DEVELOPMENT OF INDIRECT ELISA FOR HUMAN BRUCELLOSIS AND SERO-EPIDEMIOLOGICAL SURVEY OF HUMAN BRUCELLOSIS IN KARNATAKA STATE

A THESIS

Submitted by

ANNAPURNA S. AGASTHYA

in partial fulfillment for the award of the degree of

DOCTOR OF PHILOSOPHY

Department of Biotechnology
FACULTY OF HUMANITIES AND SCIENCE

Dr. MGR
EDUCATIONAL AND RESEARCH INSTITUTE UNIVERSITY
(Decl. U/S -3 of the UGC Act 1956)
CHENNAI – 600 095
OCTOBER 2008
BONAFIDE CERTIFICATE

Certified that this thesis titled “DEVELOPMENT OF INDIRECT ELISA FOR HUMAN BRUCELLOSIS AND SERO-EPIDEMIOLICAL SURVEY OF HUMAN BRUCELLOSIS IN KARNATAKA STATE” is the bonafide work of MRS. ANNAPURNA S. AGASTHYA who carried out the research under my supervision. Certified further that to the best of my knowledge the work reported herein does not form part of any other thesis or dissertation on the basis of which a degree or award was conferred on an earlier occasion of this or any other candidate.
DECLARATION

I declare that this thesis titled “DEVELOPMENT OF INDIRECT ELISA FOR HUMAN BRUCELLOSIS AND SERO-EPIDEMIIOLOGICAL SURVEY OF HUMAN BRUCELLOSIS IN KARNATAKA STATE” is the bonafide work carried out under the supervision of Dr. Prabhudas K., Director, Project Directorate on Animal Disease and Monitoring and Surveillance, Hebbal, Bangalore-560024. Declared further that to the best of my knowledge the work reported herein does not form part of any other thesis or dissertation on the basis of which a degree or award was conferred on an earlier occasion of this or any other candidate.

SIGNATURE

Annapurna S.Agasthya
Ph.D. Candidate
Dr.M.G.R. University
Chennai-600095.
ABSTRACT

Brucellosis is a disease of considerable economic and social importance caused by different species of *Brucella*. The disease is mainly an occupational disease in India and not an uncommon disease. The development of a highly sensitive, specific and definitive diagnostic for brucellosis remains as elusive target. The indirect Enzyme Linked Immunosorbent Assays can measure M, G and A immunoglobulin, which allows for a better interpretation of the clinical situation because of higher sensitivity and specificity compared to conventional serological tests.

The present work was undertaken at Project Directorate on Animal Disease Monitoring and Surveillance, Bangalore to study the sero-prevalence of human brucellosis and endemicity of infection in different parts of Karnataka state by using the conventional Rose Bengal Plate Agglutination Test (RBPT), Standard Tube Agglutination Test (STAT) and an indigenously standardised indirect Enzyme Linked Immunosorbent Assay (ELISA).

The study group was consisting of 618 professionals (Group 1), 1500 cases of Pyrexia of Unknown Origin (Group 2) and 652 individuals (Group 3) without a history of fever but with consistent joint pain, backache etc., making a total of 2770 samples.

The analysis of 2770 serum samples by RBPT and STAT had revealed 4.98% (138 positives) sero-prevalence and 11.58% by indirect ELISA (321 positives). All the sero-positive cases were distributed between 10 to 60 years of age. The sero positivity for a total of 2770 samples screened was 91.58% in males and 8.41% in females. Sero positivity was found to be more in the age group of 41-50 years (38%), followed by 31-40 years (37.07%), followed by 21-30 years (14.33%), followed by 51-60 years (6.85%), followed by 11-20 years (2.8%) with the least prevalence recorded in the age group < 10 years (0.93%).
The 618 serum samples from professionals had shown 2.26% (14 positive) of sero-positivity by RBPT and STAT and 15.69% (97 positives) by indirect ELISA. Of all the 97 sero-positive professionals, the highest rate of sero-prevalence of brucellosis was observed among the veterinary inspectors (41.23%) followed by veterinary assistants (30.92%), veterinary officers (12.37%), veterinary supervisors (6.18%), animal attendants i.e. Group D (6.18%), shepherd (2.06%) and butcher (1.03%).

The 1500 serum samples collected from Pyrexia of Unknown Origin (PUO) cases, had given a sero positivity of 8.26% (124 positives) by RBPT, STAT and 13.6% (204 positives) by indirect ELISA. The sero positivity in males was 12% (180 positives) and 1.6% (24 positives) in females. The sero-prevalence was higher in males than females. The sero-positive cases were found to be highest between 20 to 40 years of age. The age group of <20 showed 0.8% prevalence, the age group > 20 years showed 7.46 % prevalence and the age group of > 40 years showed 5.33% of prevalence. A sero-prevalence of 9.27% (139 positives) and 4.33% (65 positives) had been observed in South Karnataka and North Karnataka respectively. In individuals with no history of fever but associated with joint pain, backache etc., the sero positivity was reported only by indirect ELISA and it was 3.06%, (20 positives).

All the samples which were tested positive by RBPT were also positive by STAT with agglutination titers of ≥ 1:80. The indirect ELISA done by using the smooth lipopolysaccharide (S-LPS) antigen of *Brucella abortus* 99 and *Brucella melitensis* 16 M biotype 1 was found more sensitive than the RBPT and STAT.

The present study showed that the brucellosis is still a professional hazard in the veterinary practitioners and the cases of brucellosis may be easily misdiagnosed because of the deceptive nature of the clinical signs and symptoms. All the cases that showed the presence of antibodies to *Brucella abortus* had varied clinical manifestations of brucellosis.
The clinicians should keep in mind the possibility of an occupational or environmental exposure in cases of fever. It would also be worthwhile to create awareness about the disease in such professionals so that necessary precautions and periodic screening of such occupationallly exposed people can be done. Elimination of the infection in animals by vaccination to produce *Brucella* free animals/animal products can prevent the infection in humans.
ACKNOWLEDGEMENT

I express my profound gratitude to my guide Dr. Prabhudas K., Director, Project Directorate on Animal Disease Monitoring and Surveillance (PD_ADMAS), Hebbal Bangalore -560024, for his able guidance and suggestions at crucial junctions in bringing out the thesis study successfully. My sincere thanks to Dr. M.G.R. University for providing me this opportunity to complete my research programme.

I also express my deep gratitude to Dr. Sri Krishna Isloor, Assistant Professor of Microbiology, Veterinary College, Hebbal-560024, who helped me to make the dissertation study successfully. I also express my sincere thanks to Dr. T. Prabhu, for the valid suggestions whenever required.

I would like to thank Dr. D.V.S.S.R. Prakash, Principal, Administrative Management College, Bangalore-560083, for the encouragement to undertake this study. I would like to thank Mr. Subrahmanya Agasthya, my husband for his moral support. My sincere thanks to the Deputy Directors of the three districts (Bidar, Bagalakote and Tumkur) of Karnataka state from where the serum samples are collected from Veterinary and Para-veterinary professionals. My sincere thanks to the clinicians and laboratory personals for helping me in collecting the serum samples from different clinical conditions.

My special thanks to Dr. B.G. Mantur (Belgaum) for providing the positive serum samples and Dr. H.G. Honnappa, Veterinary College, Bangalore for helping in the statistical analysis of the research data. I express my sincere thanks to Dr. Kakoli Ahmed for her excellent technical support and PD_ADMAS staff for their co-operation in the study.

PLACE: BANGALORE

ANNAAPURNAS.AGASTHYA
ABSTRACT iii
LIST OF TABLES xiv
LIST OF FIGURES xvii
LIST OF SYMBOLS, ABBREVIATIONS Xviii

1 INTRODUCTION 1
1.1 Outline of thesis

2 LITERATURE REVIEW 6
2.1 Brucellosis – World scenario
2.2 Brucellosis – in India
2.3 General characteristics of Brucella species
2.4 Sources of infection and transmission
2.5 Pathogenesis
2.6 Clinical Features
2.7 Laboratory Diagnosis of brucellosis
2.8 Treatment and Prevention

3 OBJECTIVES OF THE PRESENT WORK 19
3.1 The objectives of the study included
3.2 The parameters of the study

4 EXPERIMENTAL WORK 20
4.1 GENERAL CONSIDERATIONS
4.2 Cultural, biochemical and serological studies.
   4.2.1 Tryptose agar media
   4.2.2 Motility test
   4.2.3 Gram’s stain
   4.2.4 Oxidase test
   4.2.5 Urease test
   4.2.6 Basic fuchsin and Thionin for dye inhibition test
   4.2.7 Tests for confirmation of smooth phase of Brucella culture
      4.2.7.1 Serum agglutination test
4.2.7.2 Acriflavin test
4.2.7.3 Crystal Violet staining of bacterial cultures
4.3 S-LPS Extraction
4.3.1 Isolation of smooth – lipopolysaccharide (S-LPS) of Brucella species
4.3.2 Cultivation of Brucella abortus 99 and Brucella melitensis 16 M biotype 1
4.3.2.1 Removal of lipid contamination
4.3.2.2 Hot phenol extraction of S-LPS
4.3.2.3 Enzymatic digestion of extract
4.4 SDS-PAGE analysis of S-LPS
4.4.1 SDS-PAGE
4.4.2 Staining SDS-PAGE gels with Coomassie brilliant blue

5 ANALYSIS PROCEDURE

5.1 Clinical material
5.1.1 Proforma (Used for collection of history of patients)
5.1.2 Study group
5.2 Serological tests
5.3 Rose Bengal Plate Agglutination Test
5.4 Standard Tube Agglutination Test (STAT)
5.5 Screening of samples by indirect ELISA
5.6 Procedure for indirect ELISA
5.6.1 Requirements
5.6.2 Optimization of Brucella antigen (S-LPS)
5.6.3 Coating the micro plate
5.6.4 Addition of test and control sera
5.6.5 Addition of the conjugate
5.6.6 Addition of substrate/Chromogen and stopping solution
5.7 Interpretation of the results for Indirect ELISA

5.8 Layout of the plate

6 RESULTS

6.1 Cultural and biochemical confirmation of *Brucellae*
   6.1.1 Morphologic and growth characteristics
   6.1.2 Biochemical properties
   6.1.3 ‘Phase’ confirmation of *Brucellae*
   6.1.4 Protein estimation of *Brucella abortus* and *Brucella melitensis* antigen

6.2 Standardisation of indirect ELISA for the assay of *Brucella* antibodies
   6.2.1 Result of checker board titration
   6.2.2 *Brucella* S-LPS control sera
   6.2.3 Protocol for indirect ELISA

6.3 Study group
   6.3.1 Sero positivity for brucellosis and its age and sex distribution
   6.3.2 Result of screening
   6.3.3 Ratio among the three serological tests

6.4 Agreement between the tests: The Kappa statistic
   6.4.1 Between indirect ELISA and RBPT
   6.4.2 Between indirect ELISA and STAT

   6.4.3 Agreement between the tests: - The Kappa statistics

6.5 Sero positivity for brucellosis in group I, II and III
   6.5.1 Result: For group: I (In professionals)
   6.5.2 Analysis of positive cases
   6.5.3 Ratio among the tests
   6.5.4 Ratio among the tests
6.5.5 Distribution of sero-positive cases on the basis of clinical signs and symptoms
6.5.6 Sero-prevalence among professionals
6.5.7 Incidence of infection in professionals
6.5.8 Inference
6.5.9 Geographical distribution of brucellosis
6.5.10 Distribution of positive serum samples according to gender
6.5.11 The influence of age in the incidence of brucellosis

6.6 Result for Group II: Sero-prevalence of brucellosis in Pyrexia of Unknown Origin cases
6.6.1 Distribution of sero-positive and sero-negative individuals based on sex
6.6.2 Sero-prevalence analysis by $X^2$ test
6.6.3 Influence of age on sero-prevalence of brucellosis
6.6.4 Analysis of age group, sero-positive and sero-negative results of brucellosis
6.6.5 The geographical distribution of sero-positive and sero-negative individuals in South Karnataka and North Karnataka
6.6.6 Sero-prevalence percentage of brucellosis in South Karnataka and North Karnataka based on sex and place
6.6.7 Relative efficiency of serological tests employed for diagnosis of brucellosis
6.6.8 Calculation of Kappa value for RBPT and indirect ELISA and STAT and indirect ELISA

6.7 Group III: Analysis of data for non-PUO cases
6.7.1 Geographical distribution of brucellosis in
Karnataka

6.7.2 Endemicity of brucellosis in Karnataka

6.7.2.1 Endemicity of brucellosis in professionals

6.7.2.2 Endemicity of brucellosis in Pyrexia of Unknown Origin cases

6.7.2.3 Endemicity of brucellosis in non-Pyrexia of Unknown Origin cases

7 DISCUSSION

7.1 Identification of study groups

7.2 Interpretation of the serological tests for 2770 samples

7.3 Group I: Interpretation of the result for the professionals

7.4 Group II: Interpretation of the result for the PUO cases

7.5 Group III: Interpretation of results for non–PUO cases

8 CONCLUSIONS AND SCOPE FOR FUTURE WORK

8.1 CONCLUSION

8.1.1 The individual category of samples has given the following observations

8.2 SCOPE FOR FUTURE WORK

9 APPENDICES

10 REFERENCES

11 LIST OF PUBLICATIONS
LIST OF TABLES

1. Table: 4. 1. Sources of *Brucella* strains. 20

2. Table: 6.1. Morphologic and growth characteristics. 40

3. Table: 6.2. OD values for positive control serum. 53

4. Table: 6.3. OD values for negative control serum. 55

5. Table: 6.4. Influence of age and sex on brucellosis occurrence. 59

6. Table: 6.5. Depicting result of serological tests for RBPT, STAT and indirect ELISA. 61

7. Table: 6.6. Agreement between indirect ELISA and RBPT. 63

8. Table: 6.7. Agreement between indirect ELISA and STAT. 49

9. Table: 6.8. Results of serological tests (RBPT, STAT and indirect ELISA). 51

10. Table: 6.9. Analysis of positive result detected by RBPT, STAT and indirect ELISA. 51

11. Table: 6.10. Ratio among the tests (RBPT, STAT and indirect ELISA). 52

12. Table: 6.11. Distribution of sero-positive individuals based on clinical signs and symptoms. 52

13. Table: 6.12. Depicts the result of screening among professionals. 53

14. Table: 6.13. Incidence of infection in different category of
professionals.


16 Table: 6.15. Depicts sex wise distribution of positive cases.

17 Table: 6.16. The Influence of age on the incidence of brucellosis among professionals.

18 Table: 6.17. Prevalence of infection based on sex for sero-positive and sero-negative result.

19 Table: 6.18. X² test for analysis of sero-prevalence.


21 Table: 6.20. Age group analysis for brucellosis results.

22 Table: 6.21. Depicts the result of screening in South Karnataka and North Karnataka.

23 Table: 6.22. Depicts sero-prevalence of brucellosis in Karnataka based on sex and place.

24 Table: 6.23. Relative efficacy of RBPT and indirect ELISA.

25 Table: 6.24. Relative efficacy of STAT and indirect ELISA.

26 Table: 6.25. Age wise distribution of sero-positive and sero – negative individuals, (non-PUO cases).


28 Table: 6.27. Endemicity of brucellosis in professionals in Karnataka (Data relevant to the study of 618 cases).
Table 6.28. Endemicity in North Karnataka, (Data relevant to the study of samples from Pyrexia of Unknown Origin cases.)

Table 6.29. Endemicity in South Karnataka (Data relevant to the study of samples from Pyrexia of Unknown Origin cases).

Table: 6.30. Endemicity in South Karnataka (Data relevant to the study of samples taken from patients with no history of fever).

Table: 6.31. Endemicity in North Karnataka, (Data relevant to the study of samples taken from patients without history of fever).

Table 7.1: Brucellosis sero-prevalence by RBPT, STAT and indirect ELISA and dot-ELISA.
LIST OF FIGURES

Figure 4.1  Bulk cultivation of *Brucella* organisms. 24
Figure 4.2  Culture during removal of lipid contamination. 26
Figure 4.3  Hot phenol extract. 27
Figure 4.4  S-LPS during dialysis. 29
Figure 4.5  Lyophiliser for lyophilisation of S-LPS. 30
Figure 4.6  SDS Page analysis of S–LPS, (Only marker proteins are eluted) 32
Figure 6.1  Titration of conjugate and S-LPS for indirect ELISA. 42
Figure 6.2  Standardised indirect ELISA. 44
Figure 6.3  ELISA reader used for taking OD values. 45
Figure 6.4  Influence of sex and age on the occurrence of brucellosis. 47
Figure 6.5  Sex wise distribution of brucellosis. 56
Figure 6.6  Influence of age on the sero-prevalence of brucellosis. 58
Figure 6.7  Geographical distribution in South Karnataka and North Karnataka. 60
Figure 6.8  Sero-prevalence based on sex and place. 62
Figure 6.9  Age wise distribution of brucellosis serology. 65
Figure 6.10  Geographical distribution of brucellosis in Karnataka. 67
Figure 6.11  Endemicity of brucellosis among professionals in Karnataka. 69
Figure 6.12  Endemicity of brucellosis in North Karnataka. 71
Figure 6.13  Endemicity of brucellosis in South Karnataka. 73
Figure 6.14  Endemicity in South Karnataka: In patients without a history of fever, (Non–PUO). 75
Figure 6.15  Endemicity in North Karnataka: In patients without a history of fever, (Non- PUO). 76
LIST OF SYMBOLS, ABBREVIATIONS

1. ASLO: Anti-Streptolysin O Test.
2. CC: Conjugate Control.
3. C*: Moderate Positive
4. C**: Strong Positive
5. C*: Strong Negative.
6. CO₂: Carbon-Dioxide.
7. CRP: C - reactive protein Test.
8. °C: Degree Celsius.
9. iELISA: Indirect Enzyme Linked Immunosorbent Assay.
11. FCR: Folin-Ciocalteu Reagent
12. IgG-HRP: Immunoglobulin G - Horse Radish Peroxidase
13. <: Less than.
14. ≤: Less than or equal.
15. MA: Maximum Possible Agreement
16. µg/ml: Microgram per milliliter.
17. >: More than.
18. MW: Molecular weight.
22. OA: Observed Agreement.
23. %: Percentage.
24. PUO: Pyrexia of Unknown Origin.
25. RA Factor: Rheumatoid Arthritis Factor
26. RBPT: Rose Bengal Plate Agglutination Test.
27. STAT: Standard Tube Agglutination Test.
28. SDS: Sodium Dodoxyl Sulphate.
29. X²: Chi Square.
CHAPTER 1

INTRODUCTION

1.1 OUTLINE OF THESIS

Brucellosis is a recognised public health problem with worldwide distribution and one of the major causes of mortality and morbidity. It is also a disease of considerable economic and social importance. Brucellosis is one of the most important reemerging zoonosis in many countries. The \textit{Brucella} species, particularly \textit{Brucella melitensis} and \textit{Brucella suis} are potential agents of biological terrorism (Meslin, 1997; Wright, 2001). Brucellosis has been present for millennia (Capasso, 2002). A high prevalence in certain geographic areas is well recognized, although largely underestimated. More than 500,000 new cases are reported each year, and according to the World Health Organization this figure underestimates the magnitude of the problem (Corbel and MacMillan, 1997).

Brucellosis is caused by Gram-negative coccobacillus belonging to genus \textit{Brucella} named after Sir David Bruce, who first isolated this microbe. The etiological agent, mode of transmission, clinical manifestations and disease epidemiology were known only in the late 19\textsuperscript{th} and early 20\textsuperscript{th} centuries as a result of pioneering work by several investigators. The cause of the disease was obscure until Bruce reported numerous small coccal organisms in stained sections of spleen from a fatally infected soldier. He named the organism \textit{Micrococcus melitensis} (Madkour and Kasper, 2001). Brucellosis has been known by several names like undulant fever, Mediterranean fever, Gibraltar fever, Malta fever etc., (Wright, 2000).

Brucellosis is mainly an occupational disease also in developed countries like U.S.A. and European Countries, where \textit{Brucella melitensis} and \textit{Brucella abortus} are frequent pathogens. The \textit{Brucella abortus} is prevalent worldwide. \textit{Brucella suis} is endemic in Southern USA, South East Asia and Latin America. \textit{Brucella canis} infection is reported from Latin America, Central Europe and Japan (Williams, 1982; Corbel and MacMillan, 1997; Young, 2000).
Human brucellosis is not an uncommon disease in India. Animal brucellosis is reported from every state in India. However, no statistical information is available about the extent of infection in man in various parts of the country (Park, 1997). The occurrence of disease has been reported from every corner of the country (Shukla, 1962; Koshi and Myers, 1967; Bal and Tyagi, 1971; Panjarathinam and Jhala, 1986).

Brucellosis is an important zoonosis in India, which accounts for loss of approximately 30 million man-days annually. The disease is predominantly an occupational disease in India, as shown by most of the sero epidemiological studies done in India. Most of the diagnosed cases of brucellosis in all the studies were found to be engaged in occupations (Veterinarians, abattoir workers, farm workers) in which they are at increased risk of developing brucellosis (Roy et al., 1965; Koshi and Myers, 1967; Nagalotimath and Joglekar, 1978). The disease prevalence in India varies from 0.8% to 24.47% as per the study done in different states in India. Brucellosis is mainly documented from northern and costal areas of Karnataka (Roy et al., 1965; Panjarathinam and Jhala, 1986; Mantur et al., 1994; Park 1997; Kadri et al., 2000).

The prevention of emergence or reemergence of brucellosis also requires the knowledge about magnitude of problem in a given geographical area. Persons infected with Brucella species, usually have signs and symptoms consistent with an influenza like or septicemic illness, often with insidious onset. The symptoms and clinical signs most commonly reported are fever, fatigue, malaise, chills, sweats, headaches, myalgia, arthralgia, weight loss (Young, 1983; Young, 1989).

