SEROEPIDEMIOLOGY OF BOVINE BRUCELLOSIS

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DECLARATION

I hereby declare that this thesis entitled **"SEROEPIDEMIOLOGY OF BOVINE BRUCELLOSIS"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that the thesis, entitled **"SEROEPIDEMIOLOGY OF BOVINE BRUCELLOSIS"** is a record of research work done independently by **Smt. Priya.P.**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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Introduction

1. INTRODUCTION

Brucellosis is an infectious bacterial disease caused by members of the genus brucella which are obligate intracellular parasites. Each brucella species has a preferred natural host that serves as a reservoir of infection and it is essentially a disease of the sexually mature animals with a prediliction for ungulate placentas, fetal fluids and testes of male animals. The disease in animals is also known as contagious abortion, infectious abortion, Bang's disease and enzootic abortion. Brucellosis is an occupational direct anthrapozoonosis. Brucellosis occurs world wide in domestic and game animals and causes a serious economic problem for the intensive and extensive animal production systems of humid tropics.

The disease was first described in man by Marston in 1861. In humans disease is also called as Undulant fever, Bang's fever, Malta fever, Cyprus fever, Rock of Gibraltar fever and Mediterranean fever. David Bruce, a Scottish physician isolated the organism *Micrococcus melitensis* from the spleen of four cases of Malta fever in 1887. The bacterium *Bacillus abortus* was isolated from the aborted foetuses of cattle in 1897 by a Danish veterinarian, Bernard Bang (Stableforth, 1959). By about 1910, Malta fever had become a major illness among British troops stationed in Mediterranean region.

Extensive studies on brucellosis in animals and man were carried out by different workers, revealed that *Brucella melitensis* is the major etiologic agent of the disease in India. First report of brucellosis in India was made by Polding (1950). In Kerala, the first report on brucellosis was by Raja *et al.* (1979).

Brucellosis in animals is transmitted through direct/indirect contact, ingestion, inhalation, penetration/invasion and insemination. Congenital infections are rare. The susceptibility of the animal depends significantly on their natural resistance, their age, level of immunity and on environmental stress. On

entry, the brucella invade the mucous membrane of pharynx and oral cavity and localizes in the adjacent lymph nodes. After infection of the regional lymph nodes, bacteraemia occurs which can last for 1-3 weeks and distribute the organisms to the lymphatic system. Presence of erythritol, a carbohydrate produced by the foetus facilitates a better establishment of the organism in the gravid uterus. It interferes with the fetal circulation, causes necrotizing placentitis and eventually leads to abortion. In non-pregnant animals, the first infection often occurs in the udder followed by the infection of the uterus after the onset of pregnancy. The various clinical symptoms of the disease include: early embryonic death, abortion in the last trimester of pregnancy, retention of placenta and birth of weak calves. In bulls, there will be inflammation of seminal vesicles and vesicular glands, chronic inflammation of epididymis, tendon sheath, synovial bursae, enlargement of regional lymph nodes. In relatively resistant indigenous animals, abortions are rare but the infection causes typical signs to a significant reduction in productivity such as long intercalving time, very low herd fertility rate and low milk production.

Laboratory diagnosis of brucellosis is best achieved by the isolation of the organism from the infected materials, such as blood, milk, urogenital discharge, aborted foetus, fetal membranes, lymph nodes, udder and uterine tissues. Since isolation of organism is cumbersome, it is generally not followed in the routine diagnostic practice. Instead, diagnosis mainly relies on the assessment of antibody responses by various serological tests. Common serological tests employed are Rapid plate agglutination test (RPT), Rose bengal plate test (RBPT), Standard tube agglutination test (STAT), 2mercaptoethanol test (2MET), Antiglobulin test (AT), Rivanol agglutination test(RAT), Indirect haemolysis test (IHT), EDTA agglutination test (EAT), Agar gel immuno diffusion test (AGID), Enzyme linked immunosorbent assay (ELISA) and Complement fixation test (CFT).

The multiplicity of the serological tests currently available for the detection of bovine brucellosis indicates that no single test can detect all infected

cattle. Hence, in practice, in an eradication programme a combination of serological tests are used and the success of the programme is based on the efficiency of these serological tests. Allergic tests are also employed for diagnosis in human practice. Molecular diagnosis of brucellosis includes the use of polymerase chain reaction and the use of DNA probes to detect the organism.

The treatment of diseased animals is not a common practice. Hence prevention and control of disease assumes paramount importance. This is best achieved by identification of reactor animals and segregation, intensive surveillance of the population, screening of new additions to herd, prompt disposal or destruction of the aborted fetuses and after births, vaccination of cattle with *Brucella abortus* live strain-19 vaccine, or alternatively with *Brucella abortus* strain 45/20 (inactivated) vaccine, pasteurisation of milk and health education. In developed countries, test and slaughter policy has helped to make a considerable progress towards the eradication of the disease in animals.

The serosurvey spread over different geographical areas would help in understanding the distribution and epidemiology of the disease. Keeping this in view, the present investigation has been carried out with the following objectives.

1. To assess the extent of prevalence of antibodies to brucella amongst bovine population in our state.

2. To find out the suitability of different serological tests to screen large number of sera samples.

Review of Literature

2. REVIEW OF LITERATURE

2.1 ETIOLOGY

Brucella are small, gram-negative bacilli or coccobacilli 0.6 to 1 μ in diameter, 1.5-2 μ in length, non-spore forming, non-motile and non-acid fast organisms. These are aerobic but may require 5-10 per cent CO_2 tension for primary isolation and are commonly seen intracellularly singly or in groups. Delicate translucent colonies of 2-3 mm in diameter grow on blood or glucose agar. *B. ovis* grows in M (mucoid) Form, *B. abortus* and *B. melitensis* grow at the beginning in S (smooth) form and later dissociate into the R (rough) and M form. The biotypes are differentiated serologically (agglutination) applying specific monosera (M and R) (Stableforth, 1959).

Corbel (1975) isolated the ribosomes from *Brucella abortus* strain 19 and 45/20 and reported that they have some role in the bovine immune responses to brucella infection or vaccination.

Dubray and Plommet (1975) analysed the components of brucella envelope and reported that it consists of outer membrane, thick stratum of peptidoglycan, periplasmic space, cytoplasmic membrane and the outer membrane of phase S consists of lipopolysaccharides (LPS) and polysaccharides linked to peptidoglycan.

