SLC11A1* GENE IN PANDHARPURI BREED OF BUFFALO (*BUBALUS BUBALIS*) AND STUDY ITS RELATIONSHIP WITH RESISTANCE AGAINST BRUCELLOSIS.

THESIS

Submitted
in partial fulfillment of the requirements for the Degree of

MASTER OF VETERINARY SCIENCE

IN

ANIMAL BIOTECHNOLOGY

BY
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Enrolment No : V/04/0199

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NAGPUR- 440 001
(INDIA)
2012

PGR - ANNEXURE – XIII
DECLARATION OF STUDENT

I hereby declare that the experimental research work and interpretation of the thesis entitled, **“Genotyping of 3' UTR of the *SLC11A1* gene in Pandharpuri breed of buffalo (*Bubalus bubalis*) and study its relationship with resistance against brucellosis.”** or part thereof has not been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis/publication of any University or scientific organization. The sources of materials used and all assistance received during the course of investigation have been duly acknowledged.

Date : / / 2012 **Signature**

Chaudhari Bhushan Subhash
Enrolment No: V/04/0199

Counter signed by

Dr. A. S. Bannalikar
Chairman, Advisory Committee,
and Professor of Microbiology,
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PGR - ANNEXURE - XIV

Declaration of Advisory Committee

MR. CHAUDHARI BHUSHAN SUBHASH has satisfactorily prosecuted her course of research for a period of not less than one semester and that the thesis entitled, **"Genotyping of 3' UTR of the *SLC11A1* gene in Pandharpuri breed of buffalo (*Bubalus bubalis*) and study its relationship with resistance against brucellosis"** submitted by him is the result of research work sufficient to warrant its presentation to the examination in the subject of **ANIMAL BIOTECHNOLOGY** for the award of **MASTER OF VETERINARY SCIENCE (M.V.Sc)** degree by the Maharashtra Animal and Fishery Sciences University, Nagpur.

We also certify that the thesis or part thereof has not been previously submitted by him for a degree of any other University.

Place: Mumbai

Date : / / 2012

Signature

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Professor
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Advisory Committee

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PGR - ANNEXURE - XV

CERTIFICATE

This is to certify that the thesis entitled, **"Genotyping of 3' UTR of the *SLC11A1* gene in Pandharpuri breed of buffalo (*Bubalus bubalis*) and study its relationship with resistance against brucellosis."** submitted by **Mr. Chaudhari Bhushan Subhash** to the Maharashtra Animal and Fishery Sciences University in partial fulfillment of the requirement for the degree of **MASTER OF VETERINARY SCIENCE** in **ANIMAL BIOTECHNOLOGY** has been approved by the Student's Advisory Committee after examination in collaboration with the External Examiner.

Name & Signature of **External Examiner**	Signature with Seal **Head of the Department**	**Dr. A. S. Bannalikar** **Chairman, Advisory Committee,** **and Professor of Microbiology**

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Dean/ Associate Dean
Dr. A. M. Paturkar

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LIST OF ABBREVIATIONS

% : Per cent
µl : Microliter
^{0}C : Degree Celsius
µg : Microgram (s)
µm : Micrometer
AGE : Agarose gel electrophoresis
BLAST : Basic local alignment search tool
bp : Base pair
C:I : Chloroform - isoamyl alcohol
CTAB : Cetyl trimethylammonium bromide
Conc. : Concentrated
DNA : Deoxyribonucleic acid
DW : Distilled water
dNTPs : Deoxynucleotide triphosphates
EDTA : Ethylene diamine tetra acetate
EtOH : Ethyl alcohol
et al. *: et alii*
gm : Gram
h : Hour
I.U. : International unit
lbs : Pound
M : Molar
Mb : Mega base pairs
mg : Milligram

min	: Minute (s)
ml	: Millilitre
mM	: Millimolar
NCBI	: National Center for Biotechnology Information
OD	: Optical density
P:C:I	: Phenol – chloroform - isoamyl alcohol
PCR	: Polymerase chain reaction
pg	: Picogram
pH	: POTENTIAL OF HYDROGEN
Psi	: Pound-force per square inch
pmole	: Picomole (s)
RBC	: Red blood cells
RBPT	: Rose bengal plate test
RNA	: Ribonucleic acid
rpm	: Rotation per minute
SDS	: Sodium dodecyl sulphate
TE	: Tris-EDTA Buffer
sec	: Seconds
STAT	: Standard tube agglutination test
Taq	*: Thermus aquaticus*
TAE	: Tris acetate EDTA buffer
U	: Unit
UV	: Ultra violet
V	: Volts
viz	: Namely
v/v	: Volume by volume
w/v	: Weight by volume

ACKNOWLEDGEMENT

This thesis has been kept me on track and been seen through to completion with the support and encouragement of numerous people including my well wishers, my friends, colleagues. At the end of my thesis I would like to thank all those people who made this thesis possible and an unforgettable experience for me.

It would not have been possible to work and write this thesis without the help, guidance, support and unflinching encouragement from my guide **Dr. AnilKumar S. Bannalikar**, Professor, Dept of Microbiology, Bombay veterinary College. Under his guidance I successfully overcame many difficulties and learned a lot. His truly scientist intuition has made him as a constant oasis of ideas and passions in science, which exceptionally inspire and enrich my growth as a student and a researcher. I could not have imagined having a better advisor and mentor for my M.V.Sc. study.

Needless to mention that Dr. Aakash Doiphode, Assistant Professor, Dept of Animal Genetics and Breeding, Bombay Veterinary College, who had been a source of inspiration, for his timely guidance, help in the completion and valuable assistance in the project work.

I would like to put into words my appreciation for Dr. A. M. Paturkar, Dean, Bombay veterinary college for providing facilities for the research in animal biotechnology cell, Department of Microbiology.

I would also like to extend my thanks to Dr. (Mrs) S. B. Majee, Associate Professor of Microbiology for her constructive suggestions and guidance during the post-graduate academic period.

I would like to thank Dr. P. P. Mhase and Dr. R. R. Pharande, Assistant professor of Microbiology for providing me encouragement, inspiration, valuable suggestions during my research work.

I thanks to Dr. (Mrs) R.S. Gandge, Associate professor, Department of Microbiology for extending her full co-operation, knowledgeable suggestions during my thesis work.

My heartful thanks to Dr. S. D. Jagdale and Dr. S. P. Londhe. I would like to thank them for being persons who always helped and supported me altruistically.

I thank all the staff of the Department Dr. Ram Dhage, Mr. Ashish Nandgaonkar, Mr. Suresh Revale mama, Mr. Arvind Dalvi mama, Mr. chetan, Mr. Patlekar who helped me during my laboratory work.

I would also like to thank Dr. B. R. Kadam and Dr. (Mrs) Nimbalkar for providing the facilities and permission in KNPVC farm.

It's my fortune to gratefully acknowledge the support of some special friends. Words fail me to express my appreciation to Dr(s) Kaustubh, Sneha, Sumit, Kiran and Rutuja. They are always beside me during my happy and hard moments to push me and motivate me.

I feel immense pleasure in expressing my sincere thanks to Dr. Laxman D. Pawar, Veterinary surgeon. Who helped, taught lots of things and gave freedom to work in clinic from last four years.

I am very thankful to Mr. Karpe, Kolhapur polyclinic and staff from KNPVC, shirwal farm who kindly helped me during collection of blood.

I thank to my fellow lab mates Dr(s) Mahesh, Priyanka, Jyotsana for their encouragement and assistance during the last two years.

Words cannot express my humble feelings to my close friends Dr(s) Manjiri, Zen, Mithila, Amit, and Prerna for their love, constant encouragement; co-operation and caring always kept me on the go.

I am indebted to my friends of my Graduation studies Dr(s) Amol, Himanshu, Akshada, Prathmesh, Deepti, Anushri, Anushree and Mukul for providing a stimulating and fun filled environment.

I have reserved my whole hearted thanks for my seniors in the department Dr(s) Mayura, Dhananjay, Sneha and Apeksha for their constant help and support.

I thank my juniors cum friends Dr(s) Mangesh, Sagar, Swapnil, Sudershan, Sushama and Charu for their great help, friendly advice, besides immense caring and understanding and giving moral support in my thesis work.

Lastly I would like to thamk my seniours cum friends Dr. Kiran Shelar and Dr. Pratik Pawar for their care and guidance in under and post graduation studies.

Last but not least, I would like to thank my parents Papa and Aai for raising me with great care and love. Their affection and prayers would continue to support me throughout my life. I would like to put across my heartiest and inexplicable gratitude to younger brother Tejas for his support & encouragement and whose smiles always make me laugh.

During my stay in the college, I was fortunate enough to receive the kind co- operation from almost everyone. In the end, I desire to convey my sincere thanks to everyone, who is directly and indirectly responsible to complete this bit of work. It is extremely difficult to thank all of them individually by name. This shortcoming may be pardoned.

Place: Mumbai
Date: July 2012 *(Dr. Bhushan Subhash Chaudhari)*

1. INTRODUCTION

Brucella are facultative intracellular pathogens that infect a wide variety of animal species and humans. Bovine brucellosis caused frequently by *Brucella abortus* and sometimes by *B. melitensis*, is characterized by abortion in pregnant cows, sterility in bulls and undulant fever in humans (Chahota *et al*., 2003). The disease has serious socio-economic implications since it attributes to enormous economic losses by reducing the productive and reproductive potential of animal and suffering in both animals and humans. Although, the disease has been eradicated from some of the developed countries viz. Australia, Canada, Cyprus, Denmark, Finland, Netherlands, New Zealand, Norway, Sweden and the United Kingdom, it is widely prevalent in most parts of the world with a very high prevalence in Middle East, Latin America and Asia. Brucellosis is the most common anthropozoonosis, with more than five million cases reported annually worldwide (Papas *et al.,* 2006).

Many developed countries have successfully controlled and eradicated brucellosis in livestock species by adopting a strict test and slaughter policy, however, the disease continues to be the major cause of economic loss and human suffering in developing countries (Al-Shamahy, 1999). Since the policy of test and slaughter has not been adopted in India due to social, economic and religious reasons, the disease has assumed endemic proportion.

Considering the serious economic and medical consequences of brucellosis, attempts have been made to develop vaccines for its immunoprophylaxis. These were initially developed on an empirical basis, but more recently attempts are focused to prepare vaccines with rational design. The first effective vaccine against *Brucella* was the live *B. abortus* strain 19, a laboratory attenuated strain. This vaccine induces good protection, but suffers from limitation of persistent serological response. Similarly, *B. melitensis* strain Rev.1 that is effective against caprine and ovine brucellosis also has a disadvantage of prolonged serological response. The killed vaccines prepared from virulent strains administered with adjuvant, induce significant protection but are unacceptable due to levels of antibodies interfering with diagnostic tests. The problem of interference with diagnostic serology was overcome to some extent when live rough strain *B. abortus* 45/20 was explored for development of vaccine, but this reverted to virulence *in vivo*. The rifampicin-resistant mutant *B. abortus* RB51 strain has proved safe and effective in the field against bovine brucellosis and exhibits negligible interference with diagnostic serology. However, this vaccine is still not in common use.

Brucella abortus is a facultative intracellular pathogen that survives and replicates within host macrophages. Hence, macrophage function plays an important role in influencing natural resistance/susceptibility to infection by this intracellular pathogen. The natural resistance associated macrophage protein 1 (*NRAMP1*; also referred as *Ity/Lsh/Bcg*), a transmembrane protein of transporter family, regulates activity of macrophages against intracellular pathogens during the early stages of infection (Blackwell *et al.,* 1994). *NRAMP1* belongs to a family of membrane proteins defined by the presence of common hydrophobic core often transmembrane domains. It also regulates macrophage function which has resistance towards intracellular microorganisms including *Mycobacterium bovis* (*Bcg*), *Salmonella typhimurium* (*Ity*) and *Leishmania donovani* (*Lsh*).