The development of a definitive diagnostic test for brucellosis remains an elusive target. Brucellosis is diagnosed either by isolation of Brucella organisms in culture or by a combination of serological tests and clinical findings consistent of brucellosis. Isolation of the Brucella organisms is the definitive means of diagnosis but in practice it is difficult due to the early tissue localization, exacting culture requirements of the organism and also prolonged time required for isolation. In practice blood cultures are positive in 10-30% of brucellosis and the remaining is diagnosed serologically (Young, 1983).
The prevalence of human brucellosis is difficult to estimate as many cases remain undiagnosed or misdiagnosed as pyrexia of unknown origin because either they are inapparent or of their protean manifestations (Meslin, 1997).

There is presently a wide battery of serological tests, which can be used for diagnosis of human brucellosis, although they each have important limitations. Their sensitivity is poor in the early stage of the disease, during which the levels of antibodies can still be low, and their specificity is reduced in areas where the disease is highly endemic, in exposed professionals, and in the frequent relapses of the disease (Kiel and Khan, 1987; Young, 1991; Arija et al., 1992).

Most patients with acute brucellosis produce antibodies of the IgM isotype within a few days of onset of the disease. These antibodies are rapidly followed and superseded by IgG, and to a lesser extent IgA, antibodies. Maximum titers are reached in the third or fourth week of disease and then slowly decline; however, antibodies usually persist throughout the active phase of the disease and in some cases for long after. In sub acute or chronic brucellosis this pattern is generally not seen, the serological response consisting of a sustained production of IgG and sometimes IgA antibodies (Arija et al., 1992).

Indirect Enzyme Linked Immunosorbent Assay (ELISA) typically uses cytoplasmic proteins as antigens. The ELISA measures class M, G, and A immunoglobulin, which allows for a better interpretation of the clinical situation and overcomes some of the shortcomings of the serum agglutination test. An evaluation of an ELISA for the diagnosis of brucellosis with the standard agglutination test carried out by Elizabeth et al in Vellore (1996) revealed that ELISA is superior to the standard agglutination test. A study carried out by Araj et al (2005) showed that ELISA in general is considered and recognized as the test of choice in case of clinical suspicion of brucellosis, even when the Coombs test is negative.

Numerous serological procedures have been tried in the diagnosis of human brucellosis but few have achieved lasting application. Until recently, the most widely used was the
Standard Tube Agglutination Test. In many laboratories this has now been superseded by the Rose Bengal Plate Test and Enzyme-Linked Immunosorbent Assay, (ELISA). Other useful tests include the 2-mercaptoethanol agglutination test, the complement fixation test, and the Coombs antiglobulin test and radioimmunoassay (Magee, 1980). The potential use of an indirect ELISA for diagnosis of human brucellosis has been reported by various investigators (Magee, 1980; Hunter et al., 1986; Araj and Kauffman, 1989; Gad and Kambal, 1998; Lucero et al., 2005; Ertek et al., 2005; Araj et al., 2005; Hussein et al., 2005; Rajaii et al., 2006)). The enzyme immunoassay is an effective method for diagnosing acute and chronic brucellosis and for detecting antibodies in CSF of patients with neurobrucellosis. The ELISA is as sensitive as Radio Immuno Assay (Corbel and MacMillan, 1997).

Keeping this view in mind, the present work is undertaken to study the sero-epidemiology of human brucellosis in different parts of Karnataka state to know the endemicity of the disease and also to carry out the standardisation of an indirect ELISA for the sero-diagnosis of human brucellosis to achieve an advancement in the disease diagnostics and also to make a comparative study of the standardized indirect ELISA with the conventional techniques like Rose Bengal Plate Agglutination Test and Standard Tube Agglutination Test. Only a little information is available about the prevalence of the infection in the state.

Although in the last few years Polymerase Chain Reaction (PCR) based laboratory tests have been proposed, but they cannot be considered as a routine diagnostic method yet. These limitations make serology the most useful tool for laboratory diagnosis of Brucella infection (Matar et al., 1996; Romero et al., 1995).

Sero-epidemiological surveys form the backbone of the work to establish disease prevalence and incidence rates. The diagnostic tools employed for sero epidemiological surveys must be highly sensitive and specific, user friendly and economical. In this reference, the development of software based indirect ELISA at Project Directorate on Animal Disease Monitoring and Surveillance, to screen Brucella antibodies is considered appropriate, cost effective and feasible for use.
This prospective study deals with the usefulness and significance of indirect ELISA in the diagnosis of human brucellosis in an endemic area in which livestock forming is the main occupation. The area involves the different parts of Karnataka state, which include the city, tahassels and villages. The focus of this work is to develop a highly sensitive and specific diagnostic test for the detection of anti *Brucella* antibodies in human serum samples and also to identify the endemic foci of *Brucella* infection in different parts of Karnataka state, to know the prevalence of the infection.

The main purpose of this work is to evaluate the roles of classical serological methods and indirect ELISA in the diagnosis of human brucellosis in the population studies, in order to design the diagnostic protocols applicable in all hospital practices and to study the endemicity of the infection in different parts of the Karnataka state. The serum samples are collected from the individuals with a history of Pyrexia of Unknown Origin, individuals in whom fever is not the predominant feature with a complaint of persistent joint pain, low backache and had shown negative result for Anti streptolysin O (ASLO), C- reactive protein test (CRP), Rheumatoid arthritis factor (RA) and from the professionals like Veterinary, Para-veterinary staff and other occupations like shepherd, animal owners and butcher.

In the first and second phase all the serum samples are screened by Rose Bengal Plate Agglutination Test (RBPT) and Standard Tube Agglutination Test. In the third phase all the serum samples are subjected to the standardised indirect ELISA by using smooth lipopolysaccharide extracted from *Brucella abortus 99 and Brucella melitensis 16 M biotype 1*. The result obtained by indirect ELISA is compared with the conventional RBPT and STAT and discussed for the higher efficiency of indirect ELISA as well as sero-prevalence of human brucellosis.
CHAPTER 2

LITERATURE REVIEW

2.1 Brucellosis – World scenario

Because of the variable clinical manifestations of brucellosis, only an estimated 4%-10% of cases are recognized and reported in the United States (Kaufmann and Wenger, 1992). Brucellosis has managed to elude eradication, even in most developed countries (Young, 1995; Corbel and MacMillan, 1997).

The highest prevalence of human brucellosis occurs in countries of the Iberian Peninsula and Mediterranean littoral in Europe. Occasional cases are reported from Althania and Yugoslavia. In northern Europe the Brucella abortus accounted for most of the cases of brucellosis in northern Europe and the bovine brucellosis were controlled. The human cases now seen are due to Brucella melitensis acquired in other countries (Corbel and Macmillan, 1997). In Mediterranean Countries the annual incidence of brucellosis varies from 1-78 cases per 100000 population and more than 550 cases per 100000 populations have been reported in the confirmed areas where no animal control measures are applied (Cosivi and Siemens, 1998). Occupational transmission of brucellosis remains a public health hazard particularly among persons exposed to swine. In United States, the incidence of human brucellosis peaked shortly after the second world war and declined there after more or less of incidentally with control of Brucella abortus in cattle. Currently about 100 cases are reported annually to CDC since 1996. In USA, the main pathogen is Brucella abortus and the infection is typically an occupational disease. In countries where the disease in animals has been brought under control, it can reappear sporadically in individuals who acquire the infection from abroad, usually by consuming illegally imported and unsafe products (Arambulo, 1998).

The population of Yemen lies in the south of the Arabian Peninsula. It has open borders for sheep, goats and Camels with Saudi Arabia and Oman, its neighboring countries. Goat and sheep breeding is more important than cattle and camel breeding in all regions of the country (Al-Shamahy, 1999).
The regions where the *Brucella melitensis* is endemic are Mediterranean Countries (Spain, Portugal, Italy, and Greece), Middle East countries (Iran, Iraq, Kuwait, Saudi Arabia, Israel and Jordan), Latin American countries (Peru, Argentina, and Mexico) and Asia including Indian subcontinent (Gotuzzo, 1999; Young, 2000).

The incidence of human brucellosis in France and Spain is moderate. France reported an incidence of 0.74 cases per 1-lakh populations and Spain reported 1.04 cases per 1 lakh population. With in France the incidence is variable ranging from 1.07 to 18-24 cases per 1 lakh population in affected areas, while many areas are free of infection (Wright, 2000; Wright, 2001). The global incidence of brucellosis is not known due to variable quality of disease reporting and notification system in many countries. Worldwide, the only countries believed to be free of brucellosis are Norway, Sweden, Finland, Denmark, Ireland, Switzerland, Romania, Canada, Czech and Slovak republic (Madkour and Kasper, 2001).

Worldwide, reported incidence of human brucellosis in endemic disease areas varies widely, from <0.01 to > 200 per 100,000 population (Boschirol et al., 2001). Brucellosis has been present for millennia (Capasso, 2002). The disease is usually present in Hispanic populations and is probably related to the illegal importation of unpasteurised dairy products from neighboring Mexico, where the disease is endemic (Fosgate et al., 2002; Chang et al., 2003).

The importance of screening house-hold members of acute brucellosis cases in endemic areas like Saudi Arabia has recently been emphasized, screening family members of an index case of acute brucellosis detected additional cases (Almuneef et al., 2004).

Brucellosis, like tuberculosis, is a chronic granulomatous infection caused by intracellular bacteria and requires combined, protracted antibiotic treatment. The disease causes much clinical morbidity as well as a considerable loss of productivity in animal husbandry in the developing world. In this era of international tourism, brucellosis has become a common imported disease in the developed world (Georgios et al., 2005).
The sero-epidemiological study on human brucellosis in Assiut Governorate conducted by Hussein et al (2005) showed that brucellosis is the most important zoonotic disease constituting a public health problem in Assiut Governorate. A study conducted by Cadmus et al (2006) in Nigeria showed that brucellosis is still a major zoonosis in Nigeria.

The study done in Nigeria confirmed the endemicity of brucellosis especially bovine brucellosis among slaughtered cattle at the abattoir; hence making it a source of occupational hazard to workers who are directly involved in cattle meat processing (Farzin et al., 2007). Public health enlightenment should be focused on the zoonotic aspect of this disease as it relates to consumption of unpasteurised milk and other food items obtained from diseased animals.

Brucellosis is known to cause debilitating conditions if not promptly treated. In some rural areas of Tanzania however, practitioners give evidence of seeing brucellosis cases with symptoms of long duration (John et al., 2007). In Germany, brucellosis has emerged as a disease among Turkish immigrants. In this population group, the infection is associated with major diagnostic delays, possible resulting in treatment failures, relapses, chronic courses, focal complication and a high case-fatality rate (Sascha et al., 2007). The importance of screening family members of an index case of acute brucellosis in South east Iran has recently been shown to detect additional cases and improve the treatment because all family members may be exposed to a common source (Sharifi et al., 2007).

A retrospective chart review was performed of 20 patients who received a diagnosis of brucellosis over a period of 13 years at a large, tertiary care children’s hospital in Dallas, Texas. Diagnostic criteria, epidemiology, clinical presentations, and outcomes were recorded. The study concluded that childhood brucellosis in the United States is now an imported disease, primarily from Mexico (Mark and Shen, 2008). In a recent study done in Thassos of Greece showed that brucellosis is a disease of public health priority in Greece (Vorou et al., 2008).
2.2 Brucellosis – in India

Wright and Smith reported 10 cases of brucellosis in British soldiers serving in India in 1897 and this is the first report of human brucellosis in India (Mathur, 1954).

Human brucellosis in India is not an uncommon disease. The occurrence of disease has been reported from every corner of the country (Bal and Tyagi, 1971, Koshi and Myers, 1967; Shukla, 1962; Panjarathinam and Jhala, 1986). However, no statistical information is available about the extent of *Brucella* infection in man, in various parts of the country. The disease is of particular concern in India, as 80% of population resides in rural areas in close contact with livestock like cattle, sheep, goat etc., (Park, 1997).

In 1962 Shukla carried out analysis of 25 cases of PUO. Four cases out of 25 showed significant titers of *Brucella* antibodies (≥ 1:160) and all were sero-positive in dilutions more than 1:20 by STAT.

In 1963, Joga Rao reported seven cases of brucellosis during a brief period of 15 months in Srikakulam district in Andhra Pradesh. In 1964, Singh and Saxena in Delhi carried out a serological study of brucellosis among 650 patients with a history of PUO and on 2014 samples received for Widal test. Out of 650 serum samples 34 were positive and out of 2014 serum samples one was positive with significant titer of > 1:125.

In Jamnagar, Roy carried out a serological study of brucellosis in 1965, which recorded a sero-prevalence of 6% among 365 PUO cases (Roy *et al.*, 1965).

In 1966 in Punjab, Chitkara and Kaur carried out a study of human brucellosis. Out of 1648 PUO cases, the prevalence was found to be 2.08% with antibody titers > 1.125 and 268 (16.26%) cases showed agglutination in dilutions < 1:125.

From 1951 – 1967, Mathur diagnosed 232 cases of brucellosis in and around Karnal. The study was done based on serology, clinical and sero-epidemiological features. Out of 85 sero-positivity cases 53 strains of *Brucella melitensis* were isolated (Mathur, 1969).

From 1968-1978 Koshi and Myers studied blood samples sent for cultures, in Vellore for the evidence of brucellosis. Six cases were diagnosed by culture and serology, two by culture alone and 12 by serology alone (Corbel, 1997).
In 1968, Anand in Kashmir studied 500 cases of PUO and the prevalence was found to be 1.4%. Koshi et al diagnosed 8 and 2 cases of brucellosis out of 1547 serum samples sent for Widal test and 247 PUO cases (Koshi et al., 1971).

Mahakur and Panda carried out a study in 1972 in Burla. 215 cases of sera collected from individuals with a history of fever as predominant feature and a prevalence of 3.72% was reported in those cases.

Nagalotimath and Joglekar carried out a study involving 10268 serum samples (1971-1975) from different patients including those of PUO cases. 90 cases were found to be positive for brucellosis among them (Nagalotimath and Joglekar, 1978).

Sen and Khama carried out a sero epidemiological survey of brucellosis in PUO cases in villages of West Bengal. The prevalence of brucellosis in those cases was reported at 3.2% (Sen and Khama, 1978).

Ramanna and co-workers carried a sero-epidemiological survey in rural population (1652 cases of PUO) of North India. They reported the prevalence of brucellosis at 4.11 % among rural population as opposed to 0.96% in urban populations (Ramanna et al., 1982).

In 1984, Umapathy conducted a sero epidemiological survey, in and around Bangalore. Out of 644 PUO cases, 1.2% prevalence was reported by STAT in agglutination titers ≥ 1:160 (Umapathy, 1984).

In 1986, Panjarathinam and Jhala studied 143 PUO cases and 205 suspected cases of brucellosis in Gujarat. (24.47%) of PUO cases and (14.15%) suspected cases of brucellosis showed Brucella agglutination in diagnostic titers. The overall prevalence of human brucellosis was reported to be 8.5% with Brucella agglutination titers of ≥ 1:80 (Panjarathanam and Jhala, 1986).

Sharma and co-workers (1979) carried out sero-epidemiological investigation on brucellosis in the State of Uttar Pradesh (U.P.) and Delhi, during the year 1976 and 1977. A sero-prevalence of 0.89% was recorded in man. In 1994, Mantur and co-workers recorded a sero positivity of 2.2% among 3752 patients attending a teaching hospital, Bijapur. The serum samples were received for various serological tests with a predominant history of fever. Of 51 blood samples cultured 14 (27.4%) showed the growth of Brucella organisms. All the isolates belonged to Brucella melitensis biotype 1 (Mantur et al., 1994).
A serological survey of brucellosis was carried out in sheep, goats and some human risk group using rapid plate test and Rose Bengal Plate Agglutination Test by Desai et al during 1995. A total of 102 serum samples collected from Veterinarians, Para-veterinarians and shepherds were subjected for investigation. The study was done in and around Bangalore and Bidar and recorded a sero-prevalence of 5.9% for human samples.

From 1992-1997 a total of 3532 patients of Pyrexia of Unknown Origin were subjected to Wright’s tube agglutination tests for brucellosis. Of the 3532 patients tested 28 (0.8%) were found sero-positive for brucellosis, by Kadri and co-workers in Kashmir. The cases were sero-positive in dilutions ≥ 1:160 (Kadri et al., 2000).

A sero-prevalence study did by Hussain et al (2000) in Assam revealed 6.77% sero-positivity by RBPT and STAT. The samples were collected from the individuals with a history of febrile condition and joint pain. In STAT the titer was found from 1:80 to 1:320 IU.

Handa et al (2000) studied the prevalence of brucellosis in North India during 1998. The study that included PUO cases and occupationally exposed individuals revealed the prevalence of 6.6 % and 14% sero positivity.

Sachdev et al (2001) reported Brucella abortus infection in a six year old male child of Indian origin, resident of New Delhi. The serum was positive for Brucella abortus at 1:160 but culture was negative.

The study carried out in Himachal Pradesh by Chahota et al (2003) in an out break of brucellosis in an organized dairy farm involving cows and the in contact human beings had 10% of sero-reactors for Brucella melitensis. Brucellosis may be more common in males in areas where the disease is an occupational hazard of farmers and shepherds, butchers or Veterinarians. Brucellosis in children can be very common in particular in areas with Brucella melitensis (Mantur et al., 2004).

Ajay Kumar and Nanu recorded 1.6% of sero-positivity for Brucella agglutinins by using various standard tests. Among the general population a prevalence of 2.45% was observed and among the Veterinary students 1.14% (Ajay Kumar and Nanu, 2005).

A total of 325 serum samples from PUO cases received in the laboratory of Department of Microbiology, Miraj from January to December 2001 for Widal test were screened simultaneously for presence of Brucella antibodies by slide agglutination by Joshi et al., (2005).This study revealed a sero-positivity of 6.76% for brucellosis.
Mantur *et al* (2006) reported brucellosis in children who were identified by testing samples referred to the Microbiology laboratory of Medical College, Bijapur during a period of 13 years. Vaishnavi and Kumar (2007) carried out a study on sero-prevalence of brucellosis in Chandigarh among 292 blood donors and reported 6.36% of sero-prevalence.

In most of the studies conducted in India, with few exceptions, the magnitude of brucellosis was found to be higher in males than females and the second, third and fourth decades of age. Brucellosis in human occurs in all age groups and both males and females are affected equally in particular when dairy is the most common source of infection (Mantur *et al*., 2004).

Most of the sero-epidemiological studies done in India have revealed that brucellosis is predominantly an occupational disease. Epidemiological evidence shows that in India brucellosis is present in different species of mammalian farm animals including cattle, goats, buffalo, yaks, camel, horses and pigs (Isloor *et al*., 1998; Renukaradhya *et al*., 2002; Mehra *et al*., 2000).

Most of the diagnosed cases of brucellosis in all the studies were found to be engaged in occupations, (Veterinarians, abattoir workers, farm workers) in which they are at increased risk of developing brucellosis (Roy *et al*., 1965; Chitkara and Kaur, 1966; Nagalotimath and Joglekar, 1978; Ramanna *et al*., 1982; Mantur *et al*., 1994; Ajay Kumar and Nanu, 2005).

Occupationally, cases of brucellosis were caused by consumption of unpasteurised goats or cow’s milk and milk products (Chitkara and Kaur, 1966; Koshi and Myers, 1967; Ramanna *et al*., 1982).

Kalorey *et al* (2000) carried out a study on sero-prevalence of brucellosis in livestock and humans in Vidarbha region and reported 9.67% of disease incidence in animal attendants by RBPT and STAT.

Thakur and Thapliyal (2002) recorded a prevalence rate of 4.97% in samples that included specimens from persons occupationally exposed to animals. The higher prevalence 2.6% was recorded among occupationally exposed individuals and 0.82% prevalence were recorded in humans with unknown history of animal contact. Sarguna *et al* (2002) reported a case for rare occurrence of brucellosis in patients infected with the human immunodeficiency virus (HIV).
A total of 175 cases were diagnosed as brucellosis during the period of six year (June 1997 to May 2003) by Kochar et al (2003) in Bikaner (Northwest India). The cases were studied for clinical profile and majority of the patients in the study were shepherds from rural area.

Mudaliar et al (2003) carried out the detection of antibodies to Brucella abortus in animal handlers in Pune and reported a sero-prevalence of 5.33% in animal handlers. In the same study a sero-prevalence of 4.51% in farm workers and 14.63% in Veterinary doctors was recorded. The titers of STAT recorded in this study ranged from 80 IU to 320 IU.

Shukla (1962) studied 25 cases of PUO in Baroda and found that all were sero-positive for brucellosis with 4 cases in agglutination titers >1:160. Brucella melitensis was isolated from 4 cases, were from cases with significant agglutinin levels, and one from a case with agglutinin titer of 1:120.

Brucella melitensis was isolated in 25.92% of sero-positive cases in a study conducted by Singh and Saxena (1964) in Delhi who reported a sero positivity of 6.6% and 0.9% among 650 PUO cases and 2014 serum samples received for Widal.

Mathur (1969) isolated 53 strains of Brucella melitensis in 85 sero-positive cases with an isolation rate of 62.35% in Karnal. Koshi and Myers (1967) isolated Brucella melitensis from one out of ten serologically diagnosed and symptomatic cases of brucellosis.

In 1985, Nagalotimath et al reported the problem of sterility, repeated abortions and prematurity etc., in patients with a history of exposure to cattle, sheep and goat which may be due to brucellosis. Out of 160 cases subjected for STAT and 2-Mercaptoethanol test 18 (11%) presented with one or the other obstetrical problems.

A serological and cultural study of brucellosis was done by Panjarathinam and Jhala (1986) among 805 aborted women. They found the sero-prevalence of brucellosis to be 6.46% in dilutions  1:80. But no Brucella species was isolated from any these cases. Mantur and co-workers (1994) isolated Brucella melitensis biotype 1, 17 strains from 51 sero-positive cases.