Jones and Berman (1975) reported that the covalent linkage of protein with lipopolysaccharide molecule in brucella accounted for its greater stability in phenol.

The composition of LPS of smooth *Brucella abortus* revealed that about one third of the composition was accounted to the lipid A component, which consists of D-glucosamine (10.1 per cent), phosphate (5.9 per cent), n-tetradecanoic acid (myristic acid, 12 per cent), n-hexadecanoic acid (Palmitic

acid, 32 per cent), n-octa decanoic acid (stearic acid, 15 per cent), 3-hydroxy decanoic acid (27 per cent) and 3-hydroxy hexa-decanoic acid (about 4 per cent). β-hydroxy myristic acid present in the lipid A fractions of many other Gram negative bacteria was notably absent (Caroff *et al.*, 1984).

2.2 GEOGRAPHICAL DISTRIBUTION

Versilova and Aslanjan (1974) analysed the epidemiological data on brucellosis from 1963-67 and reported that the major part of the infection was confined to the Asian parts of Soviet Union.

The seroprevalence of brucellosis in cattle and sheep in Eastern Sudan was reported as 22 and 13.6 per cent respectively (Weiser, 1995) and 7.42 per cent in cattle slaughtered at Nigeria (Alsanda and Agbede, 1999).

Thapliyal (1999) reported that brucellosis was endemic in many areas of India.

In France, the national control programme on brucellosis appeared successful and the situation at present is close to the eradication of brucellosis in cattle, sheep and goat (Bastuji and Delcueillerie, 2000).

Kubuafor *et al.* (2000) reported that cattle in the Akwapim South district of Ghana were infected with brucellosis with a mean seroprevalence of 6.6 per cent using RBPT.

The seroprevalence of brucellosis among bovids in Sri Lanka was reported as 4.6 per cent using ELISA (Silva *et al.*, 2000).

2.3 EPIDEMIOLOGY

2.3.1 Species

2.3.1.1 Cattle

Sreenivasan (1972) conducted a survey on the prevalence of brucellosis in endemic areas of Tamil Nadu among 29,513 cattle using serological methods and reported an overall seropositivity of 2.7 per cent.

A seroprevalence of 48.33 per cent was reported by screening 50 sera samples from cattle of coastal Karnataka (Stephen *et al.*, 1978) and 12.1 per cent was reported by screening 82 cattle sera samples in Dharwad using RBPT (Sevalgi *et al.*,1987).

Standard tube agglutination test revealed a seroprevalence of 1.9 per cent among 80 cattle in Rajasthan (Mathur *et al.*, 1979) and 44.35 per cent among 115 unvaccinated exotic cattle in Kashmir (Bachh *et al.*, 1988).

Hemashettar *et al.* (1987) tested 143 sera samples from cattle during an outbreak of brucellosis in an organised dairy farm in Karnataka using STAT and 2 MET and 12 animals were found to have brucella antibodies.

Screening of 101 sera samples of cows from two organised dairy farms revealed an overall seropositivity of 18.81 per cent, 16.83 per cent and 12.87 per cent respectively by STAT, HIT, and 2 MET (Kalimuddin *et al.*, 1990).

Chandramohan *et al.* (1992) tested 138 sera samples collected from zebu cattle with history of abortion, retained placenta, repeat breeding and pyrexia using ELISA and seropositivity was found to be 18.84 per cent.

Rampal and Dwivedi (1992) conducted a serological study among seventeen cross bred cows and three bulls using RBPT followed by STAT and observed that all the affected animals had a significant titre (1:80 to 1:320) with *Brucella abortus* plain antigen.

A serosurvey on bovine brucellosis involving 459 cattle sera samples covering 16 districts of Tamilnadu revealed a seropositivity of 9.37 per cent using rapid plate agglutination test (Suresh *et al.,* 1993).

Mrunalini and Ramasastry (1999) recorded a seropositivity of 3.8 per cent for brucellosis after analyzing sera samples from 14,895 cattle from organised farm and village backyard animals using different serological tests.

Hussain *et al.* (2000) reported that out of the 135 cattle sera samples tested for brucellosis, 38.52 per cent were positive by RBPT and 43.7 per cent by STAT.

An overall seropositivity of 9.21 per cent was detected when 139 sera sample from infertile cattle were screened for brucellosis by RBPT, STAT and 2MET (Tandle *et al., 2000).*

Sandhu *et al.* (2001) tested sera samples of 666 cows using RBPT and STAT and 67 samples were positive with a seroprevalence of 10.06 per cent.

2.3.1.2 Buffaloes

Studies conducted on the seroprevalence of brucellosis in buffaloes using standard tube agglutination test revealed a seropositivity of 2.24 per cent (Baby and Paily, 1979), 2.6 per cent (Mathur *et al.*, 1979) and 8.65 per cent (Oberoi and Kwatra, 1982).

Sevalgi *et al.* (1987) screened a total number of 464 buffaloes using RPT and STAT and reported a seropositivity of 2.1 per cent.

Kulkarni *et al.* (1991) examined the sera samples from 75 buffaloes using RBPT, STAT and ELISA and reported an overall seroprevalence of 12 per cent.

Serological testing of buffalo bulls in Punjab from different localities using STAT showed a serum titre ranging from 1:80 to 1:320 and concluded that

brucella infected buffalo bulls might be responsible for disease transmission (Rampal and Dwivedi, 1992).

Suresh *et al.* (1993) conducted a sero survey on brucellosis among 284 buffaloes in Tamil Nadu using RPT, RBPT and STAT and found an overall seropositivity of 10.92 per cent, while testing the sera samples of 11,368 buffaloes in Andhra Pradesh using the same tests showed a seropositivity of 4.14 per cent (Mrunalini and Ramasastry, 1999).

Chauhan *et al.* (2000) tested 59 sera samples (50 from aborted and 9 from pregnant buffaloes), using RBPT, STAT and ELISA and reported the occurrence of brucellosis among aborted and pregnant buffaloes as 44 and 11.11 per cent respectively.