NRAMP1 recently referred as solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1 (*SLC11A1*) (www.genecards.org), encodes a divalent cation (Fe^+ & Mn^+) transporter that localizes in phagolysosome membrane in macrophages. In cattle this gene has been associated with resistance against *Brucella abortus* infection (Feng *et al.,* 1996 and Barthel *et al.,* 2001). The role of *SLC11A1* is to prevent intracellular bacterial growth. In bovine, natural resistance to brucellosis is significantly associated with $(GT)_{13}$ allelic variant of microsatellite locus at 3' untranslated region (3' UTR) of the *NRAMP 1* / *SLC11A1* (Wyllie *et al.,* 2002; Forbes and Gros, 2003). Some experimental studies have suggested that the movement of cations may actually occur in the opposite direction, resulting in an increased concentration of iron into the phago-lysosome, which may favor bacterial killing by generating oxygen intermediates through the Fenton reaction (Zwilling *et al.,* 1999; Goswami *et al.,* 2001).

India has the distinction of possessing more than one half of the world buffalo population which contributes to more than 50% of total milk production of India and 2.03% of world meat production (FAO, 2005) earning annual foreign exchange revenue worth US$ 3122 million (APEDA, 2005). Brucellosis causes severe economic losses in buffalo and poses serious zoonotic threats (Guarino *et al*., 2000; Rathore *et al*., 2002). Although, association of natural resistance against brucellosis with polymorphisms at the bovine *NRAMP1* 3' UTR has already been established (Adams and Templeton, 1998), only a few reports are available describing polymorphic nature of 3' UTR of *NRAMP1* / *SLC11A1* in buffaloes.

A recent study on polymorphism at 3' UTR of *NRAMP1/SLC11A1* conducted in Murrah breed of buffalo (*Bubalus bubalis*) has shown existence of four allelic variants {viz., $(GT)_{13}$, $(GT)_{14}$, $(GT)_{15}$ and $(GT)_{16}$}. The $(GT)_{13}$ allele, either in homozygous $\{(GT)_{13}/(GT)_{13}\}$ or heterozygous state $\{(GT)_{13}/(GT)_n\}$, was significantly associated with increased activity of macrophages against *Brucella* suggesting its role in natural resistance against brucellosis (Ganguly *et al.,* 2008). Selection and breeding of such naturally resistant animals therefore could be an effective tool for control of brucellosis in livestock species and would be a boon to the dairy industry.

Pandharpuri is an important buffalo breed in southeast Maharashtra state of India. The breeding tract of Pandharpuri buffalo comes under scarcity zone and includes plain and sub-mountain agro-climatic zones. This breed is found in the regions of Solapur, Sangli and Kolhapur districts of Maharashtra state. The breed is famous for its high reproductive ability, producing a calf every 12 months. Under average management conditions and hot-dry climate these buffaloes yield 6-7 liters of milk per day; however under good management they are reported to yield up to 15 lit. of milk per day.

Keeping in view the above facts, the present investigation was planned to undertake genotyping of 3' UTR of the *SLC11A1* gene and study its relationship to natural resistance against brucellosis in Pandharpuri breed of buffalo.

The precise objectives of the investigation were as follows.

1. Genotyping of 3' UTR of the *SLC11A1* gene of Pandharpuri buffaloes.
2. To study the relationship of *SLC11A1* gene with resistance against brucellosis.
3. To attempt expression studies of *SLC11A1* gene by Real-Time PCR.

2. REVIEW OF LITERATURE

2.1 Brucellosis

Brucellosis, an economically important reproductive disease of livestock, is prevalent in most of the developing countries. The disease induces infertility, delayed heat, interrupted lactation, loss of calves, wool, meat and milk production and attributes to enormous economic losses to the livestock industry. Furthermore, the disease has added significance owing to its transmissibility to human beings where it causes chronic debilitating disease (WHO, 1971; Radostits *et al.*, 2008 and Corbel, 1997).

2.2 Incidence of brucellosis and its economic impact

Brucellosis is a widespread disease of economic importance affecting animals all over the world. The economic losses associated with brucellosis are primarily due to its adverse effects on the reproductive and productive potential of the affected animals, loss of calves, sterility, infertility, as well as reduction or complete loss of milk yield after the abortion (Yadav, 1982). Furthermore, the disease carries added significance owing to its transmissibility to human beings where it causes a serious, chronic debilitating disease with persistent bacteraemia resulting loss of valuable man days. The disease has been reported to be responsible for economic losses to the tune of 60 million dollars in Unites States (Cottorello *et al.,* 2002) while in India the disease causes an estimated economic loss of over 300 million Rupees annually.

Serological studies conducted in India indicate that the disease is highly endemic in many parts of the country including Assam (Hussain *et al.,* 1987), Uttar Pradesh and Delhi (Sharma *et al.,* 1984), Himachal Pradesh (Vaid *et al*., 1991) & Andhra Pradesh (Mrunalini *et al.,* 2000), Bombay (Das *et al.,* 1990), Uttaranchal (Jain *et al.* 2006), Madhya Pradesh (Mehra *et al*., 2000). The disease has been found to be prevalent in different species of domestic animals including Cattle (Chandramohan *et al*., 1992 and Bala *et al.,* 1982); Sheep (Jain *et al.,* 2006) and also reported in humans. Perusal of the available literature indicates prevalence of the disease on the basis of serological detection of antibodies ranges from 0.3 percent to 56 percent (Jain *et al*., 2006).

2.3 Genus Brucella

The *Brucella* are small, nonmotile, nonsporulating, noncapsulated, non toxigenic, non fermenting, Gram-negative coccobacilli (Salehi *et al.,* 2006). They are partially acid-fast since they do not get decolorized by 0.5% acetic acid in the modified Ziehl-Neelsen (MZN) stain. The organisms retain carbol fuschin in MZN staining and appear red-stained coccobacilli (Alton *et al.,* 1988). The organisms are aerobic and carboxyphilic and their growth is enhanced by 5-10% CO_2. Most *Brucella* species react positively in catalase, oxidase and urease tests, with the exception of *B. ovis*, which is oxidase and urease negative and *B. neotomae*, which is oxidase negative (Yagupsky *et al*., 2005). The *Brucella* are related antigenically to the members of several other genera *viz. Agrobacterium*, *Ochrobactrum*, *Phyllobacterium* and *Rhizobium* (Corbel, 1997), *Yersinia enterocolitica* (Kittelberger, 1995)*, Escherichia coli* (Imaoka *et al.,* 2007). Taxonomically the genus *Brucella* is placed under family *Brucellaceae* order Rhizobiales, class Alphaproteobacteria.

2.4 *Brucella abortus* Infection

B. abortus is an intracellular pathogen that causes chronic infections with persistent or recurrent bacteraemia in several species of ruminants and humans. The organism has a marked predilection for the gravid uterus of cattle. An important step in the pathogenesis of *Brucella* placentitis is the invasion of trophoblastic epithelium. Studies carried out in caprine model indicate that bacteremic *B. abortus* cells enter and replicate within erythrophagocytic trophoblasts and then colonize the chorioallantoic trophobolastic epithelium. Marked intracellular replication of *B. abortus* kills trophoblasts and the organisms are shed into surrounding tissues and fluids. The cycle of cell invasion, intracellular replication and cell death continues, leading to placentitis and abortion (Anderson *et al*., 1986). The spontaneous abortions induced by *B. abortus* in cattle are a cause of serious economic loss to dairy farms. Human beings often acquire infection through contact with infected animals or products contaminated with these bacteria. Brucellosis can cause a range of symptoms in humans that are similar to flu and may include fever, sweats, headaches, arthritis, back pains and physical weakness. Severe infections of the central nervous systems or lining of the heart may also occur resulting in meningitis, spondylitis, endocarditis or even death. Brucellosis can also cause long-lasting or chronic symptoms that include recurrent fevers, joint pain and fatigue. Bacteriologic culture and serological agglutination tests are used for the diagnosis of infected humans and animals (Young, 2000).

2.5 Association of *SLC11A1* 3'UTR in natural resistance against brucellosis

Brucella abortus is a facultative intracellular pathogen that survives and replicates in host macrophages. The macrophage function thus plays an important role in influencing natural resistance/susceptibilIty to infection by this intracellular pathogen. The natural resistance associated macrophage protein 1 (*NRAMP1* also referred as *Ity/Lsh/Bcg* and recently refereed as *SLC11A1*), a transmembrane protein of transporter family, regulates activity of macrophages against intracellular pathogens during the early stages of infection (Blackwell *et al*., 1994). After phagocytosis, *NRAMP1* is targeted to the membrane of the microbe-containing phagosome, where it modifies the intra-phagosomal milieu affecting microbial infection (Gruenheid *et al*., 1997). Though, physiological role of *NRAMP1* is not yet clear, it plays a significant role in inhibiting bacterial growth, production of reactive oxygen and nitrogen products, enhancing phagolysosomal fusion and regulation of cytokine production. Studies in mice have shown that a point mutation of G169D (Vidal *et al*., 1993) in *NRAMP1* gene confers resistance towards a number of antigenically different intracellular microorganisms including *Mycobacterium bovis* (Bacille Calmette-Guerin, *Bcg*), *Salmonella typhimurium* (*Ity*) and *Leishmania donovani* (*Lsh*) (Bradley *et al*., 1979; Gros *et al*., 1981; Plant and Glynn, 1974 and Skamene *et al*., 1982).

The macrophages from naturally resistant cattle, when challenged *in vitro* with virulent *B. abortus* S2308, exhibited superior ability to restrict the intracellular replication of *B. abortus* than those from susceptible cattle (Price *et al*., 1990; Qureshi *et al*., 1996; Templeton *et al*., 1990). Single-stranded conformational analysis (SSCA) revealed a highly significant ($P<0.0089$) association of a polymorphic $(GT)_n$ microsatellite (where n is 13, 14, 15, or 16) located in the 3' untranslated region (3' UTR) of bovine *NRAMP1* with natural resistance to brucellosis (Adams and Templeton, 1998). A $(GT)_{13}$ microsatellite allele at 3' UTR of *NRAMP1* gene has been reported to provide resistance.

Table 2.1: Microsatellite polymorphism of 3' UTR of *SLC11A1*

species	**Primer Sequence (5'-3') (Forward primer/Reverse primer)**	**Product Size (bp)**	**Reference**
Cattle	NRAMPF: GTGGAATGAGTGGGCACAGT NRAMPR: CTCTCCGTCTTGCTGTGCAT	210	Horin *et al.* (1999)
Cattle	AM1: AAGGCAGCAAGACGGAGGAGG AM2: ATGGAACTCACGTTGGCTG	174	Kumar *et al.* (2005)
Cattle	F: AAGGCAGCAAGACAGACAGG R: ATGGAACTCACGTTGGCTG	175	Paixao *et al.* (2006)
Buffalo	AM3F: TGTGTGTGTGAAGGCAGCA AM2R: ATGGAACTCACGTTGGCTG	193	Ganguly *et al.* (2008)
Cattle	F: GGAAGCTGTGGGCCTTCAC R: ATGCAGGAAGTCATCGGCAG	440	Ranjan *et al.* (2011)

In an *in vitro* study, susceptible murine macrophage cell line (RAW264.7) was transfected with two different gene constructs containing either $(GT)_{13}$ or $(GT)_{16}$ allele under bovine *NRAMP1* promoter. On challenge with infectious strain of *B. abortus*, cell lines transfected with $(GT)_{13}$ allele demonstrated more expression of *NRAMP1* protein and restrictive replication of *Brucella* than those transfected with $(GT)_{16}$ allele (Barthel *et al*., 2001).