Mantur and coworkers (2004) reported brucellosis in children who were identified by testing samples from children referred to the microbiology laboratory of the Patil Medical College in Bijapur during a period of 13 years. The sero-prevalence was 1.6% by STAT (≥1:160) and Brucella melitensis was isolated from 43 of these pediatric samples. Most of the pediatric patients were shepherd’s children and the habit of consuming fresh goat milk
and the close contacts with animals were the most likely risk factors contributing to infection.

Joshi et al in 2005 isolated *Brucella melitensis* from 5 cases out of 22 sero-positive cases in Miraj.

Burbudhe et al (1994) used Dot-ELISA with autoclaved extract of *Brucella abortus* S99 for the detection of *Brucella* antibodies in human sera. The dot ELISA was found to be more sensitive, economical and a rapid test for screening of human brucellosis under field conditions.

Elizabeth et al (1996) carried out the evaluation of an ELISA for the diagnosis of brucellosis by using the ELISA kits of Virotech, Dic System Diagnostika, GMBH, Germany in Vellore. The study revealed 13% of sero-positivity by IgG ELISA for 23 patients in whom the serological diagnosis of brucellosis was sought.

Kumar et al in 1997 carried out a sero-prevalence study for a total of 165 serum samples of abattoir personnel of Delhi by using Dot-ELISA and reported 23.45% of disease incidence.

Gokhale et al (2003) used indirect ELISA test for the serum samples collected from 33 patients in Mumbai. Out of this, 21 patients had a history of infective spondylitis and 12 sarcoliitis were subjected for *Brucella* serology by using commercially available kits from Diasorine s.r.1. USA, which utilized *Brucella abortus* as the antigen. Out of 33 patients 26 were positive by ELISA.

In most of the studies conducted in India, with a few exceptions, the magnitude of brucellosis was found to be higher in males than females and the second, third and fourth decades of age. Brucellosis in human occurs in all age groups and both males and females are affected equally when dairy is the most common source of infection. Brucellosis may be more common in males in areas where the disease is an occupational hazard of farmers and shepherds, butchers or Veterinarians. Brucellosis in children can be very common in particular in areas with *Brucella melitensis* (Mantur et al., 2004).

### 2.3 General characteristics of *Brucella* species

*Brucella* is Gram negative intracellular coccus; coccobacillus or short rod of size 0.5 to 0.7 x 0.6 to 1.5 µm. Arrangement may be single or less frequently in pairs, short chains and in small groups. The true capsule is not present. They are non-acid fast, but in modified ZN staining the *Brucella* have a red or orange appearance (Farrell, 1996).
They are catalase positive and usually oxidase positive. Acid production does not occur from carbohydrates in conventional media. They are indole negative, gelatin liquefaction negative; MR and VP negative. Growth on media with basic fuchsin (1:50000) is positive in *Brucella melitensis*, *Brucella abortus* (except biovar-2), *Brucella suis* biovar -3 and some strains of *Brucella suis*. Growth on media with thionin (in concentration 1:25000) present in *Brucella abortus* biovar-3, *Brucella suis* biovar 1, 3 and 4, *Brucella ovis* and *Brucella canis*. Growth on media with thionin (1:50000) present in *Brucella melitensis*, *Brucella abortus* biovars 3, 5,6,7,9, *Brucella canis*, *Brucella ovis* and *Brucella suis* (Holt et al., 1994).

**2.4 Sources of infection and transmission**

Milk and milk products are common sources of infection. The survival of *Brucella* may be prolonged in milk stored at optimal conditions to prevent souring. Milk may also neutralise the gastric acid to protect ingested bacteria in stomach. The raw milk, clotted cream and unevenly heated milk can harbor live *Brucella* organisms (Wright, 2000; Williams, 1982). Most of the cases of infections by *Brucella* arise from occupational or domestic contact with infected animals or with the environment contaminated by their discharges. The workers in dairy industry, shepherds, farm workers, abattoir workers, butchers, kitchen workers, veterinarians and family members who have contact with infected animals are all at risk of developing brucellosis (Wright, 2000; Gotuzzo, 1999). *Brucellae* are capable of prolonged survival in the environment, so that viable organisms inhaled in dust may be infective. Blood transfusion, bone marrow transplantation and possible kidney transplantation are sources of infection. Sexual transmission in semen may occur (Wright, 2000; Mantur et al., 1996). Transmission of *Brucella* species is a well recognised occurrence and laboratories in countries where *Brucella* species isolates are uncommon should be aware of this potential problem (Wright, 2001). Vaccine related accidents involving *Brucella abortus* strain 19 and *Brucella melitensis* strain Rev-1 has been reported among veterinarians. Conjunctival splashes and needle sticks are common modes of exposure. More over, persons without preexisting antibodies are at greater risk of developing brucellosis (Young, 1995).
2.5 Pathogenesis

It is generally considered that, *Brucella melitensis* and *Brucella suis* are more virulent than *Brucella abortus* and *Brucella canis*. The nutritional and immune status of the host, size of the infectious inoculum and route of transmission could be the determinants of disease (Young, 2000). Pathogenicity in human brucellosis is related to various factors. The S-LPS is a major determinant of virulence and dominates the antibody response. It is the main component responsible for conferring incomplete and short-term protection against infection in passive transfer experiments with monoclonal and polyclonal antibodies (Dubray, 1987).

The antigenic structure of *Brucellae* is complex and is greatly influenced by their phenotypic phase. The cultures occur invariably as smooth or rough phase and any mutation may result in their phase change. *Brucella abortus*, *Brucella melitensis* and *Brucella suis* are normally smooth phased while *Brucella ovis* and *Brucella canis* are rough phased. The phase variations are associated with their pathogenicity as seen in *Brucella ovis*. The phase variations are closely linked to O-side chain of LPS composition of the bacterial cell wall which influences the colony morphology and the antigenic characteristics.

A major surface antigen of smooth-phase strain is a lipopolysaccharide with lipid A containing two types of aminoglycose, distinctive fatty acids excluding β-hydroxymyristic acid, a core region containing glucose, mannose and quinovosamine and an O-chain comprising a homopolymer of 100 residues of 4-formamido-4, 6-dideoxymannose, which are linked predominantly to α-1, in ‘A’ epitope dominant strains, but with every fifth residue linked to α-1, 3 in ‘M’ dominant strains. The types that express both epitopes to a similar extent produce both LPS types. *Brucella abortus* contain 20 times of ‘A’ epitope as ‘M’. *Brucella melitensis* contain about 20 times of ‘M’ as ‘A’. *Brucella suis* show an intermediate pattern (Corbel, 1997). The strains of *Brucella abortus, Brucella melitensis* and *Brucella suis* can be A, M or A and M antigen positive (Dubray and Limet, 1987).

2.6 Clinical Features

Human brucellosis is known for presenting with protean manifestations (Mantur et al., 2006). Human brucellosis usually manifests as an acute (< 2 months) or sub acute (2-12 months) febrile illness which may persist and progress to a chronically (> 1 year) incapacitating disease with severe complications. Persons infected with *Brucella* species
usually have signs and symptoms consistent with an influenza like or septicemic illness, often with insidious onset. The symptoms and clinical signs most commonly reported are fever, fatigue, malaise, chills, sweats, headaches, myalgia, arthralgia and weight loss (Young, 1989; Mantur 2006). Relapsing or undulant fever is the classical pattern described by Bruce and usually observed two months after the disease (Young, 1983). Lack of appropriate therapy during the acute phase may result in localisation of bacteria in various tissues and lead to sub-acute or chronic disease that can have serious clinical manifestations (Young, 1983; Young, 1995).

2.7 Laboratory Diagnosis of brucellosis

Brucellosis is diagnosed either by isolation of *Brucella* organisms in culture or by a combination of serological tests and clinical findings consistent of brucellosis. Isolation of the *Brucella* organisms is the definitive means of diagnosis but in practice it is difficult due to the early tissue localization, exacting culture requirements of the organism and also prolonged time required for isolation. In practice blood cultures are positive in 10-30% of brucellosis and the remaining is diagnosed serologically (Young, 1983).

Most patients with acute brucellosis produce antibodies of the IgM isotype within a few days of onset of the disease. These antibodies are rapidly followed and superseded by IgG, and to a lesser extent IgA, antibodies. Maximum titers are reached in the third or fourth week of disease and then slowly decline; however, antibodies usually persist throughout the active phase of the disease and in some cases for long after. In sub acute or chronic brucellosis this pattern is generally not seen, the serological response consisting of a sustained production of IgG and sometimes IgA antibodies (Arija et al., 1992).

Numerous serological procedures have been tried in the diagnosis of human brucellosis but few have achieved lasting application. Until recently, the most widely used was the Standard Tube Agglutination Test. In many laboratories this has now been superseded by the Rose Bengal Plate Agglutination Test and Enzyme-Linked Immunosorbent Assay (ELISA). Other useful tests include the 2-mercaptoethanol agglutination test, the complement fixation test, and the Coombs antiglobulin test and radioimmunoassay (Magee, 1980). The potential use of an indirect ELISA for diagnosis of human brucellosis has been reported by various investigators (Araj and Kauffman, 1989; Lucero et al., 2005; Hunter et al., 1986; Ertek et al., 2005; Araj et al., 2005; Gad and Kambal, 1998; Hussein et al., 2005; Magee, 1980; Rajaii et al., 2006)). The enzyme immunoassay is an effective
method for diagnosing acute and chronic brucellosis and for detecting antibodies in CSF of patients with neurobrucellosis. The ELISA is as sensitive as Radio Immuno Assay (Corbel and MacMillan, 1997).

2.8 Treatment and Prevention

Traditionally, oral tetracycline (500 mg 4 times daily) for 6 weeks combined with intramuscular streptomycin (1 gm per day) for first 2-3 weeks was the recommended therapy for brucellosis. The use of doxycycline (200 mg per day) in combination with rifampicin (600 – 900 mg per day) administered orally for at least 6 weeks is a currently recommended treatment regimen. Trimethoprim-sulfamethoxazole in combination with an amino glycoside has been reported to be successful in treating children younger than 8 years of age in whom tetracycline is contraindicated. In many countries, the use of *Brucella abortus* strain 19 vaccine in cattle and *Brucella melitensis* strain Rev-1 vaccine in goats and sheep has resulted in the elimination or near-elimination of brucellosis in these animals. The prevention of human brucellosis depends on the elimination of the disease in domestic livestock. Brucellosis surveillance and eradication programs are not without costs for personnel, supplies and organisation. In many developing nations, such a commitment remains elusive (Young, 1995; Young, 2000).
CHAPTER 3

OBJECTIVES OF THE PRESENT WORK

3.1 The objectives of the study included

1. Development and evaluation of the indirect ELISA with conventional serological tests:
   a. Rose Bengal Plate Agglutination Test.
   b. Standard Tube Agglutination Test.

2. Assessment of the sero-prevalence of brucellosis in various groups or categories of individuals as well as endemicity in different parts of the state.

3. Study of the influence of age, sex and occupation in predisposing brucellosis.

3.2 The parameters of the study:

1. Cultural, biochemical and serological studies of *Brucella abortus* 99 and *Brucella melitensis* 16 M biotype 1.

2. Smooth lipopolysaccharide characterisation – The isolation of smooth lipopolysaccharide of *Brucella abortus* 99 and *Brucella melitensis* 16 M biotype 1.

3. Titration of the smooth lipopolysaccharide antigen of *Brucella abortus* 99 and *Brucella melitensis* 16 M biotype 1, to find out optimum titer for indirect ELISA.

4. Determination of strength of horse radish peroxidase-conjugate for the working dilution.

5. Collection of the serum samples from the individuals with a history of fever, (PUO), from individuals with a persistent joint pain, backache etc., and no history of fever (non-PUO) and professionals from different parts of Karnataka state.

6. Screening of the collected sera by RBPT and STAT to check the presence and quantification of the antibodies.

7. Screening of the samples by standardised indirect ELISA to evaluate the results obtained by RBPT and STAT.

8. Study of the disease as the occupational hazard.

9. To know the influence of age and sex in contracting brucellosis.

10. To know the endemicity of brucellosis in Karnataka and to estimate the disease prevalence.
CHAPTER 4

EXPERIMENTAL WORK

4.1 GENERAL CONSIDERATIONS

In the present study the glass wares used were either of Corning or Borosil make. The culture media, buffers and other biochemical reagents were prepared in quartz glass double distilled water. Tryptose agar and other culture media were obtained from M/S Hi-Media, Bombay. The chemicals used were of Analar or Excelar grade.

Glassware, rubber cork or screw caps were cleaned and sterilized by standard procedures. Syringes, micro centrifugation tubes and micro tips procured from Tarsons Ltd., Bangalore were used wherever required.

4.2 Cultural, biochemical and serological studies.

*Brucella* cultures are procured from the following sources:

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Brucella abortus</em></td>
<td>99</td>
<td>PD_ADMAS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bangalore-24.</td>
</tr>
<tr>
<td><em>Brucella melitensis</em></td>
<td>16 M Biotype1</td>
<td>PD_ADMAS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bangalore-24.</td>
</tr>
</tbody>
</table>

Tryptose agar medium was used for routine sub culturing and maintenance of the *Brucella* strains.

Tryptose agar media plates were streaked with the cultures and incubated for three days at 37° C. After three days of incubation, plates were held towards light source and colony morphology was studied.
4.2.1 Tryptose agar media

Tryptose agar medium procured from Hi-Media Laboratories Pvt. Ltd, Bombay in the form of dehydrated powder was used. Media was prepared as per the manufacturer’s instructions, (appendix 1).

The medium was autoclaved at 121°C at 15 lbs pressure for 15 minutes and sterilized.

4.2.2 Motility test

Motility of the organisms was examined by wet film method (Green and Scott, 1989).

4.2.3 Gram’s stain

The reagents of Gram’s staining i.e. crystal violet, Gram’s iodine, Safranine were procured locally. The cultures were stained by the standard procedures described by Cruickshank et al (1975) to study the Gram’s reaction of the organisms.

4.2.4 Oxidase test

Strips of What man, No.1 filter paper were soaked in a freshly prepared one percent solution of tetra methyl-p-phenylene-diamine dihydrochloride for about 30 seconds, dried and then stored in a dark bottle.

The cultures were used for oxidase test. Before use strip was moistened in distilled water and the colony to be tested was picked up and smeared over the moist area. A positive reaction was indicated by development of an intense deep purple colour, appearing within 5-10 seconds.

4.2.5 Urease test

The urease medium was prepared as shown in appendix 2.

A loopful of each Brucella strain was inoculated into the urease tubes and incubated at room temperature and checked for development of colour. The production of urease was indicated by the development of purple pink colour.

4.2.6 Basic fuchsin and Thionin for dye inhibition test

To melted agar a 0.1 percent stock solution of each dye made in distilled water and held in boiling water for 1 hr was added. Three concentrations of both basic fuchsin and Thionin dyes in Tryptose agar were prepared as shown in appendix 3.
Loopful *Brucella* organisms grown on Tryptose agar was used to prepare the suspension in 1 ml of sterile normal saline and homogenized in a vortex mixer. Immersing sterile cotton in the bacterial suspension, a single streak of the individual organism was made across three plates each containing different dilutions of the dye.

### 4.2.7 Tests for confirmation of smooth phase of *Brucella* culture

Requirements for confirmation of smooth phase of *Brucella* culture as shown in appendix 4.

#### 4.2.7.1 Serum agglutination test

One drop of bacterial suspension prepared in normal saline was taken on a slide and one drop of *Brucella* antiserum was added. The suspension was mixed using a toothpick and the absence or presence of agglutination was examined by using a hand lens.

#### 4.2.7.2 Acriflavin test

A small amount of culture was suspended in a drop of acriflavin (1:1000) and placed on a slide. The slide was observed under a hand lens. Clear suspension of bacterial culture indicated the smooth form while agglutination indicated the rough form.

#### 4.2.7.3 Crystal Violet staining of bacterial cultures

*Brucella* colonies 72 hrs-old on Tryptose agar plate were flooded with dilute crystal violet stain (1:40) for 20 seconds after which the excess stain was discarded into 2 percent phenol. The bacterial colonies, which retained the crystal violet, were confirmed as rough while unstained one were read as smooth colonies.

### 4.3 S-LPS Extraction

#### 4.3.1 Isolation of smooth – lipopolysaccharide (S-LPS) of *Brucella* species

Equipments and reagents were used as shown in appendices 5 and 6. S-LPS were purified from both the strains of *Brucella* species by hot phenol water extraction method according to Cherwonogrodzky and Stevens (1991).
4.3.2 Cultivation of *Brucella abortus 99* and *Brucella melitensis 16 M* biotype 1

Tryptose agar medium plates inoculated with *Brucella abortus 99* and *Brucella melitensis 16 M* biotype 1 are incubated for 72 hrs. A single smooth colony of each of the *Brucella* strain was used as seed inoculum for bulk cultivation and for S-LPS antigen purification.

The thick bacterial surface growth was scooped using a wide sized loop and gently suspended in 10 ml of sterile saline. The bacterial suspension was homogenized in vortex mixer and was over layered on Tryptose agar (140 ml) in a Roux flask. Several Roux flasks were simultaneously inoculated from the same master plate to provide the identical bacterial population originating from a single cell. The Roux flask was left for 1 hr for adsorption of bacteria to the medium. The inoculated Roux flasks were inverted and incubated for 72 hrs for bulk growth. The culture was checked for purity and 30 ml of 2% phenol saline was added and gently agitated in orbital shaker at room temperature for 48 hrs to kill and to produce a thick suspension of bacterial growth. The suspension was pooled and centrifuged at 20000 g at 4 ºC for 30 min in a refrigerated centrifuge. The pelletted cells were stored at -20ºC till further use.
Figure 4.1 Bulk cultivation of *Brucella* organisms
4.3.2.1 Removal of lipid contamination

1. 100 ml DW was added to 20 g wet weight of bacterial cells and continuously stirred on a magnetic stirrer for 60 min. to get an even suspension.

2. The cell suspension was centrifuged at 20,000xg at 4°C for 30 min. and the supernatant was discarded.

3. The retained pellet was resuspended in 95 percent ethanol and washed again as above (This step removes extra water left from step-2).

4. The pellet was washed in acetone which picks up extra ethanol left from the step 3.

5. The fourth wash was carried out in ethyl ether and this picks up the acetone left from the step 4.

The ether treated cells were ground to a fine powder with Mortar and Pestle and then 1 part of powdered dried cells was dissolved in 20 parts of Tris saline.
4.2 Culture during removal of lipid contamination

4.3.2.2 Hot phenol extraction of S-LPS

1. An equal volume of aqueous phenol was added to the above suspension and extracted at 70°C for 30 minutes with constant stirring. The preparation was centrifuged at 20,000 g at room temperature for 30 minutes and then the aqueous portion was saved.

2. Five volumes of methanol acetate (pre-chilled at 4°C) was added to the aqueous phase (step-1) and the preparation was kept at 4°C over night.
3. Next day the preparation in step 2 was centrifuged at 20,000 g for 30 min at 4°C and the supernatant was discarded.

4. The precipitate obtained in step-3 was resuspended in an equivalent volume of methanol acetate and centrifuged at 20,000 g for 30 min. The precipitate was washed twice more with methanol acetate to remove phenol.

5. Finally, the precipitate was suspended in Tris saline and dialysed against 100 fold volume of Tris saline.

Figure: 4.3 Hot phenol extract
4.3.2.3 Enzymatic digestion of extract

1. Enzymatic digestion of hot phenol extract was carried out with constant stirring by the successive addition of:
2. 25µg per ml each of RNAse and DNAse after addition of 5 mM magnesium sulphate at room temperature for one hr.
3. 25 µg of lysozyme per ml was added later and kept at room temperature for 5 hr.
4. 50 µg Proteinase K was added later and held at room temperature for 48 hr.
5. The resultant solution was thoroughly dialyzed against Tris Saline.
6. Dialyzed solution was subjected for centrifugation at 20000Xg at 4°C for 30 min. to remove any debris.
7. The resultant supernatant was ultra centrifuged at 1, 20,000 g at 4°C for 18 hr to yield a waxy pellet of S-LPS.
8. The pellet was resuspended in Tris saline and ultra centrifuged as above again.
9. The pellet was dissolved in sterile distilled water and thoroughly dialyzed in DW and finally lyophilized in small aliquots.
10. Protein content in S-LPS was estimated by FCR (Lowry, 1951).
Figure: 4.4 S-LPS during dialysis
4.3 SDS-PAGE analysis of S-LPS

Equipments and reagents were used as shown in appendices 7 and 8

Clean, grease and protein free glassware were used. Gloves were worn while handling the gel.

1. Slab gel apparatus (Bangalore Genie)
2. Glass plates of 16.5 x 17 cms one of them having a 1.5 x 13 cm notch.
3. Plexiglas comb, spacers and clamps.
4. Power pack vacuum pump and gel drier.

Buffers/Stains for SDS PAGE are shown in appendix 9

4.4.1 SDS-PAGE

1. Glass plates, spacers, rubber gasket and comb were cleaned thoroughly and dried.
2. The glass plates with spacers and rubber gasket were assembled and clamped.
3. Gel casting.
4. Resolving gel was prepared and poured in between the plates with a gentle, continuous flow avoiding air bubbles, up to about three fourths of the height of the plate (about two inches below the level of the notch). A thick layer (approx. 0.5 cm) water saturated butanol was immediately overlaid carefully avoiding mixing the two using a syringe fitted with a thin needle. The gel was allowed to set. The butanol layer was blotted off using filter paper strips. Stacking gel was prepared and poured up to the required height and the comb was placed and the gel was allowed to set.
5. The comb was removed and the slab was fixed to the electrophoresis apparatus. The running buffer was poured into both the tanks.
6. The samples and MW markers were prepared in sample buffer and boiled in a water bath for 5 min. 40 µl of each sample, containing 25 µg of protein were loaded into each well using micro pipette.
7. The samples were run at 10 mA until the dye-front reached the lower end of the gel.