In a study to detect brucella antibodies in 113 buffaloes (92 males and 21 females) using RBPT, STAT, ELISA and AGPT, 83.33 per cent of the samples showed agglutinating antibodies more than 80 IU to *Brucella abortus* (Pati *et al.*, 2000).

2.3.2 Host factors

2.3.2.1 Age

Mathur *et al.* (1979) indicated low prevalence rate of brucellosis in younger stock due to the lesser chance for antigenic exposure than adult stock.

Bachh *et al.* (1988) stated that prevalence of brucellosis was higher in cattle which are above two years of age.

A higher seroprevalence of brucellosis was observed in goats above four years of age (Masoumi *et al.*, 1992).

Susceptibility to brucellosis was found to be higher in animals above six years of age (Suresh *et al.*, 1993) and four years of age (Ghani *et al.*, 1998).

Maiti *et al.* (1999) reported that fifteen new born calves aged between five to 30 days showed serological reactivity to *Brucella abortus* infection.

The seroprevalence of brucellosis in bovids more than three years of age was twice as high as the younger age group (Silva *et al.*, 2000).

2.3.2.2 Breed

Kumar *et al.* (1974) reported that the prevalence of brucellosis is higher in non-descript bullocks and bulls than in exotic breeding bulls.

On contrary, Suresh *et al.* (1993) reported that incidence was more in exotic purebred and crossbred cattle than in native cattle and among buffaloes the rate of infection was higher in Murrah crossbreds than native breeds.

Omer *et al.* (2000) stated that cattle herds with crossbreds were more likely to be seropositive than herds with exotic pure breds.

2.3.2.3 Sex

Baby and Paily (1979) conducted a study on buffaloes and showed that males and females are equally susceptible to brucellosis.

The incidence of brucellosis was reported to be more in females than males (Suresh *et al.*, 1993, Silva *et al.*, 2000).

2.3.3 Management

A higher prevalence rate of brucellosis was observed in big herds resorted to hill grazing and kept in unhygienic conditions devoid of direct sunlight (Sreenivasan, 1972).

Various factors such as stocking density of animals, type of husbandry practices, use of maternity pens, practise of artificial insemination and type of

housing as some of the managemental factors contributing to the maintenance and spread of brucellosis (Salman and Meyer, 1984).

Samaha *et al.* (1990) noticed an incidence rate of 4.93 per cent among cows kept in cow house system.

The spread of brucellosis is also influenced by housing animals in larger area with poor management (Omer *et al.*, 2000).

Silva *et al.* (2000) reported that agroclimatic zones and system of management practised in the farms are important risk factors in the occurrence of *Brucella abortus* infection in Sri Lanka.

2.3.4 Transmission

Polding (1950) concluded that humidity, rainfall, lack of sunlight, unhygienic condition and overstocking of animals play an important role in transmission.

It was also observed that the transmission of brucellosis was related most frequently to the sources of replacement animals (Kellar *et al.*, 1976).

Nicoletti (1980) reported that community pastures have been a means for herd transmission of brucellosis.

Poor fencing, movement and contact of animals with the nomadic cattle, indiscriminate buying without adequate quarantine of animals were reported to be important factors for the spread and maintenance of brucellosis (Bale and Diaka, 1981).

Use of semen from an infected bull transmitted brucellosis to 71 per cent of the cows inseminated and the infection was transmitted to calves born to these dams (Salman and Meyer, 1984).

Dallapozza *et al.* (1997) stated that direct and indirect contacts between sheep and/or goat flocks and cattle herds represent a risk for brucellosis infection in cattle.

Thapliyal (1999) reported large sized herds and higher densities of animal population greatly facilitate the transmission of brucellosis.

Radostitis *et al.* (2000) reported that intraherd spread of brucellosis occurs by both vertical and horizontal transmission.

Ridler *et al.* (2000) concluded that *Brucella ovis* was transmitted from infected rams to stags which are grazing on the same paddock and that mode of transmission was by direct contact.

Communal grazing system and increased interaction with game animals also increase the risk of transmission of brucellosis (Kabagambe *et al.*, 2001).

2.4 CLINICAL SIGNS

Fensterbank (1978) reported hygroma in a male calf having congenital *Brucella abortus* infection.

Divekar (1979) reported orchitis, synovitis, hygroma and swelling of various joints as clinical manifestations of brucellosis in bulls.

Roberts (1986) described the occurrence of retention of placenta due to placentitis and cotyledonitis in animals suffering from brucellosis.

Brucella is the predominant bacterial species involved in bovine abortion (Das and Paranjape, 1988) and the presence of brucella agglutinins were reported in cattle with previous history of abortion and also in repeat breeders with no history of abortion (Bachh *et al.*, 1988).

Mussa *et al.* (1990) described the clinical manifestations of brucellosis in adult cattle as hygroma, arthritis and long intercalving intervals.

Maiti *et al.* (1999) reported arthritis in brucella infected new born calves.

Almeida *et al.* (2000) showed that in cattle cervical bursitis is associated with brucella infection.

Radostitis *et al.* (2000) reported abortion storm in first calf heifers after fifth month of pregnancy and orchitis, epididymitis and synovitis in bulls due to *Brucella abortus* infection.

Chand *et al.* (2002) reported epididymo-orchitis in breeding rams due to *Brucella melitensis* infection.

2.5 DIAGNOSIS

2.5.1 Bacterial Isolation

Bale and Diaka (1981) obtained eleven isolates of *Brucella abortus* from cattle in a government farm at Nigeria and eight strains were characterized.

Crawford *et al.* (1986) isolated *Brucella abortus* field strain biotype 1 from milk and utrine swab specimens of aborted heifers.

Sevalgi *et al.* (1987) isolated - *Brucella melitensis* biotype 1 from animals and human beings in Dharwad.

Brucella abortus biovar 4 was isolated from 14 dogs from the farms with brucella infected cattle of which 10 dogs were serologically positive (Forbes, 1990).

Brucella melitensis biotype 1 was isolated from an aborted foetus, a pregnant doe and her unborn foetus (Ribiero *et al.*, 1990).

Verma *et al.* (2000) isolated *Brucella abortus* biotype 3 from aborted cows and indicated that isolation and identification of brucella from clinical samples and morbid materials is possibly the most reliable method for diagnosis.

Chand *et al.* (2002) isolated *Brucella melitensis* biotype 1 from the breeding rams having epididymo-orchitis, belonging to an organised sheep farm in North India.