A few studies have been carried out in the recent past to study the polymorphism of 3' UTR of *SLC11A1* in cattle and buffaloes in India and its relationship with natural resistance to brucellosis (Table 2.1)

Price *et al.* (1990) studied the activity of macrophages against intracellular *B. abortus*. The study was conducted 2 groups of animals. Group A comprised 11 naturally resistant and 10 chronically infected susceptible cows challenged with *Brucella abortus* whereas group B included 12 heifers (mammary macrophages) and 22 bulls (blood monocyte-derived macrophages). The results revealed that the macrophages from naturally resistant cattle were significantly superior in controlling the *in vitro* intracellular replication of *B. abortus.*

Vidal *et al*. (1993) suggested that the natural resistance to infection with intracellular parasites was controlled by a dominant gene on mouse chromosome 1, called *Bcg, Lsh* or *Ity*. *Bcg* affected the capacity of macrophages to destroy ingested intracellular parasites early during infection. A 400 kb bacteriophage and cosmid contig within the genomic interval containing *Bcg* was assembled. A search for transcription units by exon amplification identified six novel genes in this contig. RNA expression studies showed that one of them, designated *Nramp*, was expressed exclusively in macrophage populations from reticuloendothelial organs and in the macrophage line J774A. *Nramp* encoded an integral membrane protein that had structural homology with known prokaryotic and eukaryotic transport systems, suggesting a macrophage-specific membrane transport function. Susceptibility to infection (Bcg^{s}) in 13 Bcg^{r} and Bcg^{s} strains tested was associated with a non conservative Gly-105 to Asp-105 substitution within predicted transmembrane domain 2 of *Nramp*.

Canonne *et al*. (1995) suggested that the susceptibility to infectious diseases was under genetic control in humans. Animal models provided an ideal tool to study the genetic component of susceptibility and to identify candidate genes that could be tested for association or linkage studies in human populations from endemic areas of disease. The *NRAMP1* gene was isolated by positional cloning the host resistance locus *Bcg*/*Ity*/*Lsh*, and mutations at this locus impaired the resistance of mice to infections with intracellular parasites, such as *Salmonella*, *Leishmania* and *Mycobacterium*.

Qureshi *et al.* (1996) isolated macrophages from cattle resistant or susceptible to brucellosis and determined their bactericidal activities against *B. abortus*, *M. bovis BCG*, *S. Dublin* and *S. Typhimurium.* The animals were characterised as resistant (n=12) or susceptible (n=22) based on recovery from an *in vivo* challenge with the *B. abortus* strain 2308 inoculated conjunctively at dose of 10^7 colony forming units (CFU). Based on the intracellular survival of *B. abortus* the animals were classified as susceptible (14) and resistant (7).

Barthel *et al.* (2001) used an *in vitro* model, murine RAW264.7 macrophage cell line (Bcg^s) to analyse the regulation of *NRAMP1* gene and its role in macrophage function. It was observed that, a polymorphism within a microsatellite in the 3' untranslated region critically affected the expression of bovine *NRAMP1* and the control of *in vitro* replication of *Brucella abortus* but not of *Salmonella enterica* serovar Dublin.

Kumar *et al.* (2005) carried out experiment on 150 *B. indicus* and *B. indicus* X *B. taurus* cattle. Out of 150 animals, 100 animals were selected randomly and subjected to sequencing studies for the presence of $(GT)_{13}$ genotype. They observed that all the animals belonged to same $(GT)_{13}$ homozygous genotype and found that this genotype did not provide enough resistance against brucellosis in naturally infected herd.

Borriello *et al.* (2006) tested 231 water buffalo cows for the presence of anti- *Brucella abortus* antibodies (by agglutination and complement fixation tests) and the *NRAMP1* genotype (by PCR-denaturing gradient gel electrophoresis). The 31% seropositive animals were of *NRAMP1* A^+ $(GT)_{33}$ genotype while 9.4% seropositive were from *NRAMP1*BB $(GT)_{36}$ genotype. B allele was associated with resistance to *B. abortus* infection. The monocytes from animals with BB $(GT)_{36}$ genotype were tested for their activity against three *Brucella* species viz., *B. melitensis*, *B. suis*, *B. abortus*. It was observed that Monocytes from BB animals had an ability to control the intracellular growth of *Brucella*.

Paixao *et al.* (2006) carried out molecular characterization of 3' UTR of *NRAMP1* of 248 pure bred cattle including Holstein and Zebu. The cattle from genetically resistant group yielded a single band of 175 bp with 13 GT pairs. However, the cattle from susceptible group showed either a single band of 177 bp, or two bands of 175 and 177 bp, or two bands of 175 and 179 bp. Out of all the animals investigated, 53.6% of cattle showed a homozygous GT_{13} genotype.

Capparelli *et al.* (2007) studied the association of *NRAMP1* with resistance to the intracellular *B. abortus* in water buffaloes. Using denaturing gradient gel electrophoresis (DGGE) 2 alleles, *NRAMP1* A and *NRAMP1* B were found. Monocytes and macrophages from animals of both the genotypes were infected *in vitro* with *B. abortus*. The monocytes from animals with genotype *NRAMP1* AA showed higher of CFU ($p<0.0001$) and were considered susceptible whereas those from animals with *NRAMP1* BB showed lower CFU ($p=0.0047$) suggesting resistance to *B. abortus* infection.

Capparelli *et al.* (2007a) tested 413 water buffalos for presence of anti *Brucella* antibodies and *NRAMP1* genotype by capillary electrophoresis. They found the BB homozygous genotype in 271 animals were serologically negative and resistant to brucellosis. Exposure of these to *B. abortus* did not produce the infection. The monocytes from the BB (resistant) animals displayed a higher basal level of *NRAMP1* mRNA and a lower number of viable intracellular bacteria than did the monocytes from AA (susceptible) genotypes. They concluded that the antibacterial protein *NRAMP1* provides the animals with BB genotype the possibility of controlling bacteria to enter inside the cell.

Paixao *et al.* (2007) carried out a study in pregnant cows experimentally infected with *B. abortus* and naturally infected cows. Among the experimentally cows 42.1% showed abortions whereas in naturally infected cows 43.1% abortions were recorded. *B. abortus* was isolated from 27 (64.3%) cows that aborted and had weak calves. They did not observe any association between intensity of pathological changes (necrotic placentitis) and genotype of the animal. *In vitro* studies on bovine monocyte-derived macrophages from different genotypes revealed presence of intracellular bacteria in macrophages derived from all the genotypes suggesting absence of association between genotype and resistance to *B. abortus*.

Ganguly *et al.* (2008) screened 65 Murrah breed of buffalo (*Bubalus bubalis*) to identify polymorphism at 3' UTR of *NRAMP1* gene and evaluated the association of polymorphisms with the macrophage function. Four allelic variants (viz., GT_{13}, GT_{14}, GT_{15} and GT_{16}) were identified. Majority of the buffaloes were of either homozygous $(GT)_{14}/(GT)_{14}$ or heterozygous $(GT)_{14}/(GT)_{15}$ with $(GT)_{14}$ allele occurring most frequently (62%). For association study, non-vaccinated and serologically negative animals were divided into three genotypic groups: group 1 (n=2) comprising animals of homozygous $(GT)_{13}$ genotype, whereas, group 2 (n=4) and group 3 (n=6) consisted animals of heterozygous $[(GT)_{13}/(GT)_n$, where $n \neq 13]$ and non-$(GT)_{13}$ $[(GT)_n/(GT)_n$, where $n \neq 13]$ genotype, respectively. Macrophages, after maturation, were challenged with *Brucella* LPS to assay the macrophage function in terms of H_2O_2 and NO production. The $(GT)_{13}$ allele, either in homozygoous $\{(GT)_{13}/(GT)_{13}\}$ or heterozygous $\{(GT)_{13}/ (GT)_n$, where n = 14, 15 or 16$\}$, was significantly ($p < 0.01$) associated with increased production of H_2O_2 and NO. It was suggested that $(GT)_{13}$ allelic variant had a significant association with the improved macrophage function in buffalo.

Martinez *et al.* (2008) genotyped the 3' UTR of the bovine *NRAMP1* in Colombian Creole Blanco Orejinegro (BON) (Bos taurus) (n = 140) and Zebu Brahman (Bos indicus) (Z) (n = 20) cattle and their crosses (BON X Zebu Brahman [B X Z] [n = 10]; Zebu Brahman · BON [Z X B] [n = 10]), and in animals from a Holstein · BON (H XB) (n = 10) cross. Direct sequencing and single-strand conformation polymorphism analysis (SSCP) was carried out to study the polymorphism. The association between resistance to brucellosis infection and SSCP genotype was evaluated using a macrophage *in vitro* killing assay employing a virulent *Brucella abortus* strain. The 3' UTR (GT) repeated polymorphism was genotyped and its association with resistance to brucellosis was evaluated. When all breeds were grouped, a high frequency in the homozygote $(GT)_{12}$ (AA genotype) (0.823) and a very low frequency in the homozygote $(GT)_{10}$ (BB genotype) (0.047) were detected. The BON (0.963), Z X B (0.60) and H X B (1.00) cattle showed high $(GT)_{12}$ allele frequencies, unlike that seen for the B X Z and Zebu cattle (0.3002 and 0.218, respectively). The $(GT)_{10}$ allele was only found in the Zebu cattle (0.391). A significant association ($p< 0.001$) was found between the *B. abortus* macrophage in vitro killing assay phenotypes and the bovine *SLC11A1* 3' UTR genotypes, suggesting association of A allele with resistance.

Pazzola *et al*. (2009) conducted a study to compare the allele frequency of 3' UTR *NRAMP1* microsatellite between local and specialized dairy cattle breeds reared in Sardinia, Italy. Blood samples of 97 Sarda, 55 Italian Brown and 36 Italian Friesian cattle were analyzed by PCR and PCR-SSCP. A total of three alleles were found, $(GT)_{13}$, $(GT)_{14}$ and $(GT)_{15}$. $(GT)_{13}$ showed the highest frequency in all the breeds: 0.874 in the Sarda, 0.973 in the Italian Brown and 1 in the Italian Friesian. For the Sarda, both $(GT)_{14}$ and $(GT)_{15}$ showed a frequency of 0.063, while for the Italian Brown 0.018 and 0.009, respectively. Homozygous $(GT)_{13}/(GT)_{13}$ was the unique genotype for the Italian Friesian and the most representative for the Italian Brown (0.964) and Sarda (0.823). The other genotypes for the Sarda were: $(GT)_{14}/(GT)_{14}$ (0.042), $(GT)_{13}/(GT)_{14}$ (0.010), $(GT)_{13}/(GT)_{15}$ (0.094) and $(GT)_{14}/(GT)_{15}$ (0.031). In Italian Brown, both $(GT)_{14}/(GT)_{14}$ and $(GT)_{13}/(GT)_{15}$ showed a genotypic frequency of 0.018. The observed heterozygosity was lower than the expected value both for the Sarda and the Italian Brown. Sarda showed a higher genetic variability than Italian Brown and Italian Friesian.

Matteis *et al.* (2009) carried out the sequencing of the whole coding region, as well as part of the introns and UTRs, of the *NRAMP1* gene in 49 Mediterranean buffaloes, including both serologically positive and negative animals to *Brucella abortus* test. They detected 12 mutations. Nineteen haplotypes were built from the detected variant alleles, demonstrating the high variability of this gene in buffalo, however, no significant differences in haplotype frequencies were found between serologically positive/negative animals.

Ruiz-Larranga *et al.* (2010) studied the polymorphisms in *SLC11A1* gene, and performed a population-based genetic association study to test its implication in susceptibillty to *Mycobacterium avium* subspecies *paratuberculosis* (MAP) infection in cattle. In all, 57 single nucleotide polymorphisms (SNP) were detected, 25 of which were newly described in *Bos taurus*. Twenty-four SNP and two 3′ untranslated region polymorphisms, previously analyzed, were selected for a subsequent association study in 558 European Holstein-Friesian animals. The SNP c.1067C > G and c.1157–91A > T and a haplotype formed by these 2 SNP yielded significant association with susceptibility to MAP infection. The c.1067C > G was a non synonymous SNP that caused an amino acid change in codon 356 from proline to alanine (P356A). It was suggested alter *SLC11A1* protein function.

It was concluded that there was an involvement of *SLC11A1* gene in susceptibillty to MAP infection in cattle.