4.4.2 Staining SDS- PAGE gels with Coomassie brilliant blue

1. The gel was carefully separated from glass mould and immersed in 5 volumes of staining solution and placed on a slowly rotating platform for 6 hr at room temperature.
2. The stain was removed and saved for future used.
3. The gel was distained by soaking in the destainer on a slowly rotating platform for 8 hr, changing the destainer several times until polypeptide bands were clearly visible.
4. After distaining, gels were stored in DW.
5. Permanent record was made by photographing the stained gel.

Figure: 4.6 SDS page analysis of S-LPS (Only marker proteins are eluted)

CHAPTER 5

ANALYSIS PROCEDURE
5.1 Clinical material

The clinical material for the study were obtained from Pyrexia of Unknown Origin cases, non pyrexia of unknown origin cases as well as from individuals who are at occupational risk like Veterinarians, Para veterinarians, farm workers, slaughter house workers etc. The following type of proforma was prepared and given to the government hospitals, private hospital laboratories and diagnostic units to collect the particulars like clinical history, age, sex etc. Request was made to clinicians and laboratory personals to provide 2 ml quantity of serum from the patient.

5.1.1 Proforma (Used for collection of history of patients)

O.P. / I.P. No : 
Name : 
Age and sex : 
Occupation : 
Clinical history: Main complaints and presenting illness and duration.
Previous investigations and medications (if any):
Investigations:

5.1.2 Study group

It Included 2770 serum samples that were collected from both sexes and between 1-60 years of age. Following are the groups identified among 2770 patients in a study group.

Group I- 618 individuals who were engaged in occupations (farm workers, Veterinarians, abattoir workers, Para veterinarians) in which they are at increased risk of developing brucellosis. The samples from the professionals were taken to study the influence of profession in contracting the disease.

Group II -1500 patients had a history of fever and considered as Pyrexia of Unknown Origin. All the Pyrexia of Unknown Origin cases were considered as probable cases of Brucellosis and the duration of clinical illness was noted down.
Group III – 652 individuals who were not having any history of fever but who complained of joint pain as a regular clinical presentation and were negative for Rheumatoid Arthritis, Anti Streptolysin O and C - reactive protein.

Control group: 50 cases of apparently healthy blood donors of both sexes. These individuals had not suffered from any febrile illness in the past one-year. Such cases were tested for syphilis, Hepatitis B Antigen, Human Immunodeficiency Virus, Malarial Parasites and results were negative.

The serum samples were collected by taking into account the inclusion and exclusion criteria for the Pyrexia of Unknown Origin cases (Appendices 10 and 11).

5.2 Serological tests

Screening of collected serum samples by Rose Bengal Plate Agglutination Test and Standard Tube Agglutination Test. (STAT) and indirect ELISA were carried out to detect the presence of Brucella antibodies.

The serum samples were analyzed in three phases. In the first phase RBPT was performed. In the second phase, the samples were analyzed by the Standard Tube Agglutination Test, (STAT). The antigens required for both tests were procured from Institute of Animal Health and Veterinary Biologicals, Bangalore. In the third phase, indirect ELISA standardized by using smooth lipopolysaccharide of Brucella abortus 99 was employed (Cherwonogrodzky and Stevens, 1991).

Indirect ELISA for S-LPS of Brucella abortus 99 and Brucella melitensis 16 M biotype 1 retested all the samples that were tested by the above methods.

5.3 Rose Bengal Plate Agglutination Test

Rose Bengal Plate Agglutination Test antigen obtained from Institute of Animal Husbandry and Veterinary Biologicals (IAH & VB), Bangalore-24, Karnataka.

Requirements for test are RBPT antigen, micropipette (30µl), glass plate, spreader, and test sera, positive and negative control sera.

30 µl quantity of Rose Bengal antigen and 30 µl of test sera were mixed on a glass slide with a spreader. The slide was then manually rotated for two minutes and immediately examined for agglutination. With each set of test sera, known positive and negative control sera were also included (Alton et al., 1975).
5.4 Standard Tube Agglutination Test (STAT)

STAT was carried out on all sera, using antigen obtained from IAH &VB Bangalore. Antigen used was heat killed suspension of pure, smooth; *Brucella abortus* strain 99 in phenol saline, standardized against anti *Brucella abortus* serum with antibody agglutination titers of 100 IU/ml to give 50% agglutination with 1:500 dilution of the serum.

Requirements of the test are *Brucella abortus* plain antigen, 0.5% phenol saline, test tubes (75mmX7.5 mm size), test tube racks, graduated 1 ml pipette, test serum, positive and negative control serum.

Serial two-fold dilutions of the test sera starting from 1:10 were done in 0.5% phenol saline. 0.5 ml *Brucella abortus* plain antigen was added to each tube of serial dilutions and mixed.

All the tubes were incubated at 37°C overnight. With each set of screening a reference tube containing antigen without immune serum was also included. The reading was taken based on clearance of the supernatant fluid, taking the highest dilutions of the serum showing 50% agglutination corresponding to antigen control tube was taken as positive. The titer so obtained was converted into International Unit of *Brucella* antibody activity as recommended by the Joint FAO/WHO expert Committee on brucellosis, since locally prepared antigens differ from laboratory to laboratory.

Highest dilution of serum at which 50% agglutination is obtained X 2= I.U. titer.

In the above procedure a titer of 1:80 or greater is taken as an index of sero positivity (Alton *et al.*, 1975).

5.5 Screening of samples by indirect ELISA

All serum samples, a total of 2770 were subjected for indirect ELISA, which used S-LPS of *Brucella abortus* 99 strain.

**Preparations of buffers for indirect ELISA are shown in appendix 12**

5.6 Procedure for indirect ELISA

5.6.1 Requirements
1. Antigen: Smooth Lipopolysaccharide (S-LPS) of *Brucella abortus* 99 and *Brucella melitensis* 16 M biotype 1 purified by hot phenol water extraction method as per Cherwonogrodzky and Stevens (1991) was used.

2. The serum collected from confirmed cases of brucellosis obtained from the B.L.D.E.A.’s Medical College, Bijapur, (Courtesy Dr. B.G. Mantur) was used as strong positive serum control.

3. The serum sample taken from the apparently healthy individuals was used as the negative control serum. The moderate positive control was prepared by diluting the strong positive serum in negative serum (1:20 diluted).

4. Reagents are procured commercially to develop the indirect ELISA. The rabbit antihuman HRP conjugate (Bangalore Genie), O-Phenylene Diamine dihydrochloride (OPD), hydrogen peroxide (H$_2$O$_2$), bovine gelatin (British Pharmacopoeia grade 4) and 96 well ELISA plate (Nunc polysorp) were used.

Optimal working dilutions of S-LPS antigen, control sera and immunoconjugate were established by a checkerboard titration for use in the indirect ELISA. Control and test sera were used at fixed 1:100 dilution.

5.6.2 Optimization of *Brucella* antigen (S-LPS)

The titration was carried out keeping the serum dilution constant at 1:100 against various dilutions of the S-LPS antigen of *Brucella abortus* and *Brucella melitensis* to determine the optimum single working dilution of *Brucella* antigen.

A serial two fold dilution of S-LPS was prepared in carbonate and bicarbonate buffer, (PH 9.6±0.05).

A constant 1:100 dilution of known strong positive control serum (C$^{++}$) and negative control serum (C$^{-}$) were prepared in 1% BGPBST.

5.6.3 Coating the micro plate

The antigen (smooth-lipopolysaccharide) stock (lyophilized) kept at 4°C is reconstituted with distilled water. Antigen is further diluted to 1:20 by adding 50µl of reconstituted antigen in 950µl of sterile distilled water. Stock solution of the antigen is stored at −20°C. Antigen from stock is added @ 5µl/ml of coating buffer to prepare the working dilution. It is mixed properly before dispensing 100µl into all 96 wells of the micro plate. The sides of the plates were tapped to ensure that the S-LPS dilution is evenly distributed over the bottom of each well. The micro plates are covered and kept for
incubation on a shaker for one hour at 37°C. Alternatively the plates can also be incubated at 4°C overnight.

5.6.4 Addition of test and control sera

Agitation of the test sera and aliquots of all 3-control sera (C++, C+, and C−) are carried out gently to ensure homogeneity. Control and test sera should be diluted 1/100 in blocking buffer separately in perplex plate, before loading to the micro plate. Remove the antigen-coated plates from shaker. Discard the contents by inverting the plates by an abrupt downward hand motion, discharge the contents of the micro plate into the sink and slap the inverted micro plates onto a lint free absorbent to remove all residual contents. The plates were washed with washing buffer three times.

Test serum and control serum were diluted, which is 1/100, dispensed @100µl in the respective wells. The plates were kept on the shaker at 37°C for one hour.

5.6.5 Addition of the conjugate

The new stock of antihuman IgG conjugated with horseradish peroxidase is used. The stock solution is prepared by adding 5µl of neat conjugate from the vial with 245µl of sterile distilled water.

The plates were removed out of the shaker and washed three times with washing buffer. Then 100µl of the working dilution of conjugate (1:3000) was added to all the wells of the plate and returned the plate to incubator on the shaker at 37°C for one hour.

5.6.6 Addition of substrate /chromogen and stopping solution

Immediately before the end of the conjugate incubation, a working dilution of substrate/chromogen solution was prepared. 40µl of 3%Hydrogen Peroxide was added to 12.5ml of chromogen solution (sufficient for one plate). The plate was taken out of shaker and washed as described before.

Then 100 µl of working dilution of substrate/chromogen solution was added to all the 96 wells of the micro plate. The plate was kept at room temperature for 10 min. until a visible colour developed in the strong positive wells. The plate was then removed and reaction was stopped by adding 100 µl of stopping solution to all 96 wells. Immediately the optical density was read in the reader at 492 nm.

The results were interpreted as per the procedure given below.
5.7 Interpretation of the results for Indirect ELISA

There are a number of methods for determination of sero-positive or sero-negative thresholds. Based on the results obtained after screening large number of samples at the PD_ADMAS laboratory using the indirect ELISA, it is recommended that a positive result can be considered when the ELISA positive-negative ratio was equal to or more than 3 (Vrielink and Vander Poel, 1994) and three standard deviation of positive and negative OD values. This means, any sample that gives an OD value three times more than the OD value of the negative control is positive and that below is negative.

5.8 LAYOUT OF THE PLATE

<table>
<thead>
<tr>
<th>Conjugate Control</th>
<th>Conjugate Control</th>
<th>Sample 1</th>
<th>5</th>
<th>9</th>
<th>13</th>
<th>17</th>
<th>21</th>
<th>25</th>
<th>29</th>
<th>33</th>
<th>37</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conjugate Control</td>
<td>Conjugate Control</td>
<td>Sample 1</td>
<td>5</td>
<td>9</td>
<td>13</td>
<td>17</td>
<td>21</td>
<td>25</td>
<td>29</td>
<td>33</td>
<td>37</td>
</tr>
<tr>
<td>Strong Positive Control</td>
<td>Strong Positive Control</td>
<td>2</td>
<td>6</td>
<td>10</td>
<td>14</td>
<td>18</td>
<td>22</td>
<td>26</td>
<td>30</td>
<td>34</td>
<td>38</td>
</tr>
<tr>
<td>Strong Positive Control</td>
<td>Strong Positive Control</td>
<td>2</td>
<td>6</td>
<td>10</td>
<td>14</td>
<td>18</td>
<td>22</td>
<td>26</td>
<td>30</td>
<td>34</td>
<td>38</td>
</tr>
<tr>
<td>Moderate Positive</td>
<td>Moderate Positive</td>
<td>3</td>
<td>7</td>
<td>11</td>
<td>15</td>
<td>19</td>
<td>23</td>
<td>27</td>
<td>31</td>
<td>35</td>
<td>39</td>
</tr>
<tr>
<td>Moderate Positive</td>
<td>Moderate Positive</td>
<td>3</td>
<td>7</td>
<td>11</td>
<td>15</td>
<td>19</td>
<td>23</td>
<td>27</td>
<td>31</td>
<td>35</td>
<td>39</td>
</tr>
<tr>
<td>Negative Control</td>
<td>Negative Control</td>
<td>4</td>
<td>8</td>
<td>12</td>
<td>16</td>
<td>20</td>
<td>24</td>
<td>28</td>
<td>32</td>
<td>36</td>
<td>40</td>
</tr>
<tr>
<td>Negative Control</td>
<td>Negative Control</td>
<td>4</td>
<td>8</td>
<td>12</td>
<td>16</td>
<td>20</td>
<td>24</td>
<td>28</td>
<td>32</td>
<td>36</td>
<td>40</td>
</tr>
</tbody>
</table>

CHAPTER 6

RESULTS
6.1 CULTURAL AND BIOCHEMICAL CONFIRMATION OF Brucellae

6.1.1 Morphologic and growth characteristics

The Brucellae studied were Gram-negative coccobacilli and non-motile organisms. All the Brucella strains grew well on Tryptose agar without the need for CO\textsubscript{2} atmosphere at 37°C. The colony morphology of this group of organisms was typical for each species described. Broadly, the colonies were pinpointed, translucent, pale honey colored and convex from above after three days of incubation.

The growth of various species of Brucella in dyes such as thionin and basic fuchsin is used as a laboratory procedure for their differentiation.

Huddleson and Abell (1928) and Huddleson (1929) had devised this method for distinguishing between Brucella abortus, Brucella melitensis and Brucella suis, based on their differing sensitivity to inhibition by certain synthetic dyes. The method is still valid but does not produce absolute definition of these species because the sensitivity varies between biotypes, particularly in case of Brucella abortus.

The most useful dyes are thionin and basic fuchsin. Thionin inhibits most strains of Brucella abortus. On the other hand Brucella melitensis is insensitive to both these dyes, when incorporated in a suitable basal medium such as serum dextrose agar, basic fuchsin at a final concentration of 20µg/ml will permit the growth of, all Brucella melitensis biotypes and all the seven biotypes of Brucella abortus except for 2 and some strains of biotype 4.

6.1.2 Biochemical properties

Brucella strains viz. Brucella abortus 99 and Brucella melitensis 16M biotype 1 were oxidase and urease positive. Their differential thionin and basic fuchsin dye inhibition patterns are shown in Table 6.1.

6.1.3 ‘Phase’ confirmation of Brucellae

Slide agglutination, acriflavin test and crystal violet staining indicated that cultures were in smooth phase.
<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>CO₂ Requirement</th>
<th>Colony Morphology</th>
<th>Gram’s Staining</th>
<th>Urease Test</th>
<th>Oxidase Test</th>
<th>Motility</th>
<th>Thionin sensitivity</th>
<th>Basic fuchsin Sensitivity</th>
<th>Slide agglutination Test</th>
<th>Acriflavin Test</th>
<th>Crystal violet Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brucella abortus</td>
<td>99</td>
<td>-</td>
<td>Translucent</td>
<td>Gram negative Cocco-bacilli</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-25000</td>
<td>-15000</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Brucella melitensis</td>
<td>16M</td>
<td>-</td>
<td>Translucent</td>
<td>Gram negative Cocco bacilli</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-25000</td>
<td>-15000</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
6.1.4 Protein estimation of *Brucella abortus* and *Brucella melitensis* antigen

The extracted *Brucella* S-LPS antigen as estimated by FCR method contained protein at the rate of 8µg/ml and 6 µg/ml. The SDS page analysis of the S-LPS has not revealed any protein band stained later. Hence it does not contain detectable protein.

6.2 Standardisation of indirect ELISA for the assay of *Brucella* antibodies

6.2.1 Result of checker board titration: For antihuman IgG–HRP conjugate and S-LPS of *Brucella abortus* 99 by using positive control serum.

<table>
<thead>
<tr>
<th>S-LPS Concentration of S-LPS</th>
<th>2.5 µg/ml of S-LPS</th>
<th>5µg/ml of S-LPS</th>
<th>7.5µg/ml of S-LPS</th>
<th>10µg/ml of S-LPS</th>
<th>12.5µg/ml of S-LPS</th>
<th>15µg/ml of S-LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:2000</td>
<td>1.84</td>
<td>1.1</td>
<td>0.726</td>
<td>0.594</td>
<td>0.418</td>
<td>0.329</td>
</tr>
<tr>
<td>1:3000</td>
<td>1.63</td>
<td><strong>0.907</strong></td>
<td>0.651</td>
<td>0.435</td>
<td>0.313</td>
<td>0.216</td>
</tr>
<tr>
<td>1:4000</td>
<td>1.02</td>
<td>0.618</td>
<td>0.44</td>
<td>0.265</td>
<td>0.205</td>
<td>0.168</td>
</tr>
<tr>
<td>1:5000</td>
<td>0.736</td>
<td>0.392</td>
<td>0.31</td>
<td>0.216</td>
<td>0.148</td>
<td>0.109</td>
</tr>
</tbody>
</table>

There was a sudden drop in OD value (0.907) at 1:3000 dilution of the antihuman IgG-HRP conjugate, against 1:100 diluted serum at 5 µg/ml S-LPS concentration. Therefore, 1:3000 dilution of the conjugate was used as optimum working dilutions in indirect ELISA procedure in the present study.
Antihuman-HRP conjugate at 1:3000 dilution gave an OD value of 0.907 while the end dilution of 1:5000 of the conjugate showed an OD value of 0.109. (S-LPS antigen coated is 5 µg/ml).

There was a sudden drop in OD value (0.907) at 1:3000 dilution of the antihuman IgG-HRP conjugate, against 1:100 diluted serum at 5 µg/ml S-LPS concentration, (Table 6.2, figure 6.1). Therefore, 1:3000 dilution of the conjugate was used as optimum working dilution in indirect ELISA procedure in the present study, (Fig. 6.2).

6.2.2 Brucella S-LPS control sera
The OD values with respect to varying dilutions of negative serum control at different concentration of S-LPS.

Table: 6.3 OD values for negative control serum

<table>
<thead>
<tr>
<th>S-LPS Concentration →</th>
<th>2.5 µg/ml of S-LPS</th>
<th>5µg/ml of S-LPS</th>
<th>7.5µg/ml of S-LPS</th>
<th>10µg/ml of S-LPS</th>
<th>12.5µg/ml of S-LPS</th>
<th>15µg/ml of S-LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conjugate↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:2000</td>
<td>0.391</td>
<td>0.388</td>
<td>0.368</td>
<td>0.346</td>
<td>0.319</td>
<td>0.322</td>
</tr>
<tr>
<td>1:3000</td>
<td>0.339</td>
<td><strong>0.321</strong></td>
<td>0.298</td>
<td>0.269</td>
<td>0.286</td>
<td>0.239</td>
</tr>
<tr>
<td>1:4000</td>
<td>0.185</td>
<td>0.155</td>
<td>0.131</td>
<td>0.125</td>
<td>0.123</td>
<td>0.114</td>
</tr>
<tr>
<td>1:5000</td>
<td>0.119</td>
<td>0.109</td>
<td>0.108</td>
<td>0.102</td>
<td>0.101</td>
<td>0.100</td>
</tr>
</tbody>
</table>
Inference: Antihuman-HRP conjugate at 1:3000 dilution gave an OD value of 0.321, for negative control serum, (Table 6.3). 1: 3000 dilution of antihuman IgG-HRP conjugate gave an OD value of 0.907 (Table 6.2) for positive control serum. This is three times higher than the OD value of negative control. It was recommended that a positive result can be considered when the ELISA positive-negative ratio was equal to or more than 3, after screening a large number of serum samples, (Vrielink and Van der Poel, 1994) and three standard deviation of positive and negative OD values. This means, any sample that gives an OD value three times more than the OD value of the negative control is positive and that below is negative.

6.2.3 Protocol for indirect ELISA

<table>
<thead>
<tr>
<th>Steps</th>
<th>Assay steps</th>
<th>Assay condition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Incubation time</td>
</tr>
<tr>
<td>1</td>
<td>Coat <em>Brucella</em> S-LPS Ag</td>
<td>1 hr</td>
</tr>
<tr>
<td>2</td>
<td>Add test sera</td>
<td>1 hr</td>
</tr>
<tr>
<td>3</td>
<td>Add HRP Conjugate</td>
<td>1 hr</td>
</tr>
<tr>
<td>4</td>
<td>Add substrate (3% H2O2) and chromogen</td>
<td>10 minutes</td>
</tr>
<tr>
<td>5</td>
<td>Add stopper</td>
<td>None</td>
</tr>
</tbody>
</table>

Take the optical density values at 492 nm by using ELISA reader, (Figure 6.3)
Figure 6.2 Standardised indirect ELISA
Figure 6.3 ELISA reader used for taking OD values
6.3 Study group

The present study of brucellosis in cases with Pyrexia of Unknown Origin (PUO) was done for 1500 patients, 652 non pyrexia of unknown origin cases and 618 professionals consisting of Veterinarians and Para veterinarians, animal owners, slaughter house worker and shepherd collected from various regions of North Karnataka and South Karnataka. Study group includes patients from all age groups. Out of 2770 patients, 1989 males and remaining 781 were females.

6.3.1 Sero positivity for brucellosis and its age and sex distribution
(For a total of 2770 samples)

Table: 6.4 Influence of age and sex on brucellosis occurrence.