2.5.2 Serological Diagnostic Tests

The precise diagnosis of brucella infection is based on isolation and identification of the organisms from the infected animals. But this is a cumbersome and time consuming due to the fact that these fastidious organisms grow slowly on primary isolation. Moreover known infected individuals sometimes fail to yield organisms at culture. Hence assessment of the antibody response employing serological tests play a major role in the routine diagnosis of brucellosis.

2.5.2.1 Rose Bengal Plate Test (RBPT)

RBPT and CFT was used to differentiate between the immunoglobulin classes in serum antibody reactions in cattle following vaccination with *Brucella abortus* Strain 19 and Killed 45/20 vaccines. They established that RBPT antibodies could be detected as IgM and IgG fractions after the primary inoculation with *B. abortus* strain 19 vaccine (Chung *et al.*, 1980).

The higher sensitivity of RBPT could be attributed to the acidified buffer used in the reaction which might have inhibited immunologically non-specific agglutinins (Sutherland, 1980).

Waghela *et al.* (1980) suggested that RBPT and AGID are useful tests for diagnosis of brucellosis in goats where facilities for CFT are not available.

The specificity of RBPT was found to be 98.9 per cent on examination of 1051 cattle sera samples from brucellosis free herds (Stemshorn *et al.*, 1984).

Das and Paranjape (1987) reported RBPT to be more sensitive than Brucella Stabilized Antigen Plate test in detecting agglutinating antibodies.

Kulkarni *et al.* (1991) reported that RBPT detected more number of positive reactors than STAT but less than ELISA.

Rose Bengal antigen was found to be more sensitive in detecting brucellosis than Tetrazolium coloured antigen (Suresh *et al.*, 1993).

Chauhan *et al.* (2000) utilized RBPT for screening 59 sera samples from buffaloes and 27 samples were found to be positive.

Under field condition RBPT can be used as a quick reliable diagnostic test in the diagnosis of brucella infection (Kalorey *et al.*, 2000).

Amin *et al.* (2001) established Rose Bengal plate test as a simple and preferred test with high sensitivity.

Sandhu *et al.* (2001) screened 2796 samples from cattle, buffaloes and goats using RBPT and reported 67 out of 666 cows and 70 out of 750 buffaloes and 56 out of 1380 goats were positive for brucellosis.

2.5.2.2 Standard Tube Agglutination Test (STAT)

Waghela *et al.* (1980) compared STAT along with AGID, RBPT, CFT in the diagnosis of caprine brucellosis and suggested that STAT adds little information when used with other tests.

Conventional serological test, STAT was found to have a relative sensitivity and specificity of 28.57 and 17.14 per cent respectively in the diagnosis of ovine brucellosis (Barbudhe *et al.*, 1994).

STAT was more reliable, sensitive, less time consuming and can be easily applied in the seroepidemiology of bovine brucellosis (Ghani, 1995).

Agarwal and Batra (1999) used STAT for screening 150 bovine sera samples and found it to be 81.81 per cent sensitive.

Pati *et al.* (2000) performed STAT on 23 buffaloes and found the presence of agglutinating antibodies ranging from 80-320 IU in five samples.

Shringi *et al.* (2002) performed STAT on 144 bovine serum samples and proved that STAT is comparable to microplate ELISA in terms of its sensitivity and specificity than any other test.

2.5.2.3 2 -Mercaptoethanol Test (2 MET)

Stemshorn *et al.* (1984) reported merceptoethanol test to be 99.8 per cent specific and 59.9 per cent sensitive.

Venkatesha and Upadhye (1989) used mercaptoethanol test to differentiate brucella vaccinated and infected cattle at first, second and third months after S19 vaccination, whereas after 45/20 vaccination no antibodies were detected.

Kalimuddin *et al.* (1990) suggested 2 MET as a useful supplementary test for diagnosis of brucellosis in dairy herds.

Shringi *et al.* (2002) evaluated the efficacy of mercaptoethanol test among various serological tests and reported a sensitivity and specificity of 90.74 and 92.22 per cent respectively.

2.5.2.4 Enzyme Linked Immunosorbent Assay (ELISA)

Fluorimetric β galactosidase ELISA was reported to be specific, sensitive and inexpensive supplement to the conventional serological methods for the screening of large numbers of sera for brucella antibodies (Aert *et al.*, 1985).

Lee *et al.* (1985) described ELISA as an effective test than CFT in diagnosing and eradicating brucellosis from an infected flock.

Two monoclonal antibodies were compared in competitive ELISA for detection of *Brucella abortus* specific antibodies in the serum of vaccinated

and/or experimentally infected cattle and found that antibodies could be detected one or two weeks after vaccination (Sutherland, 1985).

Dohoo *et al.* (1986) recommended that indirect ELISA can be used both as a screening test and confirmatory test, in the diagnosis of brucellosis in cattle.

Cho and Niilo (1987) reported ELISA as more specific, sensitive and technically advantageous than CFT as a serodiagnostic test for detection of *Brucella ovis* infection in rams.

Catherine *et al.* (1988) developed an ELISA using an SDS (Sodium dodecyl sulphate) extract of *Brucella abortus* as antigen to detect *Brucella abortus* antibodies in cattle sera.

Chand *et al.* (1989) reported dot – ELISA as simple, rapid, sensitive and specific test and hence it could be employed as a screening test.

Kulkarni *et al.* (1991) utilized ELISA for detection of brucella antibodies in bovines and visual ELISA revealed 22.5 per cent samples as positive and spectrophotometric ELISA showed 26.47 per cent positivity.

Protein G conjugate was found to reduce the background reactivity of sera from healthy goats in an ELISA for diagnosis of brucellosis (Ficapal *et al.*, 1995).

Saravi *et al.* (1995) compared the performance of ELISA with two screening tests, Rose bengal plate test and buffered plate antigen test and with two confirmatory tests, 2 MET and CFT and reported the sensitivity of ELISA as 97.1 and specificity 95.2 per cent.

A field based ELISA kit was evaluated for detection of brucella antibodies in sheep and goat. It was found to be a simple, rapid and convenient diagnostic test (Agarwal *et al.*, 1998).