Kumar *et al.* (2011) used PCR- restriction fragment length polymorphism (RFLP) in and around TM4 of *SLC11A1* gene examined the association genotypes with the incidences of brucellosis in Hariana breed (*Bos indicus*) and Holstein Friesian crossbred (*Bos indicus* X *Bos taurus*) cattle. A fragment of 954 bp encoding the TM4 was amplified, and RFLP was carried out by digestion of the amplicon independently with *AluI* and *TaqI*. Digestion with *AluI* revealed the presence of two alleles viz, A (281, 255, 79 and 51 bp) and B (541, 255, 79 and 51 bp). The frequency of A allele was estimated as 0.80 and 0.73 in Hariana and crossbred cattle, respectively. Due to presence of a polymorphic *TaqI* site at intron 5, two alleles: T (552 and 402 bp) and Q (231, 321 and 402 bp) were identified. The frequency of T allele was estimated as 0.96 and 0.97, respectively. For association study, on the basis of serological tests and history of abortion, the animals were grouped into “affected” and “non-affected”. However, no association could be established with the observed RFLPs.

Ranjan *et al.* (2011) investigated polymorphism in the 3' UTR region of the *SLC11A1* gene in the Indian cattle breeds Nimari and Kenkatha. Polymerase Chain Reaction - Single Strand Conformation Polymorphism (PCR-SSCP) of 440 bp amplicon of *SLC11A1* gene revealed three common SSCP patterns in these breeds, which was confirmed by detecting point mutation in sequences of these patterns.

Ranjan *et al*. (2011a) investigated the genetic variations in the 3' UTR (Untranslated region) of *NRAMP1* gene in the Malvi breed of (*Bos indicus*) cattle, using PCR-SSCP (Single Strand conformational polymorphism) and by sequencing. PCR-SSCP of 440 bp amplicon of *NRAMP1* gene revealed three common SSCP patterns in Malvi breed. A total of 3 SSCP patterns viz Pattern I, Pattern II and Pattern III were observed with frequency of 0.361, 0.426 and 0.213 respectively. The pattern variations were confirmed by cloning and sequencing, which showed total 6 mutations in 3 patterns.

2.6 Expression of *NRAMP1* gene by real time PCR assay

Feng *et al.* (1996) identified a bovine homolog of *murine NRAMP1,* mapped to BTA 2 which expressed in macrophages, spleen and lung. They compare human, murine and bovine *NRAMP1* protein sequences and observed the identical homology of 86.9% between murine and bovine; 88.6% between human and bovine. Also tested the different bovine tissue for macrophage population, total RNA is examined by northern blotting with ~2.3 kb was detected in macrophage, spleen and lung RNA. Thus the results indicated that bovine *NRAMP1* is expressed principally in the macrophages and reticulo-endothelial system.

Capparelli *et al.* (2007) measured the *NRAMP1* mRNA level by real time PCR before and after *in vitro* infection to macrophages from AA (susceptible) and BB (resistant) genotypes with *B. abortus.* They found that basal level of *NRAMP1* was lower in AA than BB. After infection mRNA induction was unregulated (in 4 h) in BB but not in AA macrophages. By 24 h *NRAMP1* mRNA level was up regulated in AA macrophages while in BB showed at basal level. Thus they describe the *NRAMP1* mRNA level might be regulated pos-transcriptionally by change in mRNA stabilIty.

Capparelli *et al.* (2007a) selected 10 animals which are susceptible and resistant. Non-infected BB monocytes displayed basal levels of *NRAMP1* messenger fivefold higher than those of the non-infected AA monocytes. After the infection level of *NRAMP1* messenger of BB monocytes peaked in 6 h and decline to basal level in 24 h, where as in AA monocytes, levels of AA monocytes peaked in 24 h and remain up regulated for 24 h. thus the peak level was higher in BB than AA monocytes.

2.7 Resistance of *NRAMP1* gene in other diseases

Gros *et al*. (1981) infected mice of 12 inbred strains with *Mycobacterium bovis* (*BCG*) and observed 2 distinct patterns of responses as determined by the degree of *BCG* burden in the spleens of animals at 3 wk after infection with 104 viable bacilli: susceptible (C57BL/6J and related sublines, BALB/c and DBA/IJ) and resistant (A/J, C3H/HeCr, DBA/PJ, CBA/J, C57Br, AKR). Mendelian analysis of this trait on segregating backcross and Fa populations derived from the mating of resistant and susceptible progenitors was compatible with the hypothesis that resistance to *BCG* is controlled by a single, dominant, autosomal gene designated as *Bcg.* The product of the *Bcg* gene was found to influence the early phase of host response resulting in the genetic advantage of the resistant host being demonstrable as early as 24 h after infection.

Barton *et al.* (1995) identified a candidate gene expressed in macrophages by positional cloning and full-length sequence analysis that encoded for the Natural resistance-associated macrophage protein (*Nramp*). The hypothesis that the *Nramp* gene corresponded to *Ity/Lsh/Bcg* was tested. *In vitro* transfection was used to introduce the resistant allele into the macrophage cell line RAW 264.7 derived from the recessive susceptible BALB/c mouse strain. Expression of the transgene was monitored on the background of the endogenous susceptible allele by allele-specific oligonucleotide hybridization. The Expression of the transgene correlated with three Lsh^r-associated lipopolysaccharide/interferon-y regulated macrophage activation phenotypes: respiratory burst, nitrite release, and uptake of L-arginine. Endogenous and stimulated L-arginine fluxes were inhibitable with the radical scavengers nor dihydroguaiaretic acid and butylated hydroxyanisole. The mitochondrial electron transport inhibitors, rotenone and thenoyltrifluoroacetone, inhibited respiratory burst, and rotenone suppressed L-arginine flux, implying that mitochondrial-derived oxygen radicals were important mediators in *Nramp*-regulated signal transduction pathways. It was concluded that *Nramp* was the product of the *Ity/Lsh/Bcg* gene, and pleiotropic effects of this gene on macrophage activation may require generation of oxygen radicals for intracellular signaling by mitochondria.

Vidal *et al*. (1995) identified by positional cloning a candidate gene for *Bcg, Nramp1,* which coded for a novel macrophage-specific membrane transport protein. A mouse mutant bearing a null allele at *Nramp1* was created*,* and analyzed for its effect on natural resistance to infection. Targeted disruption of *Nramp1* had pleiotropic effects on natural resistance to infection with intracellular parasites, as it eliminated resistance to *Mycobacterium bovis, Leishmania donovani* and lethal *Salmonella typhimurium* infection, establishing that *Nramp1, Bcg, Lsh* and *Ity),* are the same locus. Comparing the profiles of parasite replication in control and *Nramp1* $^{-/-}$ mice indicated that the *Nramp1*^{Asp169} allele of *Beg*^{s} inbred strains was a null allele, pointing to a critical role of this residue in the mechanism of action of the protein. Despite their inability to control parasite growth in the early non immune phase of the infection, *Nramp1* $^{-/-}$ mutants could overcome the infection in the late immune phase, suggesting that *Nramp1* plays a key role only in the early part of the macrophage- parasite interaction and may function by a cytocidal or cytostatic mechanism distinct from those expressed by activated macrophages.

Estrada-chavez *et al*. (2001) investigated the expression of *NRAMP1* in peripheral blood cells (PBC) and tuberculous granulomas of *M. bovis* infected bovines. Using Western blotting a high-level expression of *NRAMP1* proteins in peripheral blood cells and granulomas of *Mycobacterium bovis*-infected bovines was demonstrated. Immunohistochemistry of granulomatous lesions showed heavily labeled epithelioid macrophages and Langhans cells. The data suggested that *M. bovis* infection enhanced *NRAMP1* expression and that active tuberculosis could occur despite of this response.

Guilloteau *et al*. (2003) investigated the role of *NRAMP1* gene in the control of *Brucella* infection. When BALB/c mice (*NRAMP1s*) and C.CB congenic mice (*NRAMP1r*) were infected with *Brucella melitensis,* the number of *Brucella* organisms per spleen was significantly larger in the C.CB mice than in the BALB/c mice during the first week post-infection. This *NRAMP1*-linked susceptibility to *Brucella* was temporary, since similar numbers of *Brucella* were recovered from the two strains of mice 2 weeks post-infection. The effect of *NRAMP1* expression occurred within splenocytes intracellularly infected by *Brucella.* However, there was no difference between *in vitro* replication rates of *Brucella* in macrophages isolated from the two strains of mice infected *in vivo* or in *NRAMP1* RAW264 transfectants. In mice, infection with *Brucella* induced an inflammatory response, resulting in splenomegaly and recruitment of phagocytes in the spleen, which was amplified in C.CB mice. Reverse transcription-PCR (RT-PCR), performed 5 days post-infection., showed that inducible nitric oxide synthase, tumor necrosis factor alpha (TNF-α), interleukin-12 p40 (IL-12p40), gamma interferon (IFN- γ), and IL-10 mRNAs were similarly induced in spleens of the two strains. In contrast, the mRNA of KC, a C-X-C chemokine, was induced only in infected C.CB mice at this time. This pattern of mRNA expression was maintained at 14 days post-infection., with IFN-γ and IL-12p40 mRNAs being more intensively induced in the infected C.CB mice, but TNF-α mRNA was no longer induced. The higher recruitment of neutrophils observed in the spleens of infected C.CB mice was suggested to be responsible for temporary susceptibilIty of C.CB mice to *B. melitensis* infection. It was concluded that, in contrast to infections with *Salmonella, Leishmania,* and *Mycobacterium,* the expression of the *NRAMP1* gene was of limited importance for the natural resistance of mice to *Brucella*.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Glassware

The glassware, made of neutral glass required for the present investigation was obtained from M/s Borosil, India. All the glassware used during the course of research was prepared by following standard procedures including sterilization at 160^0C in hot air oven for 2 h.

3.1.2 Plastic ware

The plastic ware required for the present research viz. centrifuge tubes, microcentrifuge tubes, PCR tubes, pipette tips etc. was procured from M/s Tarsons India, Kolkata. All the plastic ware was autoclaved at 15 lbs for 15 min. before use.

3.1.3 Chemicals and reagents

The chemicals and reagents used during present investigation were obtained from following manufacturing companies.

1) M/s Bangalore Genei, Bangalore (India)
2) M/s Hi Media Laboratories Private Limited, Mumbai (India)
3) M/s Sigma Aldrich Chemicals Private Limited (USA)
4) M/s Sisco Research Laboratories Private Limited, Mumbai (India)
5) M/s Fermentas Life Science (Canada)

The chemicals and reagents required for PCR and other molecular biology work were of molecular grade.

3.1.4 Animals

The study was conducted on a total of 54 Pandharpuri buffaloes including 11 buffaloes from Satara and 43 from Kolhapur districts of Maharashtra. The animals selected for investigation were from both types of farms i.e. farms with the history of abortions due brucellosis and those known to be free of brucellosis.

3.2 Methods

3.2.1 Collection of Specimens

3.2.1.1 Whole Blood in EDTA

Blood samples were collected aseptically from the animals under investigation by jugular vein puncture using vacutainer containing EDTA and transported to the laboratory on ice. Around 5 ml whole blood was collected from each animal which subsequently was used for isolation of DNA.

3.2.1.2 Serum Samples

For obtaining sera, blood samples were collected in vaccutainers without anticoagulant and kept in an upright position at room temperature for about 2 h. The serum was separated in sterile screw capped plastic vials and stored at - 20ºC till further use.

3.2.2 Serological Tests

The serum samples of all the 54 buffaloes were tested for presence of anti-*Brucella* antibodies by Rose Bengal Plate Test (RBPT) and Standard Tube Agglutination Test (STAT). The samples were heat inactivated at 56°C for 30 min in water bath prior to serological testing.

3.2.2.1 Rose Bengal Plate Test (RBPT)

The coloured antigen required for performing RBPT was obtained from the Division of Biological Products, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh (Plate 3.1). The test was performed as per the manufacturer's instructions. Briefly, a drop of serum (30 µl) was placed on clean grease free glass slide and an equal quantity of antigen was added and mixed thoroughly with the help of inoculation loop. The mixture was observed for clumping / agglutination for one min. and the results were recorded as agglutination (+), no agglutination (-).