<table>
<thead>
<tr>
<th>Age</th>
<th>Male Positive (N=1989)</th>
<th>Percentage (Taken for 321)</th>
<th>Female Positive (N=791)</th>
<th>Percentage (Taken for 321)</th>
<th>Total 2770</th>
<th>Percentage (Taken for 321)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10</td>
<td>03</td>
<td>0.93</td>
<td>00</td>
<td>0</td>
<td>03</td>
<td>0.93</td>
</tr>
<tr>
<td>11-20</td>
<td>08</td>
<td>2.49</td>
<td>01</td>
<td>0.311</td>
<td>09</td>
<td>2.8</td>
</tr>
<tr>
<td>21-30</td>
<td>40</td>
<td>12.46</td>
<td>06</td>
<td>1.86</td>
<td>46</td>
<td>14.33</td>
</tr>
<tr>
<td>31-40</td>
<td>107</td>
<td>33.33</td>
<td>12</td>
<td>3.73</td>
<td>119</td>
<td>37.07</td>
</tr>
<tr>
<td>41-50</td>
<td>114</td>
<td>35.51</td>
<td>08</td>
<td>2.49</td>
<td>122</td>
<td>38</td>
</tr>
<tr>
<td>51-60</td>
<td>22</td>
<td>6.85</td>
<td>00</td>
<td>0</td>
<td>22</td>
<td>6.85</td>
</tr>
<tr>
<td>Total</td>
<td>294</td>
<td>91.58</td>
<td>27</td>
<td>8.41</td>
<td>321</td>
<td>11.58</td>
</tr>
</tbody>
</table>
Figure 6.4 Influence of sex and age on the occurrence of brucellosis

The sero-positivity is higher in males 91.58% and 8.41% in females. Sero positivity was found to be more in the age group of 41-50 years (38%), followed by 31-40 years (37.07%), followed by 21-30 years (14.33%), followed by 51-60 years (6.85%), followed by 11-20 years (2.8%) with the least prevalence recorded in the age group < 10 years (0.93%). No sero-positive cases were found in the age group above 61 years, (Table 6.4; figure 6.4)
6.3.2 Result of screening

Table: 6.5 Depicting result of serological tests for RBPT, STAT and indirect ELISA

<table>
<thead>
<tr>
<th>TEST</th>
<th>RBPT</th>
<th>STAT</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>138</td>
<td>30</td>
<td>37</td>
</tr>
</tbody>
</table>

The serum samples subjected for Standard Tube Agglutination Test are showing the titers as given in the above table. The maximum numbers of samples (37) have shown the highest titer of 1:160 and the minimum numbers of samples i.e. four samples had a titer of 1:2560. The highest titers with more number of samples were observed in 1:640 and beyond this titer the number of positive cases detected had declined.

6.3.3 Ratio among the three serological tests

Out of 2770 samples, 138 (4.98%) were tested sero-positive by Rose Bengal Plate Agglutination Test and Standard Tube Agglutination Test for brucellosis.

<table>
<thead>
<tr>
<th>Ratio:</th>
<th>RBPT</th>
<th>STAT</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td>2.32</td>
</tr>
</tbody>
</table>

6.4 Agreement between the tests: The Kappa statistic

If a gold standard is not available it may not be possible to assess easily a test’s validity. (i.e., sensitivity and specificity), in which circumstance agreement between different tests may be assessed, without assuming that one test is the best.

This comparison, however does not consider the agreement between the two tests that could arise just by chance. Calculating a statistic, Kappa that takes account of chance agreement, can make a more vigorous comparison. First the expected proportion of agreement by chance, EP is calculated. This is simply the sum of the expected proportion of agreement for the positive and negative results.

\[
EP = \frac{(a+b)}{n} \times \frac{(a+c)}{n}
\]

\[
EP = \frac{(c+d)}{n} \times \frac{(b+d)}{n}
\]

\[
\]
Observed agreement beyond chance, OA = OP-EP

Maximum possible agreement beyond chance MA = 1-EP

Kappa is the ratio of the observed agreement beyond chance to the maximum possible agreement beyond chance, that is

Kappa = OA/MA.

Kappa ranges from 1 (complete agreement beyond chance) to 0 (agreement is equal to that expected by chance), where as negative values indicate agreement less than is expected by chance.

6.4.1 Between indirect ELISA and RBPT

Table: 6.6 Agreement between indirect ELISA and RBPT

<table>
<thead>
<tr>
<th>Test</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>138 (a)</td>
<td>183 (b)</td>
<td>321 (a+b)</td>
</tr>
<tr>
<td>Negative</td>
<td>45 (c)</td>
<td>2404 (d)</td>
<td>2449(c+d)</td>
</tr>
<tr>
<td>Total</td>
<td>183 (a+c)</td>
<td>2587 (b+d)</td>
<td>2770 (a+b+c+d)</td>
</tr>
</tbody>
</table>

Sensitivity = a/ (a+c) = 138/183 = 75.4%
Specificity = d/ (b+d) = 2404/2587 = 92.92%
Predictive value for positive result = a/ (a+b) = 138/321 = 42.99%
Predictive value for negative result = d/(c+d) = 2404/2449 = 98.16%.

6.4.2 Between indirect ELISA and STAT

Table: 6.7 Agreement between indirect ELISA and STAT

<table>
<thead>
<tr>
<th>Test</th>
<th>STAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>138 (a)</td>
</tr>
<tr>
<td>Negative</td>
<td>45 (c)</td>
</tr>
<tr>
<td>Total</td>
<td>183 (a+c)</td>
</tr>
</tbody>
</table>

Sensitivity = a/ (a+c) = 138/183 = 75.4%
Specificity = d/ (b+d) = 2404/2587 = 92.92%
Predictive value for positive result = a/ (a+b) = 138/321 = 42.99%
Predictive value for negative result = \( \frac{d}{c+d} = \frac{2404}{2449} = 98.16\% \).

6.4.3 Agreement between the tests: - The Kappa statistics

1. Between indirect ELISA and RBPT and between indirect ELISA and STAT

\[ \text{OP} = \left[ \frac{(a+d)}{n} \right] \]
\[ = 138 + \frac{2404}{2770} = 0.9176895 \]

\[ \text{EP} = \left[ \frac{(a+b)\times(a+c)}{n} \right] + \left[ \frac{(c+d)}{n} \right] \times \left[ \frac{(b+d)}{n} \right] \]
\[ = \frac{321}{2770} \times \frac{183}{2770} \]
\[ = 0.1158444 \times 0.0660649 = 0.0076558 \]
\[ = \left[ \frac{(c+d)}{n} \right] \times \left[ \frac{(b+d)}{n} \right] \]
\[ = 2449/2770 = 0.8841155 \]
\[ = 2587/2770 = 0.933935 \]
\[ = 0.8841155 \times 0.933935 = 0.8257064 \]

\[ \text{EP} = 0.0076558 + 0.8257064 = 0.8333622 \]

\[ \text{OP} - \text{EP} = 0.9176895 - 0.8333622 = 0.0843273 \]

\[ \frac{\text{OP}-\text{EP}}{1-\text{EP}} = \frac{0.0843273}{1-0.8333622} = 0.5060514 . \]

Kappa = 0.5060514, indicates moderate agreement (Michael Thrusfield, 2005).

The calculation for STAT is similar, as the same numbers of tests have come positive.

6.5 Sero positivity for brucellosis in group I, II and III

A total of 2770 cases were classified into 3 groups: -

**Group I**: 618 Professionals who are at increased risk of developing brucellosis due to their occupation.

**Group II**: 1500 PUO cases in whom the fever, malaise, headache, joint pain etc. were the major symptoms.

**Group III**: non-PUO cases in whom the fever is not the major symptom but the history of joint pain, arthritis was predominant in most of the individuals.

6.5.1 Result: For group: I (In professionals)

Total number of sera screened = 618
### Table: 6.8 Results of serological tests (RBPT, STAT and indirect ELISA)

<table>
<thead>
<tr>
<th>Test</th>
<th>RBPT</th>
<th>STAT</th>
<th>Total Positive (RBPT and STAT)</th>
<th>Indirect ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody Titer</td>
<td>------</td>
<td>1:160</td>
<td>1:320</td>
<td>1:640</td>
</tr>
<tr>
<td>Positive</td>
<td>14</td>
<td>4</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Percentage</td>
<td>2.26</td>
<td>0.647</td>
<td>0.97</td>
<td>0.161</td>
</tr>
<tr>
<td>Negative</td>
<td>604</td>
<td>604</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>Percentage</td>
<td>97.73</td>
<td>97.73</td>
<td>------</td>
<td>Percentage</td>
</tr>
</tbody>
</table>

#### 6.5.2 Analysis of positive cases

The Table 6.9 depicts the analysis of positive result detected by RBPT, STAT and indirect ELISA for professionals.

### Table: 6.9 Analysis of positive result detected by RBPT, STAT and indirect ELISA

<table>
<thead>
<tr>
<th>Test</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBPT</td>
<td>14 (41.66)</td>
<td>604 (576.33)</td>
<td>618</td>
</tr>
<tr>
<td>STAT</td>
<td>14 (41.66)</td>
<td>604 (576.33)</td>
<td>618</td>
</tr>
<tr>
<td>ELISA</td>
<td>97 (32.33)</td>
<td>521 (576.33)</td>
<td>618</td>
</tr>
</tbody>
</table>
| Total   | 125      | 1729     | 1854 **

\[ X^2 = 174.05 \]

Significant at 99% interval

There is a significant variation between the tests and positive and negative cases detection. The significance is at P<0.01%.

#### 6.5.3 Ratio among the tests
Table: 6.10 Ratio among the tests, (RBPT, STAT and indirect ELISA)

<table>
<thead>
<tr>
<th>Tests</th>
<th>O Positive</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBPT</td>
<td>14</td>
<td>41.66</td>
</tr>
<tr>
<td>STAT</td>
<td>14</td>
<td>41.66</td>
</tr>
<tr>
<td>ELISA</td>
<td>97</td>
<td>41.66</td>
</tr>
<tr>
<td>TOTAL</td>
<td>125</td>
<td></td>
</tr>
</tbody>
</table>

\[ X^2 = 110.24 \]

2df

**Inference:** ELISA test can detect significantly more number of positive cases than RBPT and STAT. However, RBPT and STAT are equally efficacious in identifying positive cases.

6.5.4 Ratio among the tests

RBPT: STAT: INDIRECT ELISA
1: 1: 7

6.5.5 Distribution of sero-positive cases on the basis of clinical signs and symptoms

Table: 6.11 Distribution of sero-positive individuals based on signs and symptoms

<table>
<thead>
<tr>
<th>Clinical signs and symptoms</th>
<th>Intermittent fever</th>
<th>Chills, sweat</th>
<th>Fever, joint pain</th>
<th>Fever, back pain, Joint pain</th>
<th>Asymptomatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>16</td>
<td>10</td>
<td>10</td>
<td>14</td>
<td>47</td>
</tr>
<tr>
<td>Percentage</td>
<td>16.49</td>
<td>10.30</td>
<td>10.30</td>
<td>14.43</td>
<td>48.45</td>
</tr>
</tbody>
</table>

6.5.6 Sero-prevalence among professionals

Table 6.12 shows the demographic characteristics of the people with the following distribution:

In all, 97 serum samples out of 618 screened were positive giving a prevalence of 15.69%. The prevalence of brucellosis in descending order is, Veterinary inspectors (41.23%), followed by Veterinary assistants (30.92%), Veterinary officers (12.37%), Veterinary supervisors (6.18%), Group D (6.18%), shepherd (2.06%), and butcher (1.03%) respectively. However, none of the animal owners screened (7 samples) were found positive in the above tests. Percentage positive among the categories is higher in shepherd
and butcher. Statistical analysis of 97 positive cases indicated a significant variation in the percentage of incidence of brucellosis among different category of people.

Table: 6.12 Depicts the result of screening among professionals

<table>
<thead>
<tr>
<th>Category</th>
<th>Number Screened</th>
<th>Number Positive in Indirect ELISA</th>
<th>Percentage Positive among categories</th>
<th>Percentage Positive among total positives</th>
<th>X²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veterinary Officer</td>
<td>128</td>
<td>12</td>
<td>9.37</td>
<td>12.37</td>
<td>0.001</td>
</tr>
<tr>
<td>Veterinary Inspector</td>
<td>262</td>
<td>40</td>
<td>15.26</td>
<td>41.23</td>
<td>61.60</td>
</tr>
<tr>
<td>Veterinary Assistants</td>
<td>101</td>
<td>30</td>
<td>29.7</td>
<td>30.92</td>
<td>27.19</td>
</tr>
<tr>
<td>Veterinary Supervisor</td>
<td>14</td>
<td>06</td>
<td>42.85</td>
<td>6.18</td>
<td>3.18</td>
</tr>
<tr>
<td>Group D</td>
<td>103</td>
<td>06</td>
<td>5.85</td>
<td>6.18</td>
<td>3.18</td>
</tr>
<tr>
<td>Shepherd</td>
<td>02</td>
<td>02</td>
<td>100</td>
<td>2.06</td>
<td>8.70</td>
</tr>
<tr>
<td>Butcher</td>
<td>01</td>
<td>01</td>
<td>100</td>
<td>1.03</td>
<td>10.51</td>
</tr>
<tr>
<td>Animal owner</td>
<td>07</td>
<td>00</td>
<td>00</td>
<td>00.00</td>
<td>12.49</td>
</tr>
<tr>
<td>Total</td>
<td>618</td>
<td>97</td>
<td>15.69</td>
<td>15.69</td>
<td>**</td>
</tr>
</tbody>
</table>

** Significant at 99% level
X²=12.49%

6.5.7 Incidence of infection in professionals

Table: 6.13 Incidence of infection in different category of professionals

<table>
<thead>
<tr>
<th>Category</th>
<th>Total</th>
<th>Number Positive</th>
<th>Percentage (Taken for 618)</th>
<th>X²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veterinary Professionals</td>
<td>505</td>
<td>88</td>
<td>14.23</td>
<td>33.16</td>
</tr>
<tr>
<td>Supporting Staff and others</td>
<td>113</td>
<td>09</td>
<td>1.45</td>
<td>33.16</td>
</tr>
<tr>
<td>Total</td>
<td>618</td>
<td>97</td>
<td>15.69</td>
<td>**</td>
</tr>
</tbody>
</table>

X²=66.32**
1df

** Significance at 99% level.

6.5.8 Inference: Among 618 serum samples screened 505 belonged to Veterinary professionals (including Veterinary Officers, Veterinary Inspectors, Veterinary
Supervisors, Veterinary Assistants) and 113 belonged to supporting staff, which includes group D, butcher, shepherds and animal owners. It can be observed from Table 6.13 that 88 out of 618 samples (14.23%) were found to be positive among Veterinary professionals and 1.45% of the samples are found to be positive among supporting staff and others. Thus the statistical analysis of the data indicated a significantly higher percentage of brucellosis recorded in Veterinary professionals as compared to other supporting groups of people/shepherds (P>0.01). Hence it can be considered as a professional hazard.

6.5.9 Geographical distribution of brucellosis

Table: 6.14 Geographical distribution of brucellosis in Karnataka for professionals.

<table>
<thead>
<tr>
<th>Place</th>
<th>Total Screened</th>
<th>Number Positive by Indirect ELISA</th>
<th>Percentage (Taken for 618)</th>
<th>X²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bagalakote</td>
<td>279</td>
<td>78</td>
<td>12.62</td>
<td>19.30</td>
</tr>
<tr>
<td>Bidar</td>
<td>148</td>
<td>02</td>
<td>00.32</td>
<td>03.30</td>
</tr>
<tr>
<td>Tumkur</td>
<td>188</td>
<td>14</td>
<td>02.26</td>
<td>00.70</td>
</tr>
<tr>
<td>Bijapur</td>
<td>003</td>
<td>03</td>
<td>00.48</td>
<td>03.01</td>
</tr>
<tr>
<td>Total</td>
<td>618</td>
<td>97</td>
<td>15.69</td>
<td>X² =26.31*</td>
</tr>
</tbody>
</table>

Significant at 99% level

From the table 6.14 it can be found that there is significant difference in the areas. The Bagalakote shows the highest rate of incidence 12.62% compared to the other three areas. The least incidence is recorded in Bidar.

6.5.10 Distribution of positive serum samples according to gender

Table: 6.15 Depicts sex-wise distribution of positive cases

<table>
<thead>
<tr>
<th>Sex</th>
<th>Number Screened</th>
<th>Number Positive</th>
<th>Percentage (Taken for 97)</th>
<th>X²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>01</td>
<td>01</td>
<td>1.03</td>
<td>47.96</td>
</tr>
<tr>
<td>Male</td>
<td>96</td>
<td>96</td>
<td>98.97</td>
<td>47.96</td>
</tr>
<tr>
<td>Total</td>
<td>97</td>
<td>97</td>
<td>X²=95.92**</td>
<td>1df</td>
</tr>
</tbody>
</table>

** Significant at 99% level.
Out of the 97 samples found positive for brucellosis irrespective of geographical areas, 96 out of 97 (98.96%) males were found positive as against 1 out of 97 (1.03%) of females. Further analysis of the data indicated that males had a higher rate of incidence compared to females (Table 6.15).

6.5.11 The influence of age in the incidence of brucellosis

<table>
<thead>
<tr>
<th>Age</th>
<th>Positive</th>
<th>Percentage (Taken for 97)</th>
<th>X²</th>
</tr>
</thead>
<tbody>
<tr>
<td>21-30</td>
<td>07</td>
<td>7.21</td>
<td>12.65</td>
</tr>
<tr>
<td>31-40</td>
<td>32</td>
<td>32.98</td>
<td>02.54</td>
</tr>
<tr>
<td>41-50</td>
<td>44</td>
<td>45.36</td>
<td>16.58</td>
</tr>
<tr>
<td>51-60</td>
<td>14</td>
<td>14.43</td>
<td>4.66</td>
</tr>
<tr>
<td>Total</td>
<td>97</td>
<td>15.69</td>
<td>=36.23**</td>
</tr>
</tbody>
</table>

** Significant at 99% level

The Table 6.16 shows the influence of age in the occurrence of brucellosis. The highest incidence (45.36%) was found among 41-50 years age group (44 out of 97) followed by 32 out of 97 cases (32.98) in the age group of 31-40. Fourteen cases out of 97 (14.43%) were among the age group of 51-60. The least number of cases i.e. seven out of 97 cases (7.21%) were recorded in the age group of 21-30. The analysis showed the significant difference in the age group as far as the prevalence of brucellosis is considered.

6.6 Result for Group II: Sero-prevalence of brucellosis in Pyrexia of Unknown Origin cases

6.6.1 Distribution of sero-positive and sero-negative individuals based on sex

<table>
<thead>
<tr>
<th>Result</th>
<th>Male</th>
<th>Percentage</th>
<th>Female</th>
<th>Percentage (Taken for 1500)</th>
<th>Total</th>
<th>Percentage (Taken for 1500)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sero positive</td>
<td>180</td>
<td>12</td>
<td>24</td>
<td>1.6</td>
<td>204</td>
<td>13.6</td>
</tr>
<tr>
<td>Sero negative</td>
<td>829</td>
<td>55.26</td>
<td>467</td>
<td>31.13</td>
<td>1296</td>
<td>86.4</td>
</tr>
<tr>
<td>Total</td>
<td>1009</td>
<td>67.26</td>
<td>491</td>
<td>32.73</td>
<td>1500</td>
<td>X² =47.16 1df</td>
</tr>
</tbody>
</table>
In the areas out of total population screened there were 204 samples positive out of 1500 giving an overall prevalence of 13.6%. 86.4% of the population is found to be negative for brucellosis. The 55.26% of the male population was found to be negative and 31.13% of the female patients were negative. The $X^2$ test of distribution indicated there is a significant variation with respect to sero-positive and sero-negative genders tested for brucellosis, (P>0.01).

![Figure 6.5 Sex wise distribution of brucellosis](image)

6.6.2 Sero-prevalence analysis by $X^2$ test
**Table: 6.18 X² test for analysis of sero-prevalence**

<table>
<thead>
<tr>
<th>Sex</th>
<th>Number Positive</th>
<th>Percentage (Taken for 1500)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>180</td>
<td>12</td>
</tr>
<tr>
<td>Female</td>
<td>24</td>
<td>1.6</td>
</tr>
<tr>
<td>Total</td>
<td>204</td>
<td>13.6</td>
</tr>
</tbody>
</table>

X² = 119.29**

**Significant**

The sero-prevalence was significantly higher in males (12%) as compared to females (1.6%), (P>0.01).

### 6.6.3 Influence of age on sero-prevalence of brucellosis

**Table: 6.19 Influence of age on brucellosis incidence**

<table>
<thead>
<tr>
<th>Age (In years)</th>
<th>Positive</th>
<th>Percentage (Taken for 1500)</th>
<th>Negative</th>
<th>Percentage (Taken for 1500)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;20</td>
<td>12</td>
<td>0.8</td>
<td>266</td>
<td>17.73</td>
<td>278</td>
</tr>
<tr>
<td>&gt;21-40</td>
<td>112</td>
<td>7.46</td>
<td>679</td>
<td>45.26</td>
<td>791</td>
</tr>
<tr>
<td>&gt;40</td>
<td>80</td>
<td>5.33</td>
<td>351</td>
<td>23.4</td>
<td>431</td>
</tr>
<tr>
<td>Total</td>
<td>204</td>
<td>13.6</td>
<td>1296</td>
<td>86.4</td>
<td>1500</td>
</tr>
</tbody>
</table>

X² = 29.611

Table 6.19 depicts influence of age and sero-positive and sero-negative results of brucellosis among the population screened. The age group of <20 shows 0.8% prevalence, the age group > 20 years shows 7.46 % prevalence and the age group of > 40 years shows 5.33% of prevalence. The sero negativity with respect to age group <20 years was 17.73%, age group > 20 years was 45.26% and age group > 40 years was 23.4% respectively.
6.4 Analysis of age group, sero-positive and sero-negative results of brucellosis

Table 6.20 Age group analysis for brucellosis results

<table>
<thead>
<tr>
<th>Age in years</th>
<th>O</th>
<th>E</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 20</td>
<td>12</td>
<td>68</td>
<td>46.11</td>
</tr>
<tr>
<td>21-40</td>
<td>112</td>
<td>68</td>
<td>28.47</td>
</tr>
<tr>
<td>&gt;40</td>
<td>80</td>
<td>68</td>
<td>2.11</td>
</tr>
</tbody>
</table>

The \( X^2 \) distribution indicated that there is a significant variation with respect to age group and sero-positive and sero-negative case. \( (X^2 \ 2df = 29.611 \text{ and } P \leq 0.01) \). Further from the positive cases the least sero-prevalence was observed among the age group of <20 years which differ significantly as compared to the sero-prevalence being recorded among the age group of 21-40 years and >40 years. Significantly higher prevalence was recorded among age group of 21-40 years while least sero-prevalence was recorded in <20 years and the variation among the positive cases differed significantly among the positive cases studied.
6.6.5 The geographical distribution of sero-positive and sero-negative individuals in South Karnataka and North Karnataka

Table: 6.21 Depicts the result of screening in South Karnataka and North Karnataka

<table>
<thead>
<tr>
<th>Place</th>
<th>Sero-positive</th>
<th>Percentage (Taken for 1500)</th>
<th>Sero-negative</th>
<th>Percentage (Taken for 1500)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>South Karnataka</td>
<td>139</td>
<td>9.27</td>
<td>1076</td>
<td>71.73</td>
<td>1215</td>
</tr>
<tr>
<td>North Karnataka</td>
<td>65</td>
<td>4.33</td>
<td>220</td>
<td>14.66</td>
<td>285</td>
</tr>
<tr>
<td>Total</td>
<td>204</td>
<td>13.6</td>
<td>1296</td>
<td>86.4</td>
<td>1500</td>
</tr>
</tbody>
</table>

The sero-prevalence of brucellosis in South Karnataka and North Karnataka are depicted in Table 6.21. It can be seen from the above table that 9.27% and 71.73% of brucellosis cases were positive and negative among the population of South Karnataka while the figure for North Karnataka was 4.33% and 14.66 % respectively.