Agarwal and Bhatra (1999) compared an inhibition Enzyme linked immunosorbent assay with STAT and CFT and found that inhibition – ELISA had a sensitivity of 92.04 per cent.

Samartino *et al.* (1999) demonstrated competitive ELISA test as very useful and cost effective in the diagnosis of bovine brucellosis in countries where vaccination was mandatory.

The diagnostic specificity of indirect ELISA in water buffaloes was 99.6 per cent for samples from brucellosis free herds and 68.33 per cent for samples from brucella infected herds (Guarino *et al.*, 2001).

Omer *et al.* (2001) conducted serological tests such as RBPT, CFT and ELISA and concluded that ELISA is a good alternative for testing individual animals and herds for antibodies to *Brucella abortus*.

Renukaradhya *et al.* (2001) reported that the overall specificity and sensitivity of Avidin-Biotin ELISA was 98 and 98.2 per cent respectively by testing 7040 cattle and 678 buffalo sera samples.

Shringi *et al.* (2002) reported that ELISA and Dot ELISA test had highest seropositivity and sensitivity compared to Rapid plate agglutination test, Standard tube agglutination test, Mercaptoethanol test and Heat inactivation test.

Shringi *et al.* (2003) conducted a study comparing *Brucella abortus* Str-19 LPS antigen and protein antigen and reported LPS antigen had better sensitivity in assaying brucella antibodies than protein antigen.

2.5.2.5 Complement Fixation Test (CFT)

Plackett and Alton (1975) summarised that complement fixation by bovine IgG, or IgM antibodies to *Brucella abortus* was inhibited by specific non complement fixing antibodies of IgG_2 subclass which accounts for the appearance

of prozones in CF titrations of some antisera and thus responsible for occurrence of sera which are positive to the RBPT but negative to the CF test.

Two microtitre complement fixation test was used in the serological diagnosis of naturally occurring *Brucella ovis* infection in rams and recorded a sensitivity of 100 per cent and specificity of 99.99 per cent (Searson , 1982).

Sutherland and Mackenzie (1983) employed CFT in the later stages of bovine brucellosis eradication programme. They tested 177 cattle sera from which *Brucella abortus* was isolated, and observed 159 positive reaction and established CFT as a confirmatory test.

Stemshorn *et al.* (1984) compared the sensitivity and specificity of CFT to other serological tests such as Buffered plate antigen test, Standard tube agglutination test and 2-Mercaptaethanol test and reported CFT as 79 per cent sensitive and 83 per cent specific.

ELISA, CFT and Gel diffusion precipitin test was compared for diagnosis of *Brucella ovis* infection in rams and reported the sensitivity and specificity of CFT as 96.3 and 99.3 per cent respectively (Worthington *et al.*, 1984).

Dohoo *et al.* (1986) evaluated the efficacy of five serological tests viz., buffered plate antigen test, STAT, CFT, haemolysis in gel test and indirect enzyme immunoassay in sera from 1208 cattle in brucellosis free herds, 1578 cattle in reactor herds of unknown infection status and 174 cattle from which *Brucella abortus* had been cultured and stated CFT as an appropriate confirmatory test having high specificity.

Sutherland *et al.* (1986) used CFT along with ELISA in the final stage of bovine brucellosis eradication programme.

Brucella abortus and *B. melitensis* antigens were used in parallel for performing CFT in *B. abortus* infected cattle and observed that the titres to *B. melitensis* were consistently lower than those to *B. abortus* and thus the use of

dual antigens could identify herds which are infected only with one antigen and would not be reliable for classifying individual animals (Herr *et al.*, 1991).

CFT was employed to detect serological evidence of brucellosis in sheep and goat in Uttar Pradesh and 70 sheep out of 255 and 21 goats out of 289 were detected positive (Prahlad *et al.*, 1997).

Agarwal *et al.* (1999) used CFT for detection of brucella antibodies in sheep and goat and reported a sensitivity of 97.9 per cent and specificity of 89.8 per cent.

2.5.3 Molecular Diagnosis

Bricker and Halling (1994) described a PCR assay that comprises five oligonucleotide primers which could identify and differentiate between *Brucella abortus* bv. 1, 2, 4, *Brucella melitensis, Brucella ovis* and *Brucella suis* bv. 1.

Polymerase chain reaction analysis is complementary to classical serological tests for detection of the aetiological agent of brucella species infecting buffaloes, especially during the initial phase of disease (Guarino *et al.*, 2000).

Leal-Klevezas *et al.* (2000) used polymerase chain reaction to diagnose caprine brucellosis to detect *Brucella abortus* biovar 1 by collecting both blood and milk samples and 86 per cent of the blood samples were found to be positive where as only 60 per cent were positive on serological test.

PCR was evaluated for the detection of *Brucella melitensis* in semen against the traditional cultural methods. PCR detected Brucella DNA in 12 (10 per cent), while direct culture detected only seven (5.8 per cent) out of 120 semen samples (Amin *et al.*, 2001).

Cortez *et al.* (2001) employed polymerase chain reaction for detection of brucella DNA from aborted bovine foetuses and four of the 54 culture negative

samples were positive by PCR and seven samples from aborted bovine foetuses were positive by both PCR and microbiological culture.

Shrikrishna *et al.* (2001) used random amplified polymorphic DNA (RAPD) analysis for differentiation of 6 brucella species using 15 oligonucleotide random primers and reported that RAPD finger printing provided a rapid means of differentiating brucella strains.

2.6 CONTROL AND PREVENTION

Chung *et al.* (1980) reported that animals that had received *B. abortus* strain 19 vaccine responded to the 45/20 vaccine with increased titres to the smooth antigens.

Plackett *et al.* (1980) reported that, regular boosting up of animals with strain 19 vaccine is necessary to produce protective antibody response.

Vaccination with chemically modified salt extractable proteins and unmodified salt extractable proteins stimulated an antibody and CMI response to *B. abortus*, but failed to protect cattle against an experimental challenge with the organism (Confer *et al.*, 1987).

Khalaf *et al.* (1992) stated that wide spread vaccination of adult animals is the most effective method of control of brucellosis among cattle, sheep and goat.

Cheville (2000) established that *Brucella abortus* RB51 is the vaccine of choice against brucellosis of cattle.