3.2.2.2 Standard Tube Agglutination Test (STAT)

Brucella abortus plain antigen obtained from Division of Biological Products, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh (Plate 3.2) was used for performing STAT. Two-fold dilutions of test serum (starting from 1:10 to 1:640) were prepared in 0.5% phenol saline in serological test tubes, equal quantity of *Brucella abortus* plain antigen was added, mixed thoroughly and incubated at 37 ^{0}C for 24 hours (Table 3.1).

Table 3.1: Protocol for STAT

Reagent	1	2	3	4	5	6	7	8	9
Normal saline (ml)	0.8	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Test serum (ml)	0.2	0.5	0.5	0.5	0.5	0.5	0.5	0.5	- → Discard 0.5 ml
Dilution	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	Antigen control
Brucella plain antigen (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	Shake the tubes well, cover with aluminum foil and incubate at 37^{o}C in incubator for 24 h								

The reciprocal of the highest serum dilution showing 50 per cent or more agglutination (50 % clearing) was recorded as the antibody titer of the serum. Appropriate controls were included in the test protocol. The values titres so obtained were converted into International Unit (I.U.) of *Brucella* antibody activity as recommended by the joint FAO/WHO expert committee on Brucellosis (Alton and Jones, 1967). The titres of 80 I.U. per ml and above were considered as positive for bovine sera.

3.2.3 Extraction of DNA from blood samples

The extraction of genomic DNA from blood samples of seropositive and healthy Pandharpuri buffaloes was carried out as per the protocol described by Leal-Klevezas *et al*. (1995) with slight modifications. Briefly, the procedure employed was as follows.

1. A 500 µl blood sample was transferred to microcentrifuge tube and centrifuged at 12000 rpm for 8 min. at 4 ^{0}C.
2. The supernatant was discarded and 1 ml RBC lysis buffer was added to the cell pellet. The preparation was mixed by vortexing, centrifuged at 12000 rpm for 8 min. at 4 ^{0}C and supernatant was discarded. The procedure was repeated two to three times until the red colour due to the erythrocytes was minimal.
3. A 500 µl leukocyte lysis buffer was then added, mixed by vortexing followed by addition of 10 µl of proteinase K (20 mg / ml). The preparation was again mixed by vortexing and incubated at 50 ^{0}C for 40 min. in dry bath.
4. Subsequently, 500 µl saturated phenol was added, mixed by vortexing and centrifuged at 12000 rpm for 10 min. at 4 ^{0}C.
5. The upper aqueous phase of the suspension was then transferred to a fresh microcentrifuge tube, equal volume (500 µl) of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added, mixed by vortexing followed by centrifugation at 12000 rpm for 10 min. at 4 ^{0}C.
6. The upper phase of the preparation was again transferred to a microcentrifuge tube and equal volume (500 µl) chloroform: isoamyl

alcohol (24 : 1) was added, mixed by vortexing and centrifuged at 12000 rpm for 10 min. at 4 ^{0}C.

7. The upper aqueous phase was again transferred to a fresh microcentrifuge tube and 0.6 volume of chilled isopropanol was added to it & kept at - 20 ^{0}C for 30 min.
8. The suspension was centrifuged again at 12000 rpm for 15 min. at 4 ^{0}C and upper layer was discarded leaving about 20 µl suspension over the pellet.
9. One ml of chilled 70 % ethanol was then added. The suspension was centrifuged at 12000 rpm for 10 min. at 4 ^{0}C and supernatant was discarded leaving about 20 µl above the pellet.
10. A 500 µl chilled 70 % ethanol (from - 20 ^{0}C) was added. Centrifugation was carried out at 12000 rpm for 10 min. at 4 ^{0}C and the supernatant was discarded.
11. The pellet was dried at room temperature for 30 min. and was dissolved in 50 µl of sterile distilled water and stored at - 20 ^{0}C, till further use.

3.2.4 Quantification of DNA

Quantification of DNA extracted from blood samples was done spectrophotometrically at 260 nm and 280 nm using Nanodrop spectrometer (ND 1000 Thermo Scientific, USA). The $OD_{260:280}$ ratio was calculated.

3.2.5 Assessment of integrity of genomic DNA

The isolated genomic DNA samples were subjected to agarose gel electrophoresis in ethidium bromide stained agarose gel for assessing their integrity (Brown, 2007). Agarose gel (0.8 %) prepared in 1X TAE buffer was used for this purpose. The agarose was dissolved in TAE by heating in microwave oven; ethidium bromide was added to a final concentration of 0.5 µg/ml and mixed by gentle swirling. The gel thus prepared was poured in the gel casting tray and allowed to solidify. Subsequently, comb and slabs were removed and the gel with tray was transferred to electrophoresis apparatus. 1X TAE buffer was added in electrophoresis tank covering the agarose gel to a depth of about 10 mm. 2 µl of DNA sample was mixed with 0.5 µl 6X loading dye. The mixture was loaded in the slots and electrophoresis was carried out at a constant voltage of 90 V, till dye migrated about three-fourth distance of gel. The integrity of the DNA was checked by visualization of the DNA using gel documentation system.

3.2.6 PCR amplification of *SLC11A1* 3' UTR

3.2.6.1 Oligonucleotide Primers

The amplification of 193 bp region of *SLC11A1* gene of Pandharpuri buffaloes was carried out using oligonucleotide primer sequences (Table 3.2) as per Ganguly et al., 2008. The oligonucleotides were custom synthesized and supplied by M/S. Sigma Aldrich (USA).

Table 3.2: Oligonucleotide primers for amplification of *SLC11A1* 3' UTR PCR

Name	Oligonucleotide Sequence	Reference
AM3F	**5’ –TGTGTGTGTGAAGGCAGCA- 3’**	**Ganguly et al., 2008**
AM2R	**5’ –ATGGAACTCACGTTGGCTG-3’**	

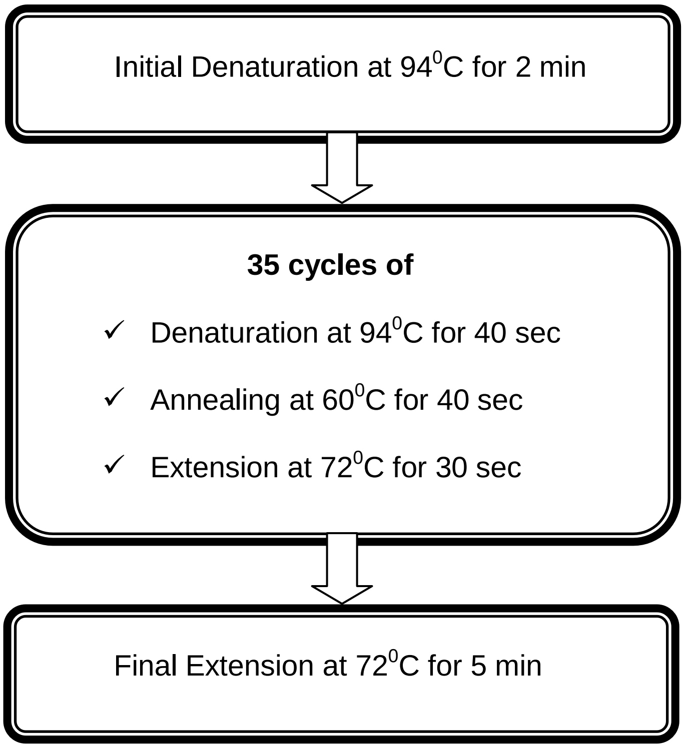
3.2.6.2 PCR procedure

The reaction was set in a total volume of 25 µl comprising reactants shown in Table 3.3. The reactions mixtures were vortexed briefly and set in to thermal cycler for cycling conditions shown in fig. 3.1. The PCR products were stored at -20^{o}C in deep freeze until further use.

Table 3.3: Reaction Mixture for *SLC11A1* gene PCR

Sr. No.	Component	Quantity (µl)	Concentration
1	10 X PCR assay buffer without MgCl$_2$	2.5	1 X
2	MgCl$_2$ (25 mM)	2	2.0 mM
3	dNTP mix (10mM)	2	100 mM
4	AM3F (10pm/µl)	1	10 pmoles
5	AM2R (10pm/µl)	1	10 pmoles
6	*Taq* DNA Polymerase	0.2	1 U
7	Templete DNA	3	30 ng
8	Sterile Distilled Water	13.3	
	Total Volume	25 µl	

Fig. 3.1: Cycling conditions for PCR



3.2.6.3 Evaluation of PCR product by agarose gel electrophoresis

The amplified products of *SLC11A1* 3'UTR were evaluated by agarose gel (1.2 %) electrophoresis in ethidium bromide stained agarose gel as described previously (3.2.5). A 100 bp DNA ladder was electrophoresed simultaneously along with the PCR products to assess the size of amplicons generated. The results of agarose gel electrophoresis were visualized and documented using automatic gel documentation system (Uvitech).

3.2.7 Sequencing

3.2.7.1 Freeze drying of PCR products

The freeze drying of the PCR products was carried out at National Institute for Research in Reproductive Health (NIRRH) Mumbai.

3.2.7.2 Sequencing of PCR product

The sequencing of the PCR products was carried out at SciGenom Labs Pvt Ltd, Cochin, (India). The sequences obtained from the ABI files were analyzed and curated using Chromas light software Version 2.01.

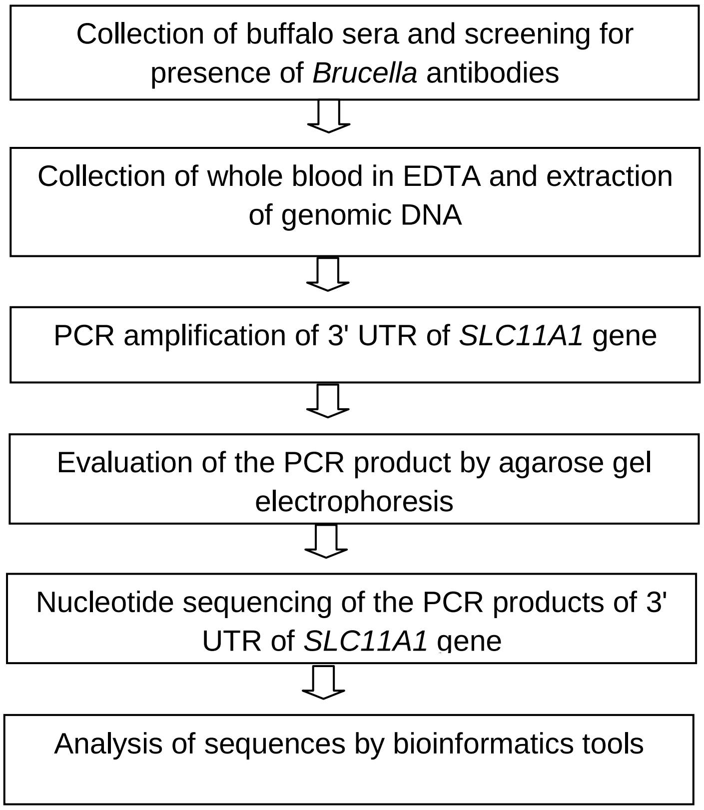
3.2.7.3 Analysis of sequences using Bioinformatics tools

The sequences of amplicons of 3' UTR of *SLC11A1* gene were analysed using bioinformatic tools available online. Sequences retrieved from SDSC Biology Workbench (http//.workbench.sdsc.edu) ABI files were subjected for correct annotation using ChromasLite software version 2.01.

4. RESULTS

The present investigation dealt with characterization of 3' UTR of the *SLC11A1* gene in relation to natural resistance against infection due to *Brucella* spp. The study was conducted on 54 Pandharpuri buffaloes from Satara and Kolhapur districts of Maharashtra state. The buffaloes were initially screened for presence of anti-*Brucella* antibodies by RBPT and STAT and characterization of 3' UTR of *SLC11A1* gene of both, *Brucella* positive and negative buffaloes was carried out. The broad strategy of the investigation was as shown in Fig.4.1.