There is a significant variation between sero-positive and sero negative and the South and North Karnataka (P≤0.01).

Further sero-prevalence of brucellosis was significantly higher (9.27%) when compared to North Karnataka region (4.33%) and the variation between South and North Karnataka region was significant, (P≤ 0.01).
Place
Figure 6.7 Geographical distribution in South Karnataka and North Karnataka
6.6.6 Sero-prevalence percentage of brucellosis in South Karnataka and North Karnataka based on sex and place

Table: 6.22 Depicts sero-prevalence of brucellosis in Karnataka based on sex and place

<table>
<thead>
<tr>
<th>Place</th>
<th>Male</th>
<th>Percentage (Taken for 1500)</th>
<th>Female</th>
<th>Percentage (Taken for 1500)</th>
<th>Total</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>South Karnataka</td>
<td>125</td>
<td>8.33</td>
<td>14</td>
<td>0.93</td>
<td>139</td>
<td>9.27</td>
</tr>
<tr>
<td>North Karnataka</td>
<td>54</td>
<td>3.6</td>
<td>11</td>
<td>0.73</td>
<td>65</td>
<td>4.33</td>
</tr>
<tr>
<td>Total</td>
<td>179</td>
<td>11.93</td>
<td>25</td>
<td>1.66</td>
<td>204</td>
<td>13.6</td>
</tr>
</tbody>
</table>

The respective percentage in South Karnataka was 8.33% in males and 0.93% in females. In North Karnataka 3.6% of males were affected by brucellosis among the population screened and 0.73 % of the female had shown sero-prevalence. The overall sero-prevalence irrespective of sex in South and North Karnataka was 4.33 % and 9.27%. 
Figure: 6.8 Sero-prevalence based on sex and place.
6.6.7 Relative efficiency of serological tests employed for diagnosis of brucellosis

Table 6.23 Relative efficacy of RBPT and indirect ELISA

<table>
<thead>
<tr>
<th></th>
<th>RBPT</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
</tr>
<tr>
<td>Positive</td>
<td>124 (a)</td>
<td>44 (b)</td>
<td>168 (a+b)</td>
</tr>
<tr>
<td>Negative</td>
<td>80 (c)</td>
<td>1252 (d)</td>
<td>1332 (c+d)</td>
</tr>
<tr>
<td>Total</td>
<td>204 (a+c)</td>
<td>1296 (b+d)</td>
<td>1500 (a+b+c+d)</td>
</tr>
</tbody>
</table>

Sensitivity = \( \frac{a}{a+c} = 60.78\% \)
Specificity = \( \frac{d}{b+d} = 96.6\% \)
Predictive value (positive) = \( \frac{a}{a+b} = 73.8\% \)
Predictive value (negative) = \( \frac{d}{c+d} = 93.99\% \).

The sensitivity of RBPT is 60.78% and specificity is 96.6%. Ability to predict the positive test is 73.8% and ability to predict negative test is 93.99%.

Table 6.24 Relative efficiency of STAT and indirect ELISA

<table>
<thead>
<tr>
<th></th>
<th>Indirect ELISA</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>STAT</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>124 (a)</td>
<td>44 (b)</td>
<td>168 (a+b)</td>
</tr>
<tr>
<td>Negative</td>
<td>80 (c)</td>
<td>1252 (d)</td>
<td>1332 (c+d)</td>
</tr>
<tr>
<td>Total</td>
<td>204 (a+c)</td>
<td>1296 (b+d)</td>
<td>1500 (a+b+c+d)</td>
</tr>
</tbody>
</table>

Sensitivity = \( \frac{a}{a+c} = 60.78\% \)
Specificity = \( \frac{d}{b+d} = 96.6\% \)
Predictive value (Positive) = \( \frac{a}{a+b} = 73.8\% \)
Predictive value (negative) = \( \frac{d}{c+d} = 93.99\% \).

The sensitivity of STAT is 60.78% and specificity is 96.6%. Ability to predict the positive test is 73.8% and ability to predict negative test is 93.99%.

6.6.8 Calculation of Kappa value for RBPT and indirect ELISA and STAT and indirect ELISA

(To find out the agreement with the result)

\[ OP = \frac{a+d}{n} \]

a = 124 and \( b = 44 \)
EP = \frac{(a+b)}{n} \times \frac{(a+c)}{n} + \frac{(c+d)}{n} \times \frac{(b+d)}{n} \quad c = 80 \text{ and } d = 1252

OP = \frac{124+1252}{1500} = 0.917

EP = \frac{a+b}{n} = 0.112
\quad \frac{a+c}{n} = 0.136
\quad \frac{c+d}{n} = 0.888
\quad \frac{b+d}{n} = 0.864

0.112 \times 0.136 = 0.015232
0.888 \times 0.864 = 0.767232
0.0152 + 0.767 = 0.7824

OP-EP = 0.917 – 0.7824 = 0.1346

Kappa = \frac{OP-EP}{1-EP} = 0.61 \text{ (Substantial agreement)} \text{ as far as predicting the positive samples is concerned (Michael Thrusfield, 2005)}

6.7 Group III: Analysis of data for non-PUO cases

Table: 6.25. Age wise distribution of sero-positive and sero-negative individuals, (Non-PUO cases)

<table>
<thead>
<tr>
<th>Age</th>
<th>Sero-positive Male</th>
<th>Sero-positive Female</th>
<th>Total</th>
<th>Sero-negative Male</th>
<th>Sero-negative Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;30</td>
<td>11</td>
<td>02</td>
<td>13</td>
<td>218</td>
<td>220</td>
<td>451</td>
</tr>
<tr>
<td>&gt;40</td>
<td>07</td>
<td>00</td>
<td>07</td>
<td>101</td>
<td>93</td>
<td>201</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
<td>02</td>
<td>20</td>
<td>319</td>
<td>313</td>
<td>652</td>
</tr>
</tbody>
</table>

The sero-positive cases from the non-PUO category after statistical analysis had shown that the sero-prevalence in the age group > 30 years was 1.99 and in the age group > 40 years it was 1.07%. The overall prevalence of brucellosis in the non-PUO cases is 3.06.
Figure: 6.9 Age wise distribution of brucellosis serology
6.7.1 Geographical distribution of brucellosis in Karnataka

Table: 6. 26 Geographical distribution of brucellosis in South and North Karnataka

<table>
<thead>
<tr>
<th>Place</th>
<th>Sero-positive</th>
<th>Sero-positive</th>
<th>Total</th>
<th>Sero-negative</th>
<th>Sero-negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
<td>Females</td>
<td></td>
<td>Males</td>
<td>Females</td>
<td></td>
</tr>
<tr>
<td>South Karnataka</td>
<td>10</td>
<td>04</td>
<td>14</td>
<td>337</td>
<td>192</td>
<td>543</td>
</tr>
<tr>
<td>North Karnataka</td>
<td>04</td>
<td>02</td>
<td>06</td>
<td>63</td>
<td>40</td>
<td>109</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>06</td>
<td>20</td>
<td>400</td>
<td>232</td>
<td>652</td>
</tr>
</tbody>
</table>

The geographical distribution of brucellosis in South Karnataka was found to be 2.14% and in North Karnataka 0.92% respectively. The male to female ratio is 2.33:1.
Figure: 6.10 Geographical distribution of brucellosis in Karnataka
6.7.2 Endemicity of brucellosis in Karnataka

6.7.2.1 Endemicity of brucellosis in professionals

Table: 6.27 Endemicity of brucellosis in professionals in Karnataka

(Data relevant to the study of 618 cases)

<table>
<thead>
<tr>
<th>Place</th>
<th>Male Positive (Taken for 97)</th>
<th>Percentage (Taken for 97)</th>
<th>Female Positive</th>
<th>Percentage (Taken for 97)</th>
<th>Total</th>
<th>Percentage (Taken for 97)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bagalakote</td>
<td>78</td>
<td>80.41</td>
<td>00</td>
<td>00</td>
<td>78</td>
<td>80.41</td>
</tr>
<tr>
<td>Bidar</td>
<td>02</td>
<td>2.06</td>
<td>00</td>
<td>00</td>
<td>2</td>
<td>2.06</td>
</tr>
<tr>
<td>Tumkur</td>
<td>13</td>
<td>13.4</td>
<td>01</td>
<td>1.03</td>
<td>14</td>
<td>14.43</td>
</tr>
<tr>
<td>Bijapur</td>
<td>03</td>
<td>3.09</td>
<td>00</td>
<td>00</td>
<td>3</td>
<td>3.09</td>
</tr>
<tr>
<td>Total</td>
<td>96</td>
<td>98.97</td>
<td>01</td>
<td>1.03</td>
<td>97</td>
<td>15.69</td>
</tr>
</tbody>
</table>
6.7.2.2 Endemicity of brucellosis in Pyrexia of Unknown Origin cases
Table: 6.28 Endemicity in North Karnataka
(Data relevant to the study of samples from Pyrexia of Unknown Origin cases)

<table>
<thead>
<tr>
<th>Place</th>
<th>Male (Taken for 204)</th>
<th>Percentage</th>
<th>Female (Taken for 204)</th>
<th>Percentage</th>
<th>Total</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bellary</td>
<td>4</td>
<td>1.96</td>
<td>3</td>
<td>1.47</td>
<td>7</td>
<td>3.43</td>
</tr>
<tr>
<td>Hospet</td>
<td>14</td>
<td>6.86</td>
<td>1</td>
<td>0.49</td>
<td>15</td>
<td>7.35</td>
</tr>
<tr>
<td>Koppal</td>
<td>9</td>
<td>4.41</td>
<td>1</td>
<td>0.49</td>
<td>10</td>
<td>4.9</td>
</tr>
<tr>
<td>Belgaum</td>
<td>6</td>
<td>2.94</td>
<td>2</td>
<td>0.98</td>
<td>8</td>
<td>3.92</td>
</tr>
<tr>
<td>Bijapur</td>
<td>20</td>
<td>9.8</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>9.8</td>
</tr>
<tr>
<td>Bidar</td>
<td>1</td>
<td>0.49</td>
<td>4</td>
<td>1.96</td>
<td>5</td>
<td>2.45</td>
</tr>
<tr>
<td>Total</td>
<td>54</td>
<td>26.47</td>
<td>11</td>
<td>5.39</td>
<td>65</td>
<td>31.86</td>
</tr>
</tbody>
</table>
Figure 6.12 Endemicity of brucellosis in North Karnataka
Table: 6.29 Endemicity in South Karnataka
(Data relevant to the study of samples from Pyrexia of Unknown Origin cases)

<table>
<thead>
<tr>
<th>Place</th>
<th>Male</th>
<th>Percentage (Taken for 204)</th>
<th>Female</th>
<th>Percentage (Taken for 204)</th>
<th>Total</th>
<th>Percentage (Taken for 204)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bangalore</td>
<td>52</td>
<td>25.49</td>
<td>3</td>
<td>1.47</td>
<td>55</td>
<td>26.96</td>
</tr>
<tr>
<td>Mandya</td>
<td>54</td>
<td>26.47</td>
<td>6</td>
<td>2.94</td>
<td>60</td>
<td>29.41</td>
</tr>
<tr>
<td>Mysore</td>
<td>3</td>
<td>1.47</td>
<td>1</td>
<td>0.49</td>
<td>4</td>
<td>1.96</td>
</tr>
<tr>
<td>Tumkur</td>
<td>11</td>
<td>5.39</td>
<td>3</td>
<td>1.47</td>
<td>14</td>
<td>6.86</td>
</tr>
<tr>
<td>Mudirangadi</td>
<td>5</td>
<td>2.45</td>
<td>1</td>
<td>0.49</td>
<td>6</td>
<td>2.94</td>
</tr>
<tr>
<td>Total</td>
<td>125</td>
<td>61.27</td>
<td>14</td>
<td>6.86</td>
<td>139</td>
<td>68.13</td>
</tr>
</tbody>
</table>

Between gender and region there is no difference. Significant variation is seen among genders. Males are more prone for brucellosis than females.
Out of 204 positive samples, 125 individuals had the history of intermittent fever with maximum temperature which usually ranged from 38°C to 41°C. The fever occurred in the evening and night with normal temperature maintained during the day over a period of 2-3 weeks. Fifty patients had high temperature with associated complaint of anorexia, weakness, severe fatigue and loss of weight; 24 patients had complained of head ache, myalgia, joint pain, arthritis and 5 patients were asymptomatic. The duration of illness was ≥ 8 weeks in all the above cases. Many individuals had the history of consuming unpasteurised milk and/or animal contact. Repeated attacks of low grade fever with depression were common manifestations in these patients.
Out of 1500 samples 124 had come positive for RBPT and STAT. The number of samples which have given the sero positivity in diagnostic titers are 32 (1:80), 36 (1:160), 29 (1:320), 17 (1:640), 6 (1:1280) and 4 (1:2560).

### 6.7.2.3 Endemicity of brucellosis in non-Pyrexia of Unknown Origin cases

#### Table: 6.30 Endemicity in South Karnataka
(Data relevant to the study of samples taken from patients with no history of fever)

<table>
<thead>
<tr>
<th>Place</th>
<th>Male</th>
<th>Percentage (Taken for 20)</th>
<th>Female</th>
<th>Percentage (Taken for 20)</th>
<th>Total</th>
<th>Percentage (Taken for 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bangalore</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
</tr>
<tr>
<td>Mandya</td>
<td>10</td>
<td>50</td>
<td>00</td>
<td>00</td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>Mysore</td>
<td>00</td>
<td>00</td>
<td>2</td>
<td>10</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Tumkur</td>
<td>00</td>
<td>00</td>
<td>2</td>
<td>10</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Mudirangadi</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
<td>00</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>10</td>
<td>50</td>
<td>04</td>
<td>20</td>
<td>14</td>
<td>70</td>
</tr>
</tbody>
</table>
Figure 6.14 Endemicity in South Karnataka: In patients without a history of fever, (Non-PUO)

Table: 6.31 Endemicity in North Karnataka, (Data relevant to the study of samples taken from patients without history of fever).

<table>
<thead>
<tr>
<th>Place</th>
<th>Male</th>
<th>Percentage (Taken for 20)</th>
<th>Female</th>
<th>Percentage (Taken for 20)</th>
<th>Total</th>
<th>Percentage (Taken for 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hospet</td>
<td>02</td>
<td>10</td>
<td>00</td>
<td>00</td>
<td>02</td>
<td>10</td>
</tr>
<tr>
<td>Bellary</td>
<td>02</td>
<td>10</td>
<td>00</td>
<td>00</td>
<td>02</td>
<td>10</td>
</tr>
<tr>
<td>Koppal</td>
<td>00</td>
<td>00</td>
<td>01</td>
<td>05</td>
<td>01</td>
<td>05</td>
</tr>
<tr>
<td>Bijapur</td>
<td>00</td>
<td>00</td>
<td>01</td>
<td>05</td>
<td>01</td>
<td>05</td>
</tr>
<tr>
<td>Total</td>
<td>04</td>
<td>20</td>
<td>02</td>
<td>10</td>
<td>06</td>
<td>30</td>
</tr>
</tbody>
</table>
Figure 6.15 Endemicity in North Karnataka: In patients without a history of fever, (Non-PUO)
CHAPTER 7

DISCUSSION

The diagnosis of brucellosis remains as one of the most challenging tests of medical knowledge and clinical acumen of the physicians. Brucellosis is diagnosed either by isolation of *Brucella* organisms in culture or by a combination of serological tests and clinical findings consistent with brucellosis. Isolation of the *Brucella* organism is the definitive means of diagnosis, but in practice it is difficult due to the early tissue localization and the exacting culture requirements of the organism. In practice, blood cultures are positive in 10-30% of brucellosis and the remainder is diagnosed serologically (Young, 1983).

It is difficult to compare sero-prevalence of brucellosis in different studies as it varies from place to place and time to time. Magnitude of problem differs from state to state in India. Even within the states in which prevalence is known it differs from place to place. The diagnosis of brucellosis also depends upon type of antigen, diagnostic techniques used and on levels of antibody titers considered as diagnostic. Selection criteria used for selection of cases for laboratory investigation for brucellosis also play an important role in determining sero-prevalence of brucellosis in particular geographical area.

In some cases, persistence of the infection cannot be confirmed with positive cultures and clinical findings and serological tests may play an important role in such circumstances. Serological tests measuring specific antibodies to *Brucella* lipopolysaccharide are of great importance in the initial diagnosis of the disease (Alton *et al.*, 1975).

ELISA is a rapid, sensitive and specific assay providing a profile of immunoglobulin classes in the diagnosis of acute and chronic brucellosis; therefore it is useful for mass screening and could be considered the method of choice for the serological diagnosis of the named disease (Araj *et al.*, 1988).
In human brucellosis the most commonly used antigens in indirect ELISA include lipopolysaccharide of *Brucella abortus* (Magee, 1980; Klerk and Anderson, 1985; Sipple *et al.*, 1982 and Saz *et al.*, 1987). In these studies, *Brucella* antigenic preparations were reported to detect antibodies against almost all *Brucella* species except *Brucella canis* and *Brucella ovis* which are only detected by using the major outer membrane protein antigens (Hunter *et al.*, 1986).

Our study group comprised of 2770 patients out of which 1989 males and remaining 781 were females.

7.1 Identification of study groups

The individuals are divided into 3 groups and the results are interpreted first for all the 2770 samples and then for the 3 groups individually. The three groups identified in our study were:

1. Group I containing 618 serum samples from professionals consisting of Veterinarians, Para veterinarians, animal owners, shepherds and slaughter house worker, who were in regular contact with the animals.

2. Group II comprising of 1500 serum samples collected from the persons with a history of fever > 37ºC, which was undiagnosed. These samples consisted of 578 Widal negative sera, 231 malaria negative sera, 222 HbsAg negative sera, 12 Weil – Felix negative sera, 414 sera from fever > 8 weeks, 23 sera from fever between 8-52 weeks and 20 sera from fever > 52 weeks.

3. Group III consisting of 652 individuals in whom the fever was not the prominent symptom but who had a history of joint pain, arthritis and backache etc. These samples consisted of 333 RA negative sera, 176 ASLO negative sera, and 143 CRP negative sera.

7.2 Interpretation of the serological tests for 2770 samples

The serum samples are subjected first to RBPT and STAT. All the 2770 serum samples are later tested by indirect ELISA by using S-LPS of *Brucella abortus* 99 and *Brucella melitensis* 16 M biotype 1.
The percentage (4.98%) of individuals showing agglutinins in the present study of 2770 patients by RBPT and STAT is comparable with that of Mahakur and Panda (1972) who reported the prevalence of brucellosis to be 3.72%. Similar sero prevalence was reported by Memish et al. (2002) in Saudi Arabia by STAT which was found to be 5% in diagnostic titer of ≥ 1:320.

The sero-prevalence in the present study is higher than the sero-prevalence reported by Mantur et al. (1994) who reported a sero-prevalence of 2.2%. Mantur et al. (2006) reported 1.9% of sero-prevalence by using RBPT by analyzing 26948 serum samples screened for serological evidence of brucellosis over a period of 16 years. Analysis of samples by STAT recorded 1.8% of sero-positivity in titers ranging from 1:10 to 1:10240. But percentage of sero positivity in agglutination titers of ≥ 1:80 has shown great variation in different studies (Mahakur and Panda, 1972, Ramanna et al., 1982, Anand, 1968). High sero positivity for brucellosis in some of the studies may be due to endemicity of the disease in those particular areas (Shukla, 1962, Panjarathinam and Jhala, 1986). This percentage is much higher than many other studies done earlier. Thakur and Thapliyal (2002) reported 1.38% sero-prevalence by RBPT and 0.82% by STAT in Uttaranchal. In the present study sero-positivity is higher than the sero-prevalence reported by Ajay Kumar and Nanu (2005), which is 1.6%. Sharma et al. have reported a sero-prevalence of 0.89% in Delhi in 1979.

In the present study, sero positivity for brucellosis in males (91.58%) was found to be higher than that of female (8.4%) in diagnostic titers, (Agasthya et al.,: appendix 13.1:2008). A sero-prevalence of 10 % by STAT and 18.75% by RBPT is reported by Barbuddhe et al. (1994). A sero-prevalence of 9.33% is reported by Katti et al., (2001), in Thiruvanthapuram by STAT in diagnostic titers and the distribution of titers were ranging from 1:10 to 1:1280. The titers observed in our study were ranging from 1:80 to 1:2560 and are also different from the observation made by Mantur et al. (2006) which showed the distribution of titers from 1:10 to 1:10240.