Vaccination with a reduced dosage of RB51 protects adult cattle against abortion or infection caused by exposure to virulent *B. abortus* during the subsequent pregnancy (Olsen, 2000).

Uzhal *et al.* (2000) concluded that multiple vaccination with strain RB51 did not induce sero conversion to brucellosis surveillance tests and 10^9 CFU of strain RB51 is safe for use in pregnant cattle.

Mahato *et al.* (2001) studied the effect of *B. abortus* strain 19 vaccine on cows and adult heifers and found that vaccination of sexually matured cattle with strain 19 vaccine could reduce abortion rate considerably.

Materials and Methods

3. MATERIALS AND METHODS

The present study was carried out in the Department of Veterinary Epidemiology and Preventive Medicine, College of Veterinary and Animal Sciences, Mannuthy during 2002-2003. The study was envisaged to assess the seroprevalence of bovine brucellosis in Kerala and the comparative evaluation of different serological tests for screening large number of sera samples for brucellosis.

In the present study a total of 1602 blood samples were collected from cattle and buffaloes maintained in various Government Farms situated in Kollam, Idukki, Palakkad, University Livestock Farm, Mannuthy; Cattle Breeding Farm, Thumburmuzhy; Livestock Research Station, Thiruvazhamkunnu; Kelappaji College of Agricultural Engineering and Technology, Thavanur; Dairy Science College, Kolahalamedu; College of Agriculture, Vellayani; Regional Agricultural Research Stations located at Pattambi, Kumarakom, Ambalavayal, Artificial Insemination Centre, Mannuthy; animals maintained in private farms located at Kozhikkode, Wayanad, Ernakulam, animals presented to University Veterinary Hospitals at Mannuthy and Kokkalai. Field samples were also collected from Wayanad, Kannur, Kozhikkode, Thrissur districts of Kerala and from the bovines slaughtered at Municipal slaughter house, Thrissur.

Glassware, Plasticware and Reagents

All glassware used were of Borosil brand. Plasticware used were of Tarson brand and chemicals were of analytical or guaranteed grade.

Rose Bengal Antigen, *Brucella abortus* Plain Antigen and Brucella positive serum were procured from Institute of Animal Health and Veterinary Biologicals, Bangalore.

Sterilisation of Glassware and Plasticware

New test tubes were kept overnight in potassium chromate solution. (Potassium chromate crystals - 80 mg, Concentrated sulphuric acid - 250 ml, Distilled water -750 ml).

The test tubes were then washed twice in ordinary tap water and also in distilled water. The test tubes were dried and sterilized by keeping in hot air oven at 160°C for 1 hour after plugging with non absorbant cotton.

Plasticware were sterilized by autoclaving at 121°C for 15 minutes at 15 pounds pressure.

3.1 COLLECTION OF BLOOD

About five ml of blood was collected aseptically by jugular puncture using an 18 G sterile hypodermic needle into test tubes having 15 ml capacity. The tubes were labeled and kept in a slanting position for clotting. Later, the clots were disrupted and the test tubes were transferred to refrigerator and kept overnight at 4°C.

Next day, sera was centrifuged at 1000 G for 10 min and transferred to cryovials of 2.5 ml capacity after heat inactivation at 56°C for 30 min, to destroy non-specific agglutinins. The vials were labeled and stored at –20°C for different serological tests.

Data regarding the sex, age, breed, gestation, lactation, reproductive problems like abortion, repeat breeding, retention of placenta and metritis and the epidemiological data regarding the managemental practices and location of districts were also collected. Samples were collected from 11 districts of Kerala.

Animals were classified into two groups based on farming systems. Animals reared under organised farms and those animals maintained by small scale rural farmers as unorganised system of farming. On the basis of altitude,

Kerala is divided into two agroecological regions such as Type I region (Low altitude region) and Type II region (High altitude, i.e., >1000 m from sea level). The districts Idukki and Wayanad were classified under Type II region and all the other districts were classified under Type I region (Rao, 2003).

Based on the districts, Kerala is divided in to three zones. The districts Malappuram, Kozhikkode, Wayanad, Kannur and Kasargode comes under Northern zone. Idukki, Ernakulam, Thrissur and Palakkad districts come under central zone and Southern zone of Kerala includes Thiruvananthapuram, Kollam Pathanamthitta, Alappuzha and Kottayam.

3.2 DIAGNOSTIC TESTS

3.2.1 Rose Bengal Plate Test (RBPT)

The test was performed based on the method described by Alton and Jones (1967).

3.2.1.1 Materials/Reagents

Clean porcelain slab, Spreaders, Test sera samples and Antigen. The antigen is a suspension of pure smooth cultures of *Brucella abortus* strain 99 in phenolised saline, the bacteria coloured by the addition of Rose Bengal dye.

3.2.1.2 Procedure

1. The clean porcelain slab was placed on a table. Both serum and antigen were thawed to room temperature.
2. One drop (25 µl) of serum was mixed with an equal quantity of *Brucella abortus* Rose Bengal coloured antigen on porcelain slab.
3. The serum and antigen were mixed with a spreader and was gently rocked by hand for about 4 minutes.

4. After 4 min, samples which showed any degree of visible agglutination were considered positive for brucella agglutinins and others regarded as negative.

3.2.1.3 Interpretation

The results were analysed on the basis of the size of particles and speed with which they appear.

Time of reaction	Character of reaction	Grading	Suggested meaning
Instantaneous	Large particulation	+++	Strong positive
2-3 minutes	Medium particulation	++	Moderate positive
4-5 minutes	Powdery particulation	+	Weak positive
10 minutes	Slight particulation	±	Doubtful
-	No particulation	-	Negative

3.2.2 Standard Tube Agglutination Test (STAT)

Method described by Alton and Jones (1967) was followed.

3.2.2.1 Materials/Reagents

Agglutination tubes and racks, Normal saline, Test sera samples, Brucella known positive and negative serum, *Brucella abortus* plain antigen. The antigen is a suspension of smooth culture of *Brucella abortus* strain 99 in phenol saline.

3.2.2.2 Procedure

1. Six agglutination tubes were set up in an agglutination rack and added 0.8 ml of normal saline to the first tube and 0.5 ml to other five tubes.