Fig. 4.1 Plan of experiment



4.1 Serological detection of brucellosis

The serum samples of all the buffaloes were screened for detection of *Brucella* antibodies by RBPT and STAT in order to find out status of brucellosis of animals under investigation.

4.1.1 <u>Rose Bengal Plate Test (RBPT)</u>

A total of 54 serum samples were screened for presence of anti-*Brucella* antibodies by RBPT (Plate 4.1) of which 10 were found positive. The overall seroprevalence of brucellosis detected by RBPT was 18.51 %. The seroprevalence of brucellosis in the Pandharpuri buffaloes from Satara was found to be higher (27.27 %) than that in buffaloes from Kolhapur (16.27 %).

Table 4.1: Detection of *Brucella* antibodies by RBPT

Sr. No.	Location	Tested	Positive	Total (%)
1	**Satara**	**11**	**3**	**27.27**
2	**Kolhapur**	**43**	**7**	**16.27**
	Total	**54**	**10**	**18.51**

4.1.2 <u>Standard Tube Agglutination Test (STAT)</u>

All the 54 sera were simultaneously subjected to STAT for quantitative detection of anti-*Brucella* antibodies (Plate 4.2). The results of STAT revealed that 6 out of the 54 sera were positive for presence of *Brucella* antibodies. The overall seroprevalence of brucellosis detected by STAT was 11.11 %. Location-wise analysis of results of STAT revealed that the seroprevalence in Kolhapur district was 11.62 % where as that in Satara district was 9.09 % (Table 4.2).

Table 4.2: Detection of *Brucella* antibodies by STAT

Sr. No.	Location	Tested	Positive	Total (%)
1	Satara	11	1	9.09
2	Kolhapur	43	5	11.62
	Total	54	6	11.11

Table 4.3: Overall seroprevalence of Brucellosis considering the two serological tests

Sr. No.	Location	Tested	Positive	Total (%)
1	Satara	11	3	27.27
2	Kolhapur	43	7	16.27
	Total	54	10	18.51

4.2 Amplification and sequencing of 3' UTR of *SLC11A1* gene

4.2.1 PCR amplification of 3' UTR of *SLC11A1* gene

The amplification of 3' UTR of *SLC11A1* gene was carried out by PCR in order to generate amplicons that could subsequently be subjected to nucleotide sequencing. The amplification products generated were evaluated by agarose gel electrophoresis and visualized under gel documentation system. All the 54 samples yielded specific amplification product of ~193 bp (Plate 4.3 and 4.4).

4.2.2 Nucleotide sequencing of the *SLC11A1* gene

Out of 54 amplicons generated from 3' UTR of *SLC11A1* gene PCR, 42 were subjected to nucleotide sequencing. The results of sequencing revealed that the sizes of amplicons of 3' UTR of *SLC11A1* gene generated from the samples ranged between 193 to 199 bp. Further, the sizes of amplicons observed in the present investigation matched to the sizes of 3' UTR of *SLC11A1* gene sequences published earlier (GenBank accession nos. DQ645387, DQ645389 and DQ645388).

4.2.3 Analysis of sequnces

The sequences of amplicons of 3' UTR of *SLC11A1* gene were analysed using bioinformatic tools available online. Sequences retrieved from SDSC Biology Workbench (http//.workbench.sdsc.edu) ABI files were subjected for correct annotation using ChromasLite software version 2.01 (figures: 4.7 and 4.8). Further, the sequences were subjected multiple sequence alignment using CLUSTALW tool (http://seqtool.sdsc.edu/cgi/BW.cgi) available online (Fig. 4.2, 4.3, 4.4, 4.5 and 4.6).

4.2.4 Identification of $(GT)_n$ microsatellite polymorphism at 3' UTR

The principal objective of the present study was to study the $(GT)_n$ microsatellite polymorphism at 3' UTR in relation to susceptibility/resistance of Pandharpuri buffaloes to brucellosis. Different alleles carrying variable number of GT repeats at the 3' UTR of the bovine *SLC11A1* gene have previously been linked to either resistance or susceptibility to *B. abortus*. The actual number of GT repeats for each allelic variant was confirmed by nucleotide sequencing and the 13, 14, 15 and 16 GT repeats were designated as $(GT)_{13}$, $(GT)_{14}$, $(GT)_{15}$, and $(GT)_{16}$ genotypes respectively.

4.2.5 Frequency of $(GT)_n$ alleles in Pandharpuri buffaloes

Out of 54 buffaloes included in the study, nucleotide sequencing of 3' UTR of *SLC11A1* gene was carried out on 42 samples. The results of distribution of buffaloes in to different genotypes (Table: 4.4) revealed that the allele $(GT)_{14}$ with a proportion of 35.71 % occurred most frequently followed by $(GT)_{15}$ with a proportion of 30.95 %. The proportion of genotype $(GT)_{13}$ was comparatively lower (23.80 %) than those of $(GT)_{14}$ and $(GT)_{15}$ where as that of $(GT)_{16}$ was lowest (9.52%). Previous studies have demonstrated that the allele $(GT)_{13}$ is associated with natural resistance where as alleles $(GT)_{14}$, $(GT)_{15}$ and $(GT)_{16}$ are associated with susceptibility to brucellosis.

.**Table 4.4: Distribution of $(GT)_n$ alleles in Pandharpuri buffaloes**

GT allele	**Number of samples showing the GT allele**	**Frequency of occurrence (%)**
$(GT)_{13}$	10	23.80
$(GT)_{14}$	15	35.71
$(GT)_{15}$	13	30.95
$(GT)_{16}$	4	09.52
Total	42	

4.2.6 Association of $(GT)_n$ alleles with brucellosis

Out of the 42 samples subjected to nucleotide sequencing, 8 were from serologically positive buffaloes. The data on genotype of buffaloes and their status of brucellosis is presented in Table 4.5. It is obvious from this data that a major proportion of 75% seropositive buffaloes belonged to genotype $(GT)_{14}$ followed by genotype $(GT)_{15}$ to which 25% buffaloes belonged. It was interesting to note that none of the sample of buffaloes belonging to genotype $(GT)_{13}$ was positive for brucellosis. The results clearly point out association of genotype $(GT)_{13}$ with resistance to brucellosis. Similarly, no sample of genotype $(GT)_{16}$ was also found positive for brucellosis. However, association of genotype $(GT)_{16}$ with resistance to brucellosis has not been previously documented. Moreover, only 4 samples of this genotype were investigated during the present work and as such to establish relationship of $(GT)_{16}$ genotype with resistance to brucellosis, investigations on greater numbers of samples would be desirable.

Table 4.5: Association of $(GT)_n$ alleles and brucellosis

Sr. No.	GT allele	Seropositive Samples	Proportion (%)	Seronegative Samples	Proportion (%)
1	$(GT)_{13}$	-	-	10	29.41
2	$(GT)_{14}$	6	75	9	26.47
3	$(GT)_{15}$	2	25	11	32.35
4	$(GT)_{16}$	-	-	4	11.76
	Total	8		34	

Table 4.6: Status of brucellosis and GT alleles

Sample No.	Location	Status of brucellosis	$(GT)_n$ allele
1	Satara	Positive	$(GT)_{14}$
2	Satara	Negative	$(GT)_{15}$
3	Satara	Negative	$(GT)_{15}$
4	Satara	Positive	$(GT)_{15}$
5	Satara	Negative	**$(GT)_{13}$**
6	Satara	Negative	$(GT)_{16}$
7	Satara	Negative	$(GT)_{15}$
8	Satara	Negative	$(GT)_{16}$
9	Satara	Negative	$(GT)_{15}$
10	Satara	Positive	$(GT)_{14}$
11	Satara	Negative	**$(GT)_{13}$**
12	Kolhapur-1	Negative	$(GT)_{14}$
13	Kolhapur-1	Negative	$(GT)_{14}$
14	Kolhapur-1	Negative	$(GT)_{14}$
15	Kolhapur-2	Negative	**$(GT)_{13}$**
16	Kolhapur-2	Negative	$(GT)_{14}$
17	Kolhapur 2	Positive	$(GT)_{14}$
18	Kolhapur 3	Negative	$(GT)_{14}$
19	Kolhapur 3	Negative	$(GT)_{15}$
20	Kolhapur 3	Positive	$(GT)_{14}$
21	Kolhapur 3	Negative	$(GT)_{15}$
22	Kolhapur-3	Negative	$(GT)_{15}$
23	Kolhapur-4	Negative	**$(GT)_{13}$**
24	Kolhapur-4	Positive	$(GT)_{15}$
25	Kolhapur-4	Negative	$(GT)_{14}$
26	Kolhapur-4	Positive	$(GT)_{14}$
27	Kolhapur-4	Positive	$(GT)_{14}$
28	Kolhapur-4	Negative	**$(GT)_{13}$**
29	Kolhapur-5	Negative	**$(GT)_{13}$**
30	Kolhapur-5	Negative	**$(GT)_{13}$**
31	Kolhapur-6	Negative	$(GT)_{14}$
32	Kolhapur-6	Negative	$(GT)_{15}$
33	Kolhapur-7	Negative	$(GT)_{14}$
34	Kolhapur-7	Negative	$(GT)_{15}$
35	Kolhapur-7	Negative	$(GT)_{15}$
36	Kolhapur-8	Negative	**$(GT)_{13}$**
37	Kolhapur-8	Negative	$(GT)_{16}$
38	Kolhapur-9	Negative	$(GT)_{15}$
39	Kolhapur-9	Negative	**$(GT)_{13}$**
40	Kolhapur-9	Negative	$(GT)_{16}$
41	Kolhapur-10	Negative	**$(GT)_{13}$**
42	Kolhapur-10	Negative	$(GT)_{14}$

The eight serologically positive animals investigated in the present study were actually derived from four different locations/farms. The total number of animals screened from these four locations was 25. At all of these locations both, *Brucella* positive and negative animals were reared together and as such there was ample opportunity for *Brucella* negative animals to acquire the infection. However, it was interesting to note that a total of 17 animals proved negative for brucellosis despite of being in the contact of *Brucella* infected animals. The genotype-wise distribution of *Brucella* negative animals at these locations was $(GT)_{13}$ - 5, $(GT)_{14}$ - 3, $(GT)_{15}$ -7 and $(GT)_{16}$ -2.

Table 4.7: Status of brucellosis and genotypes of buffaloes at four brucellosis affected farms

Sr. No.	Location	No. of animals screened	No. of animals positive		GT alleles of positive animals				GT alleles of negative animals			
			Positive	Negative	$(GT)_{13}$	$(GT)_{14}$	$(GT)_{15}$	$(GT)_{16}$	$(GT)_{13}$	$(GT)_{14}$	$(GT)_{15}$	$(GT)_{16}$
1	Shirwal	11	03	08	-	02	01	-	02	-	04	02
2	Kolhapur-2	03	01	02	-	01	-		01	01	-	-
3	Kolhapur-3	05	01	04	-	01	-		-	01	03	-
4	Kolhapur-4	06	03	03	-	02	01		02	01	-	-
	Total	25	08	17	-	06	02	-	05	03	07	02

5. DISCUSSION

Brucellosis is one of the economically important major zoonotic infections worldwide. The causative organism *Brucella abortus* is a facultative intracellular pathogen that is capable of surviving and replicating with the phagosomes inside the macrophages. Macrophages isolated from naturally susceptible and resistant animals have been found to exhibit differential ability in controlling the intracellular replications of *Brucella* organisms (Crocker et al., 1984; Qureshi *et al.* (1996); Capparelli *et al.* (2007). In bovines a $(GT)_{13}$ allele at 3' UTR of the *SLC11A1* gene has been reported to have significant association with natural resistance to brucellosis (Ganguly et al., 2008). Selection and breeding of such genetically resistant animals therefore could be an effective tool for control of brucellosis in livestock species and would be a boon to the dairy industry. Keeping in view these facts, the present investigation was planned to undertake genotyping of 3' UTR of the *SLC11A1* gene and study its relationship to natural resistance against brucellosis in Pandharpuri breed of buffalo.