The sero prevalence reported in this study by RBPT and STAT is different from the sero-prevalence reported by Thokar et al. (2000), who have recorded 21.7% of sero positivity in diagnostic titer of 1:80 to 1:1280. Present study has shown the titer wise distribution for 1:80, 1:160, 1:320, 1:640, 1:1280 and 1:2560 was 30 (21.73%), 37 (26.81%), 35 (25.36%), 18 (13.04%), 14 (10.14%) and 4 (2.89%). This is different from the observation made by the same workers who have reported the titer wise
distribution for 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560 was 45 (32.8%), 58 (42.3%),
10 (1.5%), 24 (2.7%) and 0 respectively (Thokar et al., 2000).

The study done by Ruiz-Mesa et al in 2005 showed that the Rose Bengal test was
the sole technique for the diagnosis of brucellosis in endemic areas and should be
considered very carefully in the context of patients who are exposed repeatedly to
Brucella or have a history of brucellosis.

The male-female ratio in diagnosed cases in present study is 10:1. This is in
contrast to the sero positivity reported by Thakur and Thapliyal (2002) who reported a
sero- prevalence of 5.95% in males and 3.15% in females by using RBPT, STAT and
dot-ELISA in Uttaranchal. But in present study, difference in sero positivity between
males and females is statistically insignificant. It may be due to less number of female
subjects in occupationally exposed group. This study is also different to the sero
prevalence reported by Hussein et al (2005) in Assiut Governorate which has shown a
sero-prevalence of 1.64% in female and 1.01% in male by indirect ELISA.

Brucellosis in human beings occurs in all age groups and both males and females
are affected equally in particular when dairy is the most common source of infection.
(Mantur et al., 2004). Brucellosis may be more common in male in areas where the
disease is an occupational hazard of farmers and shepherds, butchers or Veterinarians.
Brucellosis in children can be very common in particular in areas with Brucella
melitensis (Mantur et al., 2004).

Over all sero positivity for brucellosis is found to be higher in 41-50 years age
group i.e. 38%. All the sero-positive cases were distributed between 10 years to 60
years of age. The sero-prevalence is recorded as follows: 38% in the age group of 41-50
years, 37.07% in the age group of 31-40 years, 14.33%in the age group of 21-30 years,
followed by 6.85% in the age group 51-60 years, 2.8% in the age group of 11-20 years
with the least prevalence recorded in the age group < 10 years, (0.93%, Table 6.4).

The sero positivity recorded in this study is similar to the sero-prevalence reported
by Hamzic et al (2005) in Bosnia and Herzegovina who recorded the highest sero-
prevalence of 23.03% in the age group of 31-40 and 41-50.

The higher sero-prevalence of brucellosis in male between 41-60 years of age can
be attributed to increased chances of exposure to infectious agent due to active outdoor

The objective of this study was to develop a sensitive and specific diagnostic test for the detection of *Brucella* antibodies in human serum samples with the focused application of testing a large number of serum samples. Lipopolysaccharide is commonly used as an antigen in most indirect ELISA formats (Nielsen and Gall, 1994).

With the effective application of standardized indirect ELISA the sero positivity obtained for the 2770 screened serum samples is 11.58%, (appendix 13.1), which definitely is almost three times higher than the sero positivity observed by RBPT and STAT. This is due to the higher sensitivity and specificity of indirect ELISA. Indirect Enzyme Linked Immunosorbent Assays (iELISA) with LPS are held to be more sensitive and specific as the combination of other serological tests of which detect antibodies to the LPS (Diaz and Levieux, 1972).

If a sero-epidemiological survey was made by using RBPT and STAT only, the low percentage sero-prevalence would have been observed. ELISA test has the advantage of being highly sensitive and specific. Indirect ELISA used in the present study can detect significantly more number of positive cases than RBPT and STAT, i.e. the ratio among the tests is being 1:1:2.32 (RBPT: STAT: iELISA). The total number of cases diagnosed by RBPT and STAT in our study is 138 while indirect ELISA had diagnosed 321 numbers of samples as positive out of 2770 total sera samples.

In present study, the analysis of the 2770 samples by the RBPT: STAT: indirect ELISA has shown that the ratio between the tests is 1:1:2.32.

Compared to indirect ELISA the RBPT is 75.4% sensitive and 92.92% specific. The predictive value for positive result is 42.99% and predictive value for negative result is 98.16%. The analysis of the tests has indicated that the agreements between the RBPT, STAT and indirect ELISA have shown moderate agreement. Compared to RBPT, STAT the indirect ELISA is 100% sensitive and specific.

The observed sero-prevalence of 11.58 is in close accordance with the observation made by Barbuddhe *et al* (1994) who reported 16.25% of sero positivity by using dot-ELISA, by using autoclaved extract of *Brucella abortus* S 99. Our result is different from the sero prevalence reported by Batra *et al* (2004) who reported 235 samples positive by using dot-ELISA kit.
Review of the serological studies done by using the conventional tests and indirect ELISA, a large number of information is available. Following is the sero prevalence reported in different parts of the world by using indirect ELISA.

Saz et al (1987) in Spain reported that the indirect ELISA was the most sensitive test with 97% sensitivity and 96% of specificity in 1985, and was the only positive test in 6% of the patients in whom brucellosis had been confirmed by culture out of 208 patients..

Fernandex -Lago and Diaz (1986), demonstrated that indirect ELISA has the higher sensitivity compared to radial immunodiffusion, by using LPS ELISA detecting IgG class of antibodies.

Elias Krambovitis et al (1992) used the indirect ELISA for detecting brucellosis antibodies. 11.6% (34/293) of subjects with no history of brucellosis gave a positive test result. This study is in accordance with the present observation.

A sero-prevalence of 13.39% has been reported by Magee in 1980 indicating the higher sensitivity and specificity of ELISA and interpreted to be more rapid and simpler than the battery of agglutination tests commonly used.

A study conducted by Gad EI-Rab and Kambal (1998), in Saudi Arabia revealed that the indirect ELISA test in addition to measuring the antibody classes directly also detects the incomplete antibodies and it can efficiently replace the conventional test. This saves considerable laboratory cost and time. The study showed that 30 culture positive cases of brucellosis gave significant result in indirect ELISA, while only 10% of these gave readings less than 1:160 by Standard Agglutination Test.

Hussein et al (2005) have reported a sero prevalence of 30.4% for IgG antibodies and 9.8% of IgM antibodies in Assiut Governorate by screening a total of 7154 peripheral blood samples by indirect ELISA.

Rajaii et al (2006) carried out the comparison of indirect ELISA and STAT for diagnosis of brucellosis. The study revealed that indirect ELISA was more sensitive when compared with Standard Tube Agglutination Test. The indirect ELISA in addition could detect 15 samples positive which were detected negative by STAT. The evaluation of the methods used for the serologic diagnosis of brucellosis has revealed 83.7% of sero positivity for IgG ELISA by Sirmatel et al in Gaziavitep in 2002.

A specific antibody profile in human brucellosis is being studied by Arija et al (1992) and this study revealed 89% for IgG sero-prevalence in 761 serum samples.
In a study carried out by Araj et al in Kuwait in 2005, by using indirect ELISA a sero prevalence of 97% was reported in sera from patients in different stages of brucellosis, indicating that ELISA with its IgG, IgM and IgA profile is the test of choice in the diagnosis of patients with brucellosis.

Table 7.1 depicts the result of various sero-prevalence studies done by various investigators in different part of India by using the conventional, dot ELISA and indirect ELISA. The present study at PD_ADMAS is done by using indigenously standardized indirect ELISA.

**Table 7.1: Brucellosis sero-prevalence by RBPT, STAT and indirect ELISA and dot-ELISA**

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Author, Place of study and Year of study</th>
<th>Total Cases</th>
<th>Sero-prevalence By</th>
<th>By</th>
<th>By</th>
<th>By</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>RBPT</td>
<td>STAT</td>
<td>Indirect ELISA</td>
<td>Dot ELISA</td>
</tr>
<tr>
<td>1</td>
<td>Agasthya et al PD_ADMAS, Bangalore, 2004-2008</td>
<td>2770</td>
<td>4.98%</td>
<td>4.98%</td>
<td>11.58%</td>
<td>------</td>
</tr>
<tr>
<td>2</td>
<td>Mantur et al Bijapur, 1994</td>
<td>3152</td>
<td>2.2%</td>
<td>2.2%</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>3</td>
<td>Barbuddhe et al Izat Nagar, 1994</td>
<td>80</td>
<td>19.48%</td>
<td>10.38%</td>
<td>------</td>
<td>16.25%</td>
</tr>
<tr>
<td>4</td>
<td>Katti et al 2001 Thiruvananthapurnam</td>
<td>75</td>
<td>------</td>
<td>9.33%</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>5</td>
<td>Thakur and Thapliyal Uttaranchal, 2002</td>
<td>352</td>
<td>1.38%</td>
<td>0.82%</td>
<td>------</td>
<td>4.97%</td>
</tr>
<tr>
<td>6</td>
<td>Sharma et al Uttar Pradesh and Delhi 1979</td>
<td>1685</td>
<td>0.89%</td>
<td>------</td>
<td>------</td>
<td>------</td>
</tr>
<tr>
<td>7</td>
<td>Elizabeth et al Vellore, 1996</td>
<td>23</td>
<td>------</td>
<td>3.85%</td>
<td>13%</td>
<td>------</td>
</tr>
</tbody>
</table>

7.3 Group 1: Interpretation of the result for the professionals

Out of 618 professionals 14 have shown the presence of *Brucella* antibodies in their serum by RBPT and STAT showing 2.26% prevalence. This is in close accordance with the findings of Ajay Kumar and Nanu (2005), who reported sero prevalence 1.14% in Veterinarians and also to the study conducted by Araj and Azzam in 2000, in Lebanon who reported 1% of prevalence among veterinarians. A higher rate of prevalence was reported by Rana et al (1979), which revealed 27.7%. A sero-prevalence
of 5.9% has been reported by Desai et al in 1995 which is much higher than the present
observation. Hemashettar and Patil (1991) reported a sero-prevalence of 8.2% by STAT
in Belgaum.

In 2000, Kalorey et al reported 9.67% in Vidarbha region by RBPT and STAT
among in contact animal attendants and is much higher than the present report which
indicates a higher rate of prevalence in the endemic area. Mudaliar et al (2003) reported
5.33% of sero-positivity in animal handlers in Pune. In the same study a sero-
prevalence of 14.63% was recorded in Veterinary doctors.

The classical RBPT and STAT test have shown similar results of 2.26 percentage
positivity. RBPT test is often used as a rapid screening test. Chadda et al (2004)
reported 8% of sero-prevalence among the high risk group. The study group consisted
of shepherds, farmers and animal attendant. In the present study, the titers reported in
STAT are in a range from 1:160 through 1:1280 IU. This is different from the
observation made by Thokar et al (2000) who reported the sero-prevalence in titer wise
distribution from 1:80 through 1:1280. Hussain et al reported a sero-prevalence of 6.7%
in individuals closely associated with animal husbandry practice in 2000, in Assam, and
in STAT the titer was found from 1:80 to 1:320.

All the 618 samples collected from professionals showed total 97 sera positive for
of the brucellosis is 15.69% when the samples were subjected for indirect ELISA. The
analysis of RBPT: STAT: ELISA has shown that indirect ELISA test can detect
significantly more number of positive cases than RBPT and STAT. However, RBPT
and STAT are equally efficacious in identifying positive cases. Ratio among the test is
1:1:7.

The 47 (48.45%) of 97 are asymptomatic; 'at risk' individuals screened were sero
positive for Brucella antibodies. This is much higher than the sero prevalence reported
by Handa et al (2000).The antibody titers in the cases of chronic brucellosis are often
no greater than those of asymptomatic patients, with the history of either occupational
exposure to Brucella or following recovery from acute infection or this reason, it has
been concluded that, the diagnosis of chronic brucellosis had to be based on
epidemiological and clinical grounds. However, it is desirable to obtain an objective
evidence of infection by serological tests (Corbel, 1997).
A total of 165 serum samples of abattoir personnel of Delhi were tested and higher sero-prevalence of 20.6% by RBT and 12.75% by STAT has been reported by Kumar et al (1997). The same workers reported a sero prevalence of 25.45% by Dot ELISA.

The present observation is similar to the sero-prevalence reported by Thakur and Thapliyal (2002) who recorded a sero-prevalence of 17.39% by using dot ELISA which revealed markedly higher prevalence among field Veterinarians. The screening of 352 human serum samples yielded a sero positivity of 1.38% by RBPT, 0.82% by STAT and 4.97% by dot-ELISA. A sero positivity of 16.25% has been reported by Barbuddhe et al in 1994 by dot ELISA. Our study has revealed 15.69%, which is similar to this study.

In the group I, i.e. in risk group individuals the sero positivity is higher in males (98.96%) compared to females (1.03%). This is different from the finding of Kapoor et al (1985) who reported higher sero prevalence in female compared to male. In the present study the difference between males and females is statistically insignificant. It may be due to less number of female subjects in occupationally exposed group.

The sero positive cases were found between 21-60 years of age. The highest prevalence is seen in the age group of 41-50 years, (45.36%). This is different from the sero prevalence studies of Rana et al (1979), who reported that the age group from 31-40 years is more affected. This study is in accordance with the observation made by Kapoor et al (1985) who reported that the age groups of 15-30 years and less than 15 years are less affected than the age groups above 30 years (Kapoor, 1985). The sero-prevalence study conducted by Chadda et al (2004) indicated that the age group of 16-40 years is more susceptible to infection. This is different from our observation.

The higher rate of asymptomatic (sero positive) among the professionals may be attributed to the development of acquired immunity, which may present these subjects from developing overt disease following infection (Young, 1983).

### 7.4 Group II: Interpretation of the result for the PUO cases

Out of 1500 cases, 124 have shown the presence of *Brucella* antibodies in diagnostic titers ranging from 1:80 to 1:2560, giving a sero prevalence of 8.26%. Panjarathinam and Jhala in Gujarat in 1986 reported 8.5% sero- positivity and our study is in accordance with this observation. However, this finding is higher than the observation (2.7%) done by Balbir and Saxena in 1964, Chitkara et al in 1966, (2.08%)
and Singh and Saxena (1964) who reported a sero-prevalence of 5.23%. Roy et al (1965) reported 6% sero-prevalence for the PUO cases in Jamnagar.

The sero positivity obtained for the 1500 samples by RBPT and STAT is same i.e. 8.26%. This is different from the observation made by Barbuddhe et al (2000) who reported 10% sero-prevalence by RBPT and 28.7% by STAT indicating differing sensitivity of RBPT and STAT. The result obtained in the STAT shows a high degree of correlation with those obtained by testing serum in RBPT (Arija et al 1992), which is observed in our study. A sero-prevalence of 3.8% is reported by Elizabeth et al in Vellore by STAT in 1996.

In the present study, sero positivity for brucellosis in males is 12% as compared to females 1.6%, (P>0.01) as shown in Table 6.18. The male/female ratio in diagnosed cases in the present study of PUO cases is 7.5:1 and this is different from the observation made by Nagalotimath et al in 1978, (1.6:1). This is also different from the observation made by Kadri et al (2000) who reported the ratio of male to female to be 3:1 in 2000. In the present study the difference in sero positivity between male and female is statistically insignificant. It may be due to less number of female subjects available in the study.

Over all sero positivity for brucellosis found to be higher between <20 to > 40 years. The higher sero prevalence of brucellosis in males between 20-50 years of age can be attributed to increased chances of exposure to infectious agent due to active outdoor life. The highest sero prevalence is seen in the age group of 21-40 years (7.46%, Table 6.19). Sen and Khama (1978) reported 7.5% of sero reactors in the age group of 15-44 years. Our study is different from the observation made by Kadri et al (2000), who reported the highest number of positive males (42.85%) belonged to the age group 21-30 years.

The classical Rose Bengal Test is often used as a rapid screening test. In STAT the titers reported are in a range from 1:80 –1:2560 IU. Katti et al (2001) reported a sero-prevalence of 9.33% in 7 patients. Serum samples subjected for STAT showed the titers ranging from 1:10 to 1:1280. Mantur et al (2006) have reported a sero-prevalence of 1.8% in patients who had a predominant history of fever. The titers in STAT recorded were ranging from 1:10 to 1:10240. This is different from our study.
Sensitivity of RBPT was 60.78% and specificity was 96.6% in the present study. Kiel and Khan (1987) reported that the sensitivity of RBPT is very high but the specificity can be disappointingly low. Our observation is different from this.

The subsequent analysis of all the 1500 serum samples of the Pyrexia of Unknown Origin have shown total 204 samples positive for Brucella antibodies. The overall sero-prevalence of the brucellosis is 13.6% when the samples were subjected for indirect ELISA, (Agasthya et al, appendix 13.3:2009). Elizabeth et al (1996) reported 13% of sero-positivity by IgG ELISA for 23 patients in whom the serological diagnosis of brucellosis was sought, by using the ELISA kits of Virotech, Dic System Diagnostika, GMBH, Germany in Vellore. Our study is in accordance with this observation for the PUO cases.

The sero-prevalence of 61.9% has been reported by Sirmatel et al (2002) Gaziatip by using indirect ELISA in chronic brucellosis cases. A sero prevalence of 11% has been reported by Hunter et al in 1986, by using indirect ELISA in 11 patients with a history of 1-29 weeks after on set of brucellosis, out of 101 sera from suspected cases of brucellosis by using outer membrane protein as antigen. The study conducted by Sippel et al (1982) had revealed that the ELISA is an excellent method for screening large populations for Brucella antibodies and for differentiation between the acute and chronic phases of the disease.

In the present study the geographical distribution of brucellosis shown an increased prevalence in South Karnataka (9.27%) compared to North Karnataka (4.33%). In South Karnataka a sero-prevalence of 1.27% has been recorded by Umapathy in 1984, by STAT and the present sero-prevalence is much higher than the study done by Umapathy (1984). This could be attributed to higher sensitivity of ELISA and serum samples mostly collected from persons closely associated with Animal Husbandry practices and higher prevalence of infection.

In North Karnataka (4.33%) of sero positivity is observed. This is different from the observation made by Mantur et al (1994) who reported 2.2% of sero prevalence among the patients attending B.L.D.E.A.’s hospital, Bijapur. Nagalotimath and Joglekar in 1971-1975 reported 90 cases out of 10268 samples (0.87%) in Belgaum by STAT (Nagalotimath and Joglekar, 1978).
7.5 Group III: Interpretation of results for non-PUO cases

These individuals have not given any history of either the consumption of unpasteurised milk and milk products and had no risk of developing brucellosis, but the chances of animal contact could not be ruled out as some of the individuals are from the rural areas. The constant complaint in this category was the persistent joint pain. The collected sera were negative for the Rheumatoid Arthritis, C-reactive protein and Anti Streptolysin O tests. When subjected for the serological analysis out of 652 samples none were positive by RBPT and STAT but 20 samples had come positive by Indirect ELISA.

The overall sero-prevalence of brucellosis in this category of individuals is 3.06% by indirect ELISA. The statistical analyses of the samples have shown that the sero-prevalence is high in the age group > 30 years (1.99%) followed by age group > 40 years (1.07%), (Table 6.25).

Nagalotimath and Joglekar (1978) have reported 30% diagnosed cases, which are not linked to any high-risk occupation in 1978. In India, Mathur (1969) has reported many outbreaks of brucellosis in families and Institute, which he attributed to consumption of raw milk and ice cream.

The geographical distribution of brucellosis in South Karnataka is found to be 2.14% and North Karnataka 0.92%.

The sero-positivity for brucellosis found to be higher among occupationally exposed individuals (15.69%) by ELISA, when compared to group II and group III. The sero-positivity in group I shows statistically significant difference from that of group II and III. This finding is in accordance with many other studies, showing that brucellosis is an occupational disease in India (Nagalotimath and Joglekar, 1978; Hemashettar et al 1991; Kalorey et al 2000; Kochar et al 2003; Mudaliar et al 2003; Chadda et al 2004; Desai et al 1995).

The percentage of diagnosed cases in-group I (professionals) and group - II (Pyrexia of Unknown Origin) are significantly higher when tested by indirect ELISA. This shows that there is a change in epidemiological trend of brucellosis from occupational disease to a food borne disease in many developed countries (USA, Europe) and also in countries where brucellosis is endemic. Importance of raw milk and milk products in transmission of disease has been shown in many other studies in India (Mathur, 1954). Chadda et al (2004) reported an incidence of brucellosis in individuals
who had habit of raw meat ingestion. Others recorded in the same study were shepherds and farmers who were having the habit of taking raw milk.

The difference between percentages of sero positivity (in agglutination titers of ≥ 1:80) of brucellosis found to be higher in group I when compared to group II and Group III. Higher percentage of sero-positivity in group I may be due to constant exposure to infective source leading to sustained production of agglutinins (Kerr, 1968). This kind of constant exposure to infectious agent leading to development of acquired immunity may prevent these subjects from developing overt disease following infection (Young, 1995).

Among the individuals with no history of fever the sero positivity is found to be 3.06%, when compared to risk group individuals and patients with history of fever. The sero positivity is detected only by indirect ELISA indicating the need of tests with higher sensitivity as no cases are detected positive by RBPT and STAT in this group.

Serological survey of apparently healthy individuals was undertaken to find out levels of *Brucella* agglutinations, which were collected in the group III. Serum from 50 blood donors was screened. We observed none of them were tested positive by RBPT, STAT and ELISA. This is different from the observation made by Vaishnavi et al (2007) who reported 0.36% of sero-prevalence in blood donors in Chandigarh. Also 25 samples received for VDRL test in which fever was not a predominant symptom did not show any sample positive with significant titers either by RBPT, STAT or Indirect ELISA. This is in contrast to the observation made by Singh and Saxena (1964), who reported sero prevalence of brucellosis in serum samples received for VDRL as 3.37%. Chitkara et al (1966) studied 1004 serum samples from blood donors and samples sent for VDRL. They reported sero positivity of 12.55% among them. The reason for not finding any positive sample may be due to screening of less number of serum samples in the present study. The difference in sero positivity for brucellosis among apparently healthy population may be due to difference in degree of endemicity of brucellosis in those regions.