2. 0.2 ml of the test serum was added to the first tube, mixed thoroughly and transferred 0.5 ml to the second tube, from which after mixing 0.5 ml was transferred to the third tube. Serial two fold dilutions of serum were made upto tube No.6 and then discarded 0.5 ml from sixth tube. Thus the dilutions in each tubes were 1:5, 1:10, 1:20 and so on.

3. 0.5 ml of *Brucella abortus* plain antigen was added to each tube and mixed well. (Final dilution of serum in each tubes were 1:10, 1:20 and so on).

4. The following control tubes were also maintained.

   a) Positive control with 0.5 ml known positive serum and 0.5 ml of *Brucella abortus* plain antigen.

   b) Negative control with 0.5 ml of known negative serum and 0.5 ml of *Brucella abortus* plain antigen.

   c) 50 per cent control with 0.75 ml normal saline and 0.25 ml of *Brucella abortus* plain antigen.

   d) Antigen control with 0.5 ml *Brucella abortus* plain antigen and 0.5 ml of normal saline.

5. All the tubes including the control tubes were incubated at 37°C, overnight.

6. The degree of agglutination was determined by observing the clarity of supernatent without shaking the tubes. The highest serum dilution showing 50 per cent or more agglutination that is 50 per cent clearing was taken as the end point.

To express in unit system, twice the serum titre showing 50 per cent agglutination was taken as international units (IU) per ml of serum.

*3.2.2.3 **Interpretation***

Cattle, buffaloes	-	80 IU or above: Positive
		40 IU : Doubtful
Breeding bulls	-	20 IU : Doubtful

3.2.3 2-Mercaptoethanol Test (2-MET)

This test was performed as per the method given by Alton and Jones (1967).

3.2.3.1 Reagents/Materials

Agglutination tubes and racks, sterile normal saline, 0.1 M solution of 2 Mercaptoethanol (prepared by taking 7.07 ml of 2 Mercaptoethanol (14.139 M) and made up to 1 litre with normal saline, Stored at 4°C and prepared fresh every two to three weeks), Test sera samples, Brucella Positive serum, *Brucella abortus* Plain antigen,

3.2.3.2 Procedure

1. Preparation of phenol free *Brucella abortus* plain antigen

   100 ml of antigen was mixed thoroughly and transferred to a clean sterile centrifuge tube and centrifuged at 3000 rpm for 15 minutes. The supernatent was removed and the cells were reconstituted in sterile normal saline and centrifuged as before. The cells were washed twice with normal saline and finally reconstituted to 100 ml with normal saline.

2. Six agglutination tubes were set up in a row in an agglutination rack. Added 0.8 ml of 0.1 M 2 Mercaptoethanol to first tube and 0.5 ml to other five tubes.

3. 0.2 ml of the test serum was added to the first tube, mixed thoroughly and transferred 0.5 ml to the second tube, from which after mixing, 0.5 ml was transferred to the third tube. Thus two fold dilutions of serum were made up to tube No.6 and then 0.5 ml was discarded from the sixth tube. Thus the dilutions in each tubes were 1:5, 1:10, 1:20 and so on.

4. 0.5 ml of phenol free *Brucella abortus* plain antigen was added to each tube and mixed well.

5. The following control tubes were also maintained.

a) Positive control - 0.5 ml known positive serum and 0.5 ml phenol free *Brucella abortus* plain antigen.

b) Negative control – 0.5 ml of known negative serum and 0.5 ml of phenol free *Brucella abortus* plain antigen.

c) 50 per cent control - 0.75 ml 0.1 M 2 Mercaptoethanol and 0.25 ml of phenol free *Brucella abortus* plain antigen.

d) Antigen control - 0.5 ml phenol free *Brucella abortus* plain antigen and 0.5 ml of 0.1 M 2-Mercaptoethanol.

The degree of agglutination was determined by reading the degree of clarity of supernatent without shaking the tubes. The highest serum dilution showing 50 per cent or more agglutination that is 50 per cent clearing was taken as the end point.

3.2.3.3 Interpretation

Cattle, buffaloes	-	80 IU or above : Positive
		40 IU : Doubtful
Breeding bulls	-	20 IU : Doubtful

3.2.4 Avidin – Biotin ELISA (A-B ELISA)

Avidin-Biotin ELISA was performed as per the method described by Rajasekhar (1998).

3.2.4.1 Materials Required

1. 96 well microtitre plates and Micropipettes

3.2.4.2 Reagents/Buffers

3.2.4.2.1 Phosphate buffered saline (PBS) of 0.01 M, pH 7.4 ± 0.2

Sodium chloride (NaCl)	-	7.02 g
Potassium Chloride (KCl)	-	0.2 g
Sodium dihydrogen phosphate ($NaH\ PO_4\ 2H_2O$)	-	0.35 g
Disodium hydrogen phosphate ($Na_2\ H\ PO_4\ 2H_2O$)	-	1.1 g
Distilled water	-	1000 ml

3.2.4.2.2 Antigen Coating Buffer

0.05 M Carbonate/Bicarbonate buffer, pH 9.6 ± 0.05

Solution A

Sodium Carbonate	-	1.6 g
Distilled water	-	50 ml

Solution B

Sodium bicarbonate	-	0.84 g
Distilled water	-	50 ml

Coating Buffer

Solution A	-	3.5 ml
Solution B	-	8.5 ml
Distilled water	-	50 ml

3.2.4.2.3 Blocking Buffer

Bovine Gelatin (Rallis, India Ltd., Bangalore)	-	1 g
Tween 20	-	0.1 ml
Phosphate buffered saline (0.1 M)	-	100 ml

3.2.4.2.4 Wash Buffer

Phosphate buffered saline (0.01 M)	-	200 ml
Distilled water	-	800 ml

This makes phosphate buffered saline of 0.002 M of pH 7.4 ± 0.2

3.2.4.2.5 Chromogen/Substrate

O-Phenylene diamine dihydro chloride (30 g tablet)	1
Distilled water	75 ml

Three per cent Hydrogen Peroxide Liquid (Stored at +4°C)

Forty eight µl of three per cent hydrogen peroxide was added to 12 ml of the OPD-distilled water solution for the use in one microplate.