5.1 Serological detection and prevalence of brucellosis

The study was conducted on a total 54 Pandharpuri buffaloes from Satara and Kolhapur districts of Maharashtra. In order to find out status of *Brucella* infection of buffaloes, Rose Bengal Plate test (RBPT) and Standard Tube Agglutination test (STAT) were employed. Serological tests have been widely employed for diagnosis since they are simple, relatively inexpensive and rapid. The sensitivity of RBPT and STAT is known to be variable therefore a buffalo showing positive reaction even by a single test was considered as *Brucella* infected. Out of 43 sera samples of buffaloes from Kolhapur, 07 (16.27 %) were found positive whereas of the 11 buffaloes investigated from Satara, 3 (27.27%) proved positive for brucellosis. Although the studying serological prevalence of brucellosis was not the aim of present study, the data recorded suggests a greater seroprevalence of brucellosis in Satara compared to Kolhapur. Several researchers have studied the prevalence of brucellosis in different parts of

Maharashtra using different serological tests and recorded variable degree of prevalence. Londhe (2009) investigated the seroprevalence of brucellosis in Western Maharashtra and Konkan regions and observed 70% prevalence in Satara and 34.78% in Kolhapur. The observations recorded in the present investigation corroborate with those of Londhe (2009). Similarly, Madale (2011) also studies the prevalence of brucellosis in Western Maharashtra and Konkan regions and found a relatively lower (10.16%) serological prevalence in Satara district. Aher (2010) examined the bovine sera for presence of brucella antibodies by RBPT, STAT and i-ELISA. He recorded seroprevalence of 4% in Satara district.

The comparison of results of RBPT and STAT revealed that the RBPT could detect greater proportion samples (18.51%) than STAT (14.81%). Both of these tests have widely been employed for diagnosis of brucellosis and the results of higher sensitivity of RBPT recorded in present investigation are in agreement with the findings of several researchers (Aher, 2010; Madale, 2011).

5.2 Genotyping of 3' UTR of *SLC11A1* gene

Bovine *SLC11A1* gene, the homologue of a murine gene controlling natural resistance to antigenically unrelated intracellular pathogens, has been identified as one of the major candidates for controlling natural resistance and/or susceptibility to brucellosis in cattle (Feng *et al*., 1996). *SLC11A1* is also known as *NRAMP1.* Several studies (Borriello *et al*., 2006; Capparelli *et al*., 2007; Capparelli *et al*., 2007a; Ganguly *et al*., 2006) have shown a strong association of $(GT)_n$ microsatellite polymorphisms within the *SLC11A1* 3' UTR with the resistance/susceptibility against brucellosis in water buffalo and Murrah buffalo. Considering the potential of this region in resistance to *Brucella abortus*, the nucleotide sequence of 3' UTR of *SLC11A1* gene in the Pandharpuri buffalo was determined. Initially, the amplification of a fragment of 3' UTR of *SLC11A1* gene was carried out using locus specific primer sequences (Ganguly *et al*., 2008). All the amplified products obtained were in the range of 193-199 bp encompassing a region of 1804-1996 nucleotide position. The results observed in the present investigation are consistent with the observations of Ganguly *et al*., 2008.

Direct sequencing of amplified PCR products enabled us to read the $(GT)_n$ repeat at nucleotide position 1908 (Ganguly *et al*., 2008). The sequencing results exhibited four alleles viz. $(GT)_{13}$, $(GT)_{14}$, $(GT)_{15}$ and $(GT)_{16}$. The allelic variants observed during present work were concordant with the alleles reported previously (GenBank Accession No. U27105). Borriello *et al*. (2006) reported only two allelic variants viz, $(GT)_{33}$ and $(GT)_{36}$ in the 3' UTR of *SLC11A1* gene (NCBI accession number DQ095780 and DQ095781) by PCR-DGGE in the Italian water buffaloes, the alleles were termed as A and B, respectively. The explanation for this difference in the $(GT)_n$ repeats as suggested by Ganguly *et al*., 2008 could be amplification a larger fragment (between nucleotide position 1745-1955 bp) by, Borriello *et al*., (2006) and differentiation of the alleles on the basis of total number of GT repeats occurring at various nucleotide positions viz, 1781, 1876 and 1912. In the present study, we have amplified a fragment of 3' UTR of *SLC11A1* gene between nucleotide position 1804 to 1996, therefore considering nucleotide position 1912, the alleles A and B reported by Borriello *et al*., 2006 could be those containing $(GT)_{15}$ and $(GT)_{14}$ repeats respectively. However Capparelli *et al*. (2007a) amplified different region of 3' UTR of *SLC11A1* and detected the presence of four alleles termed as Nramp1A, -B, -C and –D by capillary electrophoresis in the Italian water buffaloes. In India the first report of polymorphism in the Murrah breed of buffalo has been documented by Ganguly *et al*., 2008 who observed presence of four allelic variants viz, $(GT)_{13}$, $(GT)_{14}$, $(GT)_{15}$ and $(GT)_{16}$. Our results are in agreement with the findings of Borriello *et al*. (2006) and Ganguly *et al*. (2008).

In present investigation the sequencing results revealed all the individuals to be of homozygous genotypes with respect to either of the four allelic variants viz, $(GT)_{13}/(GT)_{13}$, $(GT)_{14}/(GT)_{14}$, $(GT)_{15}/(GT)_{15}$ and $(GT)_{16}/(GT)_{16}$. None of the animal was found to be heterozygous. The heterozygous genotypes (AB, $(GT)_{15}/(GT)_{14}$) in addition to the AA, $(GT)_{15}/(GT)_{15}$ and BB, $(GT)_{14}/(GT)_{14}$ were observed by Borriello *et al*. (2006). The four different alleles observed by Capparelli *et al*. (2007a) behaved as co-dominant based on family data, whereas four animals displayed CD and two animals displayed BD genotypes.

The polymorphism of 3' UTR of *SLC11A1* gene in case of cattle in Brazil exhibited homozygous $(GT)_{13}/(GT)_{13}$ and $(GT)_{14}/(GT)_{14}$ and heterozygous $(GT)_{13}/(GT)_{14}$ or $(GT)_{13}/(GT)_{15}$ genotypes. The most frequent genotype observed in Nelore and Gir breeds of cattle was $(GT)_{13}/(GT)_{14}$ whereas in Guzera animals $(GT)_{14}$ was most common. Holsteins were found with most homogenous $(GT)_{13}$ genotype (Paixao *et al*., 2006).

The frequency of A, $(GT)_{15}$ and B, $(GT)_{14}$ alleles observed by Borriello *et al*. (2006) was 0.47 and 0.53, respectively. Whereas Ganguly *et al*. (2008) observed allelic frequency of $(GT)_{13}$ as 0.10 and that of $(GT)_{14}$, $(GT)_{15}$ and $(GT)_{16}$ as 0.62, 0.26 and 0.02, respectively. The Pandharpuri buffaloes in the present investigation revealed frequencies of 0.238, 0.3571, 0.3095 and 0.0952 respectively for $(GT)_{13}$, $(GT)_{14}$, $(GT)_{15}$ and $(GT)_{16}$ alleles (Table 5.1). The genotype frequency was same as that of respective allele frequency, since all the animals were homozygous for either of the alleles.

Table 5.1: Allele and genotype frequencies of $(GT)_n$ polymorphism at 3' UTR of *SLC11A1* gene in Pandharpuri buffalo

	Genotype			
	$(GT)_{13}/(GT)_{13}$	$(GT)_{14}/(GT)_{14}$	$(GT)_{15}/(GT)_{15}$	$(GT)_{16}/(GT)_{16}$
Allele	$(GT)_{13}$	$(GT)_{14}$	$(GT)_{15}$	$(GT)_{16}$
frequency	0.2380 (10)	0.3571 (15)	0.3095 (13)	0.0952 (04)

Numbers within parenthesis indicate the numbers of animals.

This is the first study reporting $(GT)_n$ microsatellite polymorphism at 3' UTR of *SLC11A1* gene in Pandharpuri breed of buffalo. We could detect four allelic variants viz, $(GT)_{13}$, $(GT)_{14}$, $(GT)_{15}$ and $(GT)_{16}$ in the animals under study.

5.3 Association of genotypes with seroprevalence

Table 5.2: Comparison between Brucellosis and various Genotypes

Sr. No.	Brucella positive buffaloes	Genotype
1	0	$(GT)_{13}$
2	6 (14.28 %)	$(GT)_{14}$
3	2 (4.78%)	$(GT)_{15}$
4	0	$(GT)_{16}$

The results presented in table 5.2 indicate that the animals with genotypes $(GT)_{14}$ and $(GT)_{15}$ showed seropositivity for brucellosis of 14.28% and 4.78 % respectively. It was interesting to note that none of the sample of buffaloes belonging to genotype $(GT)_{13}$ was positive for brucellosis. The results clearly point out association of genotype $(GT)_{13}$ with resistance to brucellosis. The seronegativity conferred to buffaloes by $(GT)_{13}$ allele may be because of presence of this polymorphic variant (Ganguly *et al*., 2008; Feng *et al*., 1996). Ganguly *et al.,* 2008 reported that the macrophages of Murrah buffaloes with $(GT)_{13}$ allele in homozygous or heterozygous condition had an improved macrophage function. The activity was observed in terms of H_2O_2 and NO production. These macrophages when challenged with different concentrations of *Brucella* LPS, produced more amount of H_2O_2 and NO ($p < 0.01$) as compared to the macrophages of other genotypes (Ganguly *et al*., 2008). The findings of the present study also suggest possibility of a similar improvement in macrophage function of Pandharpuri buffaloes possessing $(GT)_{13}/(GT)_{13}$ genotypes.

The monocyte culture (from BB, $(GT)_{14}/(GT)_{14}$ animals) when infected with *B. abortus*, *B. mellitensis* and *B. suis* were found to control intracellular growth of Brucella species, the BB animals also remain seronegative even after prolonged exposure to pathogens (Borriello *et al*., 2003). Macrophage function test of the seronegative and non vaccinated Murrah buffaloes of $(GT)_{14}$ heterozygous and of non $(GT)_{14}$ genotype did not yield much difference among animals (Ganguly *et al*., 2008). However in the present investigation we observed that 7.14 % of $(GT)_{14}/(GT)_{14}$ Pandharpuri buffaloes were seropositive by RBPT and STAT.

The results obtained from the study on monocytes from BB genotype (Capparelli *et al*., 2007a) viz, significantly higher levels of Nramp1 when infected with *B. abortus,* reduction in number of intracellular bacteria when infected with GFP-*B. abortus* and higher reactive oxygen and nitrogen intermediates when not infected, indicated protective effect of the Nramp1 BB genotype against *B. abortus* in the water buffaloes. Since, Capparelli *et al*., 2007a amplified different regions of 3' UTR of *NRAMP1/SLC11A1* our findings cannot be compared with these results.

5. SUMMARY AND CONCLUSIONS

Brucella abortus, the causative agent of economically important zoonotic disease brucellosis, is a facultative intracellular pathogen that is capable of surviving and replicating within the phagosomes inside the macrophages. The macrophage function thus plays an important role in natural resistance against brucellosis. The solute carrier family 11 (protein coupled divalent metal ion transporters) number 1, (*SLC11A1*) has an important role in intracellular killing of *Brucella.* The present investigation dealt with characterization of 3' UTR of the *SLC11A1* gene in relation to natural resistance against infection due to *Brucella* spp. The study was conducted on 54 Pandharpuri buffaloes from Satara and Kolhapur districts of Maharashtra state. The buffaloes were initially screened for presence of anti-*Brucella* antibodies by RBPT and STAT and characterization of 3' UTR of *SLC11A1* gene of both, *Brucella* positive and negative buffaloes was carried out.

Out of the 54 serum samples screened by RBPT 10 were found positive. The overall seroprevalence of brucellosis detected by RBPT was 18.51 %. The seroprevalence of brucellosis in the Pandharpuri buffaloes from Satara was found to be higher (27.27 %) than that in buffaloes from Kolhapur (16.27 %).