Higher agglutinin titers found among 50 subjects in control group was 1:40 and the diagnosed cases of brucellosis in present study showed agglutination titers of 1:80 and above. Hence, agglutination titer of ≥1:80 can be taken as diagnostic in this particular area. But, before concluding so, there is a need for extensive community based surveillance.
CHAPTER 8

CONCLUSIONS AND SCOPE FOR FUTURE WORK

8.1 CONCLUSION

The present study of brucellosis is done among 2770 individuals with 618 professionals forming the first group I which consist of the individuals who are in regular contact with the animals, included the Veterinarians, Para Veterinarians, shepherd, slaughter house worker and animal owners. The group II had 1500 patients with a history of Pyrexia of Unknown Origin attending various hospital laboratories and private diagnostic units. The group III had 652 individuals with a history of joint pain, arthritis, back ache and all were negative for ASLO, RA, CRP etc., Also included 50 blood donors and 25 samples collected for VDRL, without a prominent history of fever. The control group consisting of 50 healthy populations who had no history of animal contact or consumption of raw milk or any history of fever.

The analysis of 2770 serum samples by RBPT and STAT had revealed 4.98% (138 positives out of 2770 samples) sero-prevalence and 11.58% by indirect ELISA, (321 positives out of 2770 samples). The number of samples which have given the sero positivity in diagnostic titers are 30 (1:80), 37 (1:160), 35 (1:320), 18 (1:640), 14 (1:1280) and 4 (1:2560). All the samples which were tested positive by STAT with agglutination titers of ≥ 1:80 were also tested positive by RBPT.

The overall prevalence of infection in Karnataka is 4.98% by RBPT and STAT and 11.58% by indirect ELISA. The ratio among the three serological tests was found to be 1:1:2.32. The sensitivity for RBPT was found to be 75.4% and specificity 92.92%. Similar was the observation recorded in STAT. Analysis of the samples by Kappa statistic was found to be 0.5060514 which indicated moderate agreement between indirect ELISA and RBPT and between indirect ELISA and STAT.

All the sero-positive cases were distributed between 10 to 60 years of age. The sero positivity for a total of 2770 samples screened in the present study was 91.58% in males and 8.41% in females. There is no statistically significant difference between male and female sero positivity.
Sero positivity was found to be more in the age group of 41-50 years (38%), followed by 31-40 years (37.07%), followed by 21-30 years (14.33%), followed by 51-60 years (6.85%), followed by 11-20 years (2.8%) with the least prevalence recorded in the age group < 10 years (0.93%).

Out of 2770 samples the 3 groups identified are professionals, Pyrexia of Unknown Origin and the individuals without a history of fever. Group I, engaged in occupations in which they are at increased risk of contracting brucellosis, group II with a history of fever which was not diagnosed for more than 3 weeks and the group III which did not present fever as the major symptom but had a persistent history of joint pain, arthritis and backache. Sero-prevalence of brucellosis was highest in group I i.e. 2.26% by RBPT, STAT and 15.69% by indirect ELISA. There was statistically significant difference between group 1, group 2 and 3.

Further analysis of the result detected by STAT and indirect ELISA revealed the significant variation between the tests and positive and negative cases detection. The significance is at p<0.01%. Indirect ELISA test could detect significantly higher number of positive cases than RBPT and STAT. In the analysis the STAT and RBPT were found equally efficacious in detecting positive cases.

8.1.1 The individual category of samples has given the following observations

**Group I:** The 618 serum samples from professionals had shown 2.26% (14 positive out of 618 samples) of sero positivity by RBPT and STAT and 15.69% (97 positives out of 618 samples) by indirect ELISA. Of all the 97 sero-positive professionals, the highest rate of sero prevalence of brucellosis was observed among the Veterinary inspectors (41.23%) followed by Veterinary assistants (30.92%), Veterinary officers (12.37%), Veterinary supervisors (6.18%), animal attendants (6.18%), shepherd (2.06%), and butcher (1.03%). The diagnostic titer recorded in all the positive cases was ≥ 1:80. All the samples that had come positive by RBPT also had come positive by STAT.

Among 618 serum samples screened 505 belonged to Veterinary professionals (including Veterinary Officers, Veterinary Inspectors, Veterinary Supervisors, Veterinary Assistants) and 113 belonged to supporting staff, which includes group D, butcher, shepherds and animal owners. After screening, 88 out of 618 samples (14.23%) were found to be positive among Veterinary professionals and 9 out of 618 samples (1.45%) were found to be positive among supporting staff and others. Higher percentage of
brucellosis recorded in Veterinary professionals as compared to other supporting groups of people/shepherds (P>0.01). Geographical distribution of brucellosis in Karnataka for group I was found to be highest in Bagalakote (12.62%) compared to other three areas i.e. Bidar, Tumkur and Bijapur, which recorded 0.32%, 2.26% and 0.48% of sero-prevalence respectively, (Table 6.14). Males outnumbered females. The sero-prevalence in males was 98.97% and in females 1.03%. The significance was at 99% level, (Table 6.15).

The highest incidence (45.36%) was found among 41-50 years age group (44 out of 97) followed by 32 out of 97 cases (32.98) in the age group of 31-40. Fourteen cases out of 97 (14.43%) were among the age group of 51-60. The least number of cases i.e. 7 out of 97 cases (7.21%) were recorded in the age group of 21-30.

In group II, for PUO cases 124 samples out of 1500 have shown the presence of antibodies by RBPT and STAT in diagnostic titers ranging from 1:80 to 1:2560, giving a sero-prevalence of 8.26% and 13.6% (204 positives out of 1500 samples) by indirect ELISA. The result obtained in STAT shows a high degree of correlation with those obtained by testing serum in RBPT. The sero positivity in males was 12% (180 out of 1500 samples) and 1.6% (24 out of 1500 samples) in females, (p>0.01). The sero-prevalence was higher in males than females. The sero-positive cases were found to be highest between 20 to 40 years of age. The age group of <20 showed 0.8% prevalence, the age group > 20 years showed 7.46 % prevalence and the age group of > 40 years showed 5.33% of prevalence, (Table 6.19 ). A sero-prevalence of 9.27% (139 out of 1500 samples) and 4.33 % (65 out of 1500 samples) had been observed in South Karnataka and North Karnataka respectively, (Table 6.21).

The sensitivity of RBPT and STAT is 60.78% and specificity is 96.6%. Ability to predict the positive test is 73.8 and ability to predict negative test is 93.99.

The serum samples were also tested by the S-LPS of *Brucella melitensis* 16 M biotype 1. The indirect ELISA is standardized for the *Brucella melitensis* S-LPS antigen but our study has not revealed any difference in the sero positivity for brucellosis caused by *Brucella abortus* and *Brucella melitensis*. The conjugate used in the development of indirect ELISA has shown the same dilution determined by checkerboard titration required for the positive reaction, with the similar concentration of antigen at a rate of 5µg / ml.

As we could not find the difference in the result for indirect ELISA by using the S-LPS of *Brucella abortus* and *Brucella melitensis* S-LPS by screening of PUO samples. This is due to the fact that the three classical species of the *Brucellae, Brucella abortus,*
*Brucella melitensis* and *Brucella suis* represent a group of closely related bacteria of medical and veterinary importance with similar morphological, biochemical, immunological and pathological characteristics (Stableforth and Jones 1963, Hoyer and McCullough 1968).

In individuals with no history of fever the seropositivity is found to be 3.06% only by indirect ELISA while the conventional tests have come negative. The statistical analysis has shown that the sero-prevalence in the age group > 30 years is 1.99 and in the age group > 40 years it is 1.07%. The geographical distribution of brucellosis in South Karnataka was found to be 2.14% and in North Karnataka 0.92% respectively.

The diagnosis of Pyrexia of Unknown Origin (PUO) remains as one of the most challenging tests of medical importance and clinical acumen of the physicians. With large number of possible etiologies, it is obvious that no single plan can be outlined for the systematic study of PUO cases. Infections have been and continue to be, the single most important cause of PUO in Indian subcontinent and many other developing countries. Brucellosis is one of the most misdiagnosed diseases and considered to be a classical cause of PUO.

The study done at Project Directorate on Animal Disease Monitoring and Surveillance showed that Brucellosis is a major zoonotic disease with a worldwide distribution with higher prevalence in South Karnataka compared to North Karnataka. There is a strong occupational predisposition and most adults living in contact with or dealing with animals, animal excreta and animal products run a greater risk of contracting the disease. Non-occupational exposure can result consumption of raw milk or milk products and even handling of meat. Brucellosis is not an uncommon disease among patients presenting with Pyrexia of Unknown Origin. All the patients with history of fever and appropriate epidemiological background should be considered as possible cases of brucellosis. The lack of vaccines for human beings and awareness among livestock owners, farmers and rural public about brucellosis is a major limitation in control of this disease in human population. Vaccines developed to prevent this disease in humans have had limited efficacy and have been associated with serious medical reactions.

In present study, the results obtained suggested that the alertness of clinicians and close collaboration with microbiologists are essential even in endemic areas to diagnose and treat brucellosis. While effective control measures still need to be implemented, Medical doctors, Veterinarians and other health workers may help patients and risk groups
to prevent brucellosis by educating them on essential methods to prevent exposure to the pathogen such as boiling of milk and to avoid the consumption of dairy products prepared from unpasteurised milk. The screening of family members of index cases of acute brucellosis in an endemic area should be undertaken to pick up additional unrecognized cases. The history of travel to endemic regions along with exposure to animals and exotic foods are usually critical to making the clinical diagnosis. Rapid and reliable, sensitive and specific easy to perform and automated detection systems for *Brucella* species are urgently needed to allow early diagnosis and adequate antibiotic therapy in time to decrease morbidity/mortality. Routine serological surveillance is not practiced even in *Brucella*-endemic areas and from this study it is suggested that this should be a part of laboratory testing coupled with a high index of clinical suspicion to improve the level of case detection. It is necessary that reference laboratories for brucellosis in India are aware that many patients go undetected or misdiagnosed. Attempts at continuing medical education can be rewarding in such cases. An indirect ELISA test for brucellosis may be performed on patients with prolonged fever. A prospective evaluation of the test in patients with fever and response to treatment is warranted to establish the epidemiology and therapeutic efficacy. The Veterinarians and physicians need to work jointly to eradicate human brucellosis in India.

Prevention of human brucellosis focuses mainly on elimination of infection in hosts like goats, cows and sheep along with hygiene, vaccine, and effecting heating of dairy products and related foods. Vaccines developed to prevent and control livestock infection are effective in reducing the incidence of human brucellosis. The magnitude of human *Brucella* infection can serve as a barometer of the prevalence of the disease in domestic animals. Eradication of brucellosis in animals is the key to prevention in humans. Nevertheless, public health education assumes an important role in preventing the transmission of brucellosis from animals to humans.

### 8.2 SCOPE FOR FUTURE WORK

1. Validation of the kit in National Research Laboratories.
2. Commercialisation of the kit.
3. Utilisation of the kit as an effective tool kit for studying the sero-prevalence of brucellosis in different parts of the country and to undertake the prophylactic measures wherever required.
Appendix: 1 Composition of Tryptose agar media:

- Tryptose agar: 41 gms.
- Agar –agar type: 5 gms
- Yeast extract: 5 gms
- Double distilled water: 1000 ml.

Appendix: 2 Composition and Preparation of Urease medium.

a. Twenty percent urea solution:
   This was prepared by dissolving 5 gms of urea in 25 ml DW and the solution was sterilized by filtration using 0.45 µ filter.

b. Christensen basal agar media:
   This was prepared by using 2.4 gms of urea agar base Christensen to 90 ml of distilled water. This medium was sterilized by autoclaving and cooled to 50°C to which 10 ml of 20 percent urea solution was added and mixed thoroughly. Medium was then transferred to Screw Cap test tubes and slanted. The sterility of the tubes was checked before use.

Appendix: 3 Preparation of Tryptose agar with basic fuchsin and thionin dyes:

1. 1:25000 Basic fuchsin/Thionin
   - 0.1 percent stock solution: 4ml
   - Tryptose agar medium: 100ml

2. 1:50000 Basic fuchsin/Thionin
   - 0.1 percent stock solution: 1 ml
   - Tryptose agar medium: 100 ml.

3. 1:100000 Basic fuchsin/Thionin
   - 0.1 percent stock solution: 4ml
   - Tryptose agar medium: 100 ml.
Appendix: 4 Requirements for confirmation of smooth phase of *Brucella* culture:

1. Normal saline solution (NSS).
   Normal saline solution was prepared by dissolving 0.85 gms of sodium chloride in 100 ml of DW.

2. Anti *Brucella* serum
   Anti *Brucella* serum raised against *Brucella abortus* 99 in calf at PD_ADMAS, Bangalore was used in slide agglutination test.

3. A 1:1000 concentration of neutral acriflavin was made up by dissolving 5 mg of acriflavin in 5 ml of DW on the day of test.

4. Crystal violet stain:
   Solution A:
   2 gms of crystal violet was dissolved in 20 ml of absolute alcohol to prepare Solution A.
   0.8 gms of ammonium oxalate was dissolved in 80 ml of distilled water to prepare Solution B.

   The stock solution was prepared by mixing 20 ml of solution A and 80 ml of solution B and stored in a tightly stoppered bottle. This stock solution was diluted 1:40 in distilled water just before use.

Appendix: 5 Equipments

1. Ultracentrifuge (Beckman Company).

2. SDS Page Instrument (Bangalore Genie).

3. Lyophiliser (Martin Christ, Germany).

Appendix: 6 Reagents.

1. Phenol saline (2 percent)

2. Absolute ethanol

3. Acetone

4. Ethyl ether

5. Tris saline
   TRIS- Hcl, PH 7.0, 10mM
   Sodium chloride 1 percent (W/V)
   Sodium azide 0.02 percent (W/V)

6. Aqueous phenol
   90 percent phenol in DW (W/V)
7. Methanol acetate
   1 percent sodium acetate in methanol
8. RNase A (Pancreatic).
   1 percent stock solution of RNase was prepared as below.
   RNase A (USB, USA) 10mg
   DW 1 ml
   Solution was dispensed into aliquot and stored at -20°C.
9. DNAse
   1 percent stock solution of DNAse was prepared as below
   DNAse (USB, USA) 10 mg
   DW 1 ml
10. Lysozyme (Bangalore Genie, Bangalore)
11. Proteinase K
   Proteinase K (USB, USA) 20 mg
   DW 1 ml
   Aliquots of 100 µl each were made and stored at -20°C.

1. M magnesium sulphate 2.465 g
2. TRIS saline 10 ml.

Appendix: 7 Equipments:
Clean, grease and protein free glassware were used. Gloves were worn while handling the gel.

   1. Slab gel apparatus (Bangalore Genie)
   2. Glass plates of 16.5 x 17 cms one of them having a 1.5 x 13 cm notch.
   3. Plexiglas comb, spacers and clamps.
   4. Power pack vacuum pump and gel drier.

Appendix: 8 Reagents:
1. Acrylamide and bisacrylamide stock.
   This was prepared in distilled water by dissolving 29 g of Acrylamide (Sigma, USA) and 1 g of N-methylene bisacrylamide (Sigma, USA) in 100 ml and then filtering the solution through Whatman no.1 filter paper. It was stored at 4°C.
2. Tris-Hcl 1.5 M, pH 8.8, 1.5 M, pH 8.3 and 1.0M PH 6.8.
The desired amount of tris was dissolved in DW and the required PH was adjusted using concentrate HCl.

3. SDS 10 and 20 percent (Sigma, USA)
   This was prepared in DW and stored at laboratory temperature

4. N, N, N1-tetramethylene diamine dihydrochloreide (TEMED), (Sigma, USA)

5. Ammonium per sulphate 10 percent in DW

6. 2-Mercaptoethoanol (Sigma, USA)

7. Glycerol

8. Bromophenol blue, 0.2 percent in ethanol (BioRad, USA)

9. Glycine (Merck, Germany)

10. Pre-stained MW standard (GIBCO-BRL, USA):
    
    Marker                  Daltons
    Myosin (H chain)        200,000
    Phosphorylase B         97,000
    BSA                     68,000
    Ovalbumin               43,000
    Carbonic anhydrase      29,000
    β- lactoglobulin        18,400
    Lysozyme                14,300

    The protein mixture was reconstituted in sample buffer.

Appendix: 9 Buffers and stains used in SDS-PAGE.

1. Separation gel

<table>
<thead>
<tr>
<th></th>
<th>Stacking (5 percent)</th>
<th>Resolving (10 percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 percent Acrylamide and Bisacrylamide</td>
<td>1.7 ml</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>Tris – Hcl, 1.5. M, PH 8.8</td>
<td>----</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>Tris- Hcl, 1.0 M PH 6.8</td>
<td>1.25 ml</td>
<td>--</td>
</tr>
<tr>
<td>10 percent SDS</td>
<td>0.1 ml</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01 ml</td>
<td>0.012 ml</td>
</tr>
<tr>
<td>10 per cent Ammonium Perasullhate,</td>
<td>0.1 ml</td>
<td>0.3 ml</td>
</tr>
<tr>
<td>DW</td>
<td>6.8 ml</td>
<td>11.9 ml</td>
</tr>
</tbody>
</table>
2. Sample buffer

2- Mercaptoethanol 0.5 ml
20 per cent SDS 1 ml
Tris – Hcl, 1.5 M. PH 6.8 0.166 ml
Glycerol 2 ml
Bromophenol blue, 0.2 percent 60 µl
DW up to 10 ml

3. Running buffer

Glycine 28.8 g
Tris base 6.0 g
10 percent SDS 10ml
DW up to 1000 ml

4. Staining Solutions:

Coomassie brilliant blue stain.

Coomassie brilliant blue stain R 250 (Sigma, USA) 0.25 g
Glacial acetic acid 10 ml
Methanol 45 ml
DW 45 ml

The stain was filtered through Whatman No. 1 filter to remove any particulate matter.

5. Destainer for Coomassie brilliant blue

Glacial acetic acid 10 ml
Methanol 45 ml
DW 45 ml

Appendix: 10 Inclusion criteria:

a. All patients with temperature higher than 38.3°C.
B. Duration of fever more than 2 weeks.
C. Failure to reach a diagnosis.
Appendix: 11 Exclusion Criteria:
1. All the patients in whom fever (temperature > 38.3°C) is not a predominant symptom.
2. Duration of fever less than two weeks.

Appendix: 12 Composition of buffers used in indirect ELISA.

1. Coating buffer (Stock solution)

<table>
<thead>
<tr>
<th>Sol. A.</th>
<th>Sol. B.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Carbonate</td>
<td>1.06 gm</td>
</tr>
<tr>
<td>Sodium Bi-Carbonate</td>
<td>0.84 gm</td>
</tr>
<tr>
<td>Dist. Water</td>
<td>50 ml</td>
</tr>
<tr>
<td>Dist. Water</td>
<td>50 ml</td>
</tr>
<tr>
<td>ADD</td>
<td></td>
</tr>
<tr>
<td>Sol. A.</td>
<td>7ml</td>
</tr>
<tr>
<td>Sol. B.</td>
<td>17ml</td>
</tr>
<tr>
<td>Dist. Water</td>
<td>76 ml</td>
</tr>
</tbody>
</table>

2. Phosphate buffer saline (PH to 7.4 + 0.02)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl₂</td>
<td>7 gm</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2 gm</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>0.353 gm</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>1.09 gm</td>
</tr>
<tr>
<td>Dist. Water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Store at 4°C</td>
<td>for longer periods.</td>
</tr>
</tbody>
</table>

3. Phosphate buffer saline tween-20 (PBST)

1. PBS (pH to 7.4 +/- 0.02) 100ml

2. Tween 20 0.15ml

4. Washing buffer (Prepare freshly before use.)

PBS (pH to 7.4 +/- 0.02) 100ml

Distilled water 400ml
5. **Blocking buffer**

Bovine Gelatin 1.2 gm  
PBST buffer 100 ml  

The same has to be kept in the water bath until bovine gelatin melts (5-10min). After taking it out of the water bath add 0.15ml of Tween 20.

7. **Stopping solution (1mH$_2$SO$_4$)**

Concentrated H$_2$So$_4$ 5.5ml  
Distilled water 94.5ml  

8. **Chromogen solution**

Dissolve one 5mg O-Phenylene Diamine dihydrochloride tablet into 12.5ml of distilled water. Make 5ml aliquots and store in -20°C until it is used. Mix 40µl of 3%H$_2$O$_2$ to 12.5ml aliquot and use.

**Appendix: 13 List of Publications:**


11. REFERENCES


12. LIST OF PUBLICATIONS

I. NATIONAL CONFERENCE

1. **Best poster award** at X annual congress of Indian Association of Medical Microbiologists Bijapur, 2006: **Annapurna S. Agasthya**, Shrikrishna Isloor and Prabhudas K., “Development of software based indirect ELISA for the serodiagnosis of human brucellosis in Karnataka”.


II. INTERNATIONAL CONFERENCE

1. **Team Tech 2004**: **Annapurna S. Agasthya**, Shrikrishna Isloor and Prabhudas k., International Conference on total Engineering, Analysis and Manufacturing Technologies, IISC, Bangalore, presented the poster entitled “sero-epidemiology of human brucellosis in Karnataka State”.

2. **Team Tech 2006**: **Annapurna S. Agasthya**, Shrikrishna Isloor and Prabhudas k, International Conference on Total Engineering, Analysis and Manufacturing Technologies, IISC, Bangalore, presented the paper entitled “Comparison of software based Indirect ELISA with the conventional serological tests for the serodiagnosis of human brucellosis and sero epidemiological survey of human brucellosis in Karnataka”.

III. NATIONAL JOURNAL


IV. INTERNATIONAL JOURNAL