3.2.4.2.6 Stopping Solution

Concentrated sulphuric acid (EG)	-	5.5 ml
Distilled water	-	94.5 ml

(Stored at room temperature)

3.2.4.3 Biologicals

3.2.4.3.1 Antigen

Smooth Lipopolysaccharide (S-LPS). Hot water/Phenol extract of *Brucella abortus* S99, freeze dried, stored at +4°C.

The freeze dried contents of the vial was reconstituted with one ml of double glass distilled water and stored at 4°c

3.2.4.3.2 Control Sera

a. Strong anti *Brucella abortus* antibody positive (C++)

b. Moderate anti *Brucella abortus* antibody positive (C+)

c. Anti- *Brucella abortus* antibody negative (C-)

The freeze dried bovine sera was reconstituted with one ml of double glass distilled water and stored at –20°C.

Test sera were diluted 1 in 100 in blocking buffer. For this five µl of each sera were added to 500 µl of blocking buffer separately in Perspex plate.

Control sera were diluted 1 in 100 in blocking buffer. To make 1 in 100 dilution, five µl of each control were added to 500 µl of blocking buffer separately in perspex plate.

3.2.4.3.3 Immunoconjugates

Biotinylated antibovine IgG (B-anti Ig G) was reconstituted with one ml of double glass distilled water and mixed gently until completely dissolved. Stored at +4°C.

Preparation of working dilution of biotin-antibovine IgG

Working dilution of biotin antibovine IgG was prepared in blocking buffer. Twelve millilitres of working dilution was required per microplate. This was prepared by adding 5 µl of Bovine-anti IgG per ml of blocking buffer.

Avidin – Horse radish peroxidase (A-HRP) freezedried contents of the vial was mixed with one ml of double glass distilled water and stored at +4°C.

Preparation of working dilution of Avidin-Horse radish peroxidase (A-HRP) conjugate

Working dilution of Avidin-HRP was prepared in blocking buffer. Twelve millilitres of working dilution was needed per microplate. This was prepared by adding 5 µl of A-HRP from the stock solution per one ml of blocking buffer.

3.2.4.4 Procedure

Coating of microplates

A working dilution of smooth Lipopolysaccharide (S-LPS) was prepared in coating buffer. Eleven millilitres of working dilution was required per micro plate. For this 200 µl of reconstituted antigen was added to 50 ml of coating buffer and mixed well. From this 100µl each was immediately dispensed into all 96 wells of the microplate. The sides of the plate were gently tapped to ensure even distribution of S-LPS antigen. The microplate was covered using sealing tape and incubated at 37°C for one hour.

Washing the microplate

The antigen coated microplate was removed from the incubator, and the contents were discarded by inverting the microplate and then the inverted microplate was gently tapped on to a lint free absorbent towel to remove all the residual contents. The procedure was repeated two or more times after washing the microplate with wash buffer.

Addition of test and control sera

The test sera and control sera diluted 1 in 100 were dispensed in 100 µl quantity to the respective wells in microplate in duplicate except to conjugate control wells. 100 µl of diluent buffer was added to the conjugate control wells. The microplate was covered and incubated for one hour at 37°C on an orbital shaker.

Washing the plate and addition of conjugate

After one hour of serum incubation, the microplate was removed from the incubator, and the contents were discarded. The plates were washed with wash buffer three times and the microplate was dried by tapping on to a lint free absorbent towel. Added 100 µl of the working dilution of biotin-antibovine IgG conjugate to all 96 wells of the microplate and tapped to ensure uniform dispersion. Microplate was covered and incubated at 37°C for one hour on an orbital shaker.

Washing the plate and addition of A-HRP conjugate

After one hour of incubation, the microplate was removed from the incubator and the contents were discarded. The plates were washed with wash buffer three times and the microplate was dried by tapping on to a lint free absorbent towel. To all the 96 wells of the microplate, 100 µl of working dilution of A-HRP was added. The microplate was covered and incubated for 20 minutes at 37°C on an orbital shaker.

Washing the plates and addition of substrate-chromogen

After 20 minutes of incubation removed the plates from the incubator and discarded the contents. Washed three times with wash buffer and plates were dried.

To all the 96 wells of the microplate, 100 µl of working solution of substrate chromogen was added. The plates were kept at room temperature for 10 minutes.

Addition of stop solution

To all 96 wells of the microplate, 50 µl of the stop solution was added and gently tapped the microplate to ensure thorough mixing.

Preparation of a blank plate

A clean microplate not coated with antigen was used as blank plate. Fifty microlitres of stop solution was added to each well of the first column of the blanking plate.

Measurement of colour development

The microplate ELISA reader (Lab Systems, Multiscan® Plus Microplate Reader with an interference filter of 492 nm) was allowed to warm up for 15 minutes before reading to ensure uniformity in reading of all plates. Wiped the bottom of plate with a clean cloth to remove condensation and smudges.

Colour development was read by placing the blank plate followed by the test plate in the ELISA reader.

3.2.4.5 Interpretation

Readings were used in two types of data analysis.

1. Per cent positivity (PP) values used for quality assurance (QA)

$$PP = \frac{\text{Replicate OD value of each control}}{\text{Median OD value of C++}} \times 100$$

2. Per cent Positivity (PP) values used for acceptance of test sera data and for diagnostic interpretation.

$$PP = \frac{\text{Replicate OD value of test serum}}{\text{Median OD value of C++}} \times 100$$

Acceptance of control data

The data expressed in OD values and PP values for the C++ control and the data expressed in PP values for the three other controls (C+, C- and CC) were used to determine whether the test was performed within acceptable limits of variability.

Acceptance criteria for control data

Serum Controls		Upper control limit (UCL)	Lower control limit (LCL)
C++	OD values	1.2	0.7
C++	PP values	126	74
C+	PP values	63	37
C-	PP values	23	0
CC	PP values	10	-3

Microplate acceptance (First level)

Strong positive (C++ control serum)

Compared the two intermediate OD values of the C++ control to the lower and upper control limits and the values falling within these limits were accepted.

Microplate acceptance (Second level)

Compared the replicate PP values of strong positive (C++), weak positive (C+), negative (C-) and conjugate control (CC) to the UCLs and LCLs.

Diagnostic threshold PP values

Methods for the determination of diagnostic threshold PP values were (a) double the mean PP of the disease free group, (b) mean PP value plus 3 standard deviations, (c) Mean PP value of the 100 per cent. The diagnostic threshold PP value was determined