All the 54 sera were also tested by STAT for quantitative detection of anti-*Brucella* antibodies. A total 6 out of the 54 sera were positive for presence of *Brucella* antibodies. The overall seroprevalence of brucellosis detected by STAT was 11.11 %. Location-wise analysis of results of STAT revealed that the seroprevalence in Kolhapur district was 11.62 % where as that in Satara district was 9.09 %.

The amplification of 3' UTR of *SLC11A1* gene was carried out by PCR and amplification products generated were evaluated by agarose gel electrophoresis and visualized under gel documentation system. All the 54 samples yielded specific amplification product of ~193 bp.

Out of 54 amplicons generated from 3' UTR of *SLC11A1* gene PCR, 42 were subjected to nucleotide sequencing. The results of sequencing revealed that the sizes of amplicons of 3' UTR of *SLC11A1* gene generated from the samples ranged between 193 to 199 bp. Further, the sizes of amplicons observed in the present investigation matched to the sizes of 3' UTR of *SLC11A1* gene sequences published earlier (GenBank accession nos. DQ645387, DQ645389 and DQ645388).

The sequences of amplicons of 3' UTR of *SLC11A1* gene were analysed using bioinformatic tools and actual number of GT repeats for each allelic variant was confirmed. The alleles showing 13, 14, 15 and 16 GT repeats were designated as $(GT)_{13}$, $(GT)_{14}$, $(GT)_{15}$, and $(GT)_{16}$ genotypes respectively.

The results of distribution of buffaloes in to different genotypes revealed that the allele $(GT)_{14}$ with a proportion of 35.71 % occurred most frequently followed by $(GT)_{15}$ with a proportion of 30.95 %. The proportion of genotype $(GT)_{13}$ was comparatively lower (23.80 %) than those of $(GT)_{14}$ and $(GT)_{15}$ where as that of $(GT)_{16}$ was lowest (9.52%).

Out of the 42 samples subjected to nucleotide sequencing, 8 were from serologically positive buffaloes. The data on genotype of buffaloes and their status of brucellosis indicated that a major proportion of 75% seropositive buffaloes belonged to genotype $(GT)_{14}$ followed by genotype $(GT)_{15}$ to which 25% buffaloes belonged. It was interesting to note that none of the sample of buffaloes belonging to genotype $(GT)_{13}$ was positive for brucellosis.

The results of the present study suggest association of genotype $(GT)_{13}$ of Pandharpuri with natural resistance to brucellosis which could be due to improved macrophage function and intracellular killing of *B. abortus*.

Following conclusions could be derived out of the present investigation.

1. The overall seroprevalence of brucellosis in Kolhapur and Satara districts was 18.51%.
2. The seroprevalence of brucellosis in Satara district was higher (27.27%) than Kolhapur (16.27 %).
3. The 3' UTR of *SLC11A1* of Pandharpuri buffaloes could be successfully amplified by PCR generating amplicons of sizes ranging from 193 to 199 bp.
4. Four alleles 3' UTR of *SLC11A1* viz. $(GT)_{13}$, $(GT)_{14}$, $(GT)_{15}$ and $(GT)_{16}$ could be identified on the basis of sequencing.

5. All the 4 alleles viz. $(GT)_{13}$, $(GT)_{14}$, $(GT)_{15}$ and $(GT)_{16}$ were homozygous.
6. The animals with genotypes $(GT)_{14}$ and $(GT)_{15}$ showed seropositivity for brucellosis of 14.28% and 4.78 % respectively.
7. None of the animal of genotype $(GT)_{13}$ was serologically positive for brucellosis suggesting association of genotype $(GT)_{13}$ with natural resistance to brucellosis.
8. Interestingly $(GT)_{16}$ animals (n=4) revealed seropositivity by RBPT and STAT, however for a definitive conclusion a large number of $(GT)_{16}$ animals needs serological testing.
9. Further studies in this respect should focus on investigating the anti-brucellar activity of macrophages of different genotypes against B. abortus by real time PCR of *SLC11A1*.

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ABSTRACT

The present investigation dealt with characterization of 3' UTR of the natural resistance associated macrophage protein (*NRAMP1* also referred as *SLC11A1*) gene in relation to natural resistance against brucellosis. A total of 54 Pandharpuri buffaloes from Satara and Kolhapur districts of Maharashtra state were screened for presence of anti-*Brucella* antibodies by RBPT and STAT and characterization of 3' UTR of *SLC11A1* gene of both, *Brucella* positive and negative buffaloes were carried out.

Out of the 54 sera samples 10 were found positive for presence of *Brucella* antibodies with an overall seroprevalence 18.51 %. The seroprevalence of brucellosis in the Pandharpuri buffaloes from Satara was found to be higher (27.27 %) than that in buffaloes from Kolhapur (16.27 %).

The amplification of 3' UTR of *SLC11A1* gene by PCR yielded specific amplification product of ~193 bp in all the samples. Out of 54 amplicons generated from 3' UTR of *SLC11A1* gene PCR, 42 were subjected to nucleotide sequencing. The results of sequencing revealed that the sizes of amplicons of 3' UTR of *SLC11A1* gene generated from the samples ranged between 193 to 199 bp. Further, the sizes of amplicons observed in the present investigation matched to the sizes of 3' UTR of *SLC11A1* gene sequences published earlier (GenBank accession nos. DQ645387, DQ645389 and DQ645388).

Analysis of sequences of amplicons of 3' UTR of *SLC11A1* gene for presence of GT repeats revealed existence of 4 alleles showing 13, 14, 15 and 16 GT repeats that were designated as $(GT)_{13}$, $(GT)_{14}$, $(GT)_{15}$, and $(GT)_{16}$ genotypes respectively. The allele $(GT)_{14}$ with a proportion of 35.71 % occurred most frequently followed by $(GT)_{15}$ with a proportion of 30.95 %. The proportion of genotype $(GT)_{13}$ was comparatively lower (23.80 %) than those of $(GT)_{14}$ and $(GT)_{15}$ where as that of $(GT)_{16}$ was lowest (9.52%). This is the first study reporting $(GT)_n$ microsatellite polymorphism at 3' UTR of *SLC11A1* gene in Pandharpuri breed of buffalo.

The data on genotype of buffaloes and their status of brucellosis indicated that a major proportion of 75% seropositive buffaloes belonged to genotype $(GT)_{14}$ followed by genotype $(GT)_{15}$ to which 25% buffaloes belonged. It was interesting to note that none of the sample of buffaloes belonging to genotype $(GT)_{13}$ was positive for brucellosis.

The results of the present study suggest association of genotype $(GT)_{13}$ of Pandharpuri with natural resistance to brucellosis which could be due to improved macrophage function and intracellular killing of *B. abortus*.

APPENDICES

Appendix – I

Reagents used for DNA extraction

1) Equilibration of phenol

Warm the phenols stored at – 20^0C to room temperature, and melt it at 60^0C. Add Hydroxy quinoline to final concentration of 0.1 % to the melted phenol, add an equal volume of 0.5 M tris-CL (pH 8.0). Stir the mixture for 15 minutes; decant the aqueous phase after clear separation of two phases. Repeat the extraction with 0.1 M tris-CL (pH 8.0) till the pH of phenolic phase becomes more than 7.8. After removing the final aqueous phase add 0.1 volume of 0.1 M tris-CL (pH 8.0) containing 0.2 % β – mercaptoethanol and store at 4^0C in amber color bottle.

2) 1 M Tris HCL (pH 8.0)

Dissolve 24.288 g Tris buffer in 120 ml distill water and adjust pH to 8 with concentrated HCl. Adjust the final volume to 200 ml with distilled water. Filter through Whatman filter paper no. 1 and sterilize by autoclaving at 15 lbs for 15 min. Store at refrigeration temperature.

3) 1 M Tris HCL (pH 7.65)

Dissolve 24.288 g Tris buffer in 120 ml distill water and adjust pH to 7.65 with concentrated HCl. Adjust final volume to 200 ml with distilled water. Filter through Whatman filter paper no. 1 and sterilize by autoclaving at 15 lbs for 15 min. Store at refrigeration temperature.

4) 10% SDS

Dissolve 5 g SDS in 40 ml distilled water at 68 ^{O}C and adjust final volume to 50 ml with distilled water. Filter through Whatman filter paper no. 1and store at room temperature.

5) CTAB – NaCl solution (10 % solution in 0.7M NaCl))

Dissolve 2 g CTAB (cetyle hexadecyle ammonium bromide, in 15 ml 0.7M NaCl at 60 ^{O}C; adjust final volume to 20 ml with 0.7M NaC and store at room temperature.

6)1 M NaCl

NaCl : 5.844 g
Distilled water : 100 ml

7) 5 M NaCl

NaCl : 29.22 g
Distilled water : 100 ml

8) TE buffer

Tris HCL (pH 8.0) :10 mM
EDTA (pH 8.0) :1 mM

9) RBC lysis buffer

Tris HCl (pH 7.65) :17 mM
NH_4Cl :140 mM

10) Leukocyte lysis buffer

Triton X 100 : 2 %
SDS : 1%
NaCl : 100 mM
Tris HCl (pH 8) : 10 mM

12) NET buffer (pH 7.6)

NaCl	: 50 mM
EDTA	: 125 mM
Tris HCl (pH 7.6)	: 50 mM

13) Proteinase K (20 mg / ml)

Proteinase K	: 25 mg
Distilled water	: 1.250 ml

14) P:C:I

Phenol: Chloroform: Isoamyl alcohol (25: 24: 1)

15) C: I

Chloroform: Isoamyl alcohol (24: 1)

16) 70% Ethanol

Absolute ethanol 70 ml + distilled water 30 ml

17) Lysozyme (10 mg/ml)

Lysozyme	: 10 mg
Distlled water	: 1 ml

.

Appendix – II

Reagents used for Agarose Gel Electrophoresis (AGE)

1) Ethidium bromide (10 mg / ml)

Ethidium bromide : 20 mg
Distilled water : 2 ml

2) 0.5 M EDTA (pH 8.0)

Dissolve 18.612 g EDTA $2H_2O$ in 80 ml distill water and adjust pH to 8.0 using NaOH pellets. Adjust the final volume to 100 ml. Filter through Whatman filter paper no. 1 and sterilize by autoclaving at 15 lbs for 15 min. Store at room temperature.

3) Tris – Acetate – EDTA (TAE) stock solution (50 X)

Tris base : 24.2 g
Glacial acetic acid : 5.7 ml
0.5 M EDTA, pH 8.0 : 10 ml
Distilled water to make : 100 ml

(For preparing the working solution (1X), dilute the stock TAE in distilled water)

4) 6X Loding dye (Type IV)

Sucrose : 40% w/v in DW
Bromophenol blue : 0.25% w/v in DW
Store the solution at 4 ^{0}C.

Appendix – III

List of equipments

Some of the important equipments used during the study were as shown below.

Sr. No.	Name	Manufacture
1	Micropipettes	Eppenndorf
2	Microcentrifuge	REMI
3	Vortex	Genei
4	Microwave oven	L.G.
5	Nanodrop spectrophotometer	ND 1000 Thermo Scientif
6	Ice maker	Sanyo, Japan
7	Deep freeze – 20 ^{o} C	Voltas TATA
8	Power pack	Consort
9	Water bath	Biotechnics India
10	Thermal cycler	Eppenndorf
11	Submarine gel electrophoresis apparatus	Superfit
12	Gel documentation system	Uvitec
13	Mini centrifuge	Genei
14	pH meter	Toshniwal Instruments, India
15	Weighing balance	Contech
16	Sequencer	ABI Applied Biosystems

VITA

The author of this manuscript Dr. Bhushan Subhash Chaudhari was born on 6th August 1987 in Bhusawal of Jalgaon district, Maharashtra. He did his schooling at Ganesh Vidya Mandir, Kalyan and passed his Secondary School Certificate Examination in first class in the year 2002 and Higher Secondary School Certificate Examination in the year 2004 from Model Junior College, Kalyan.

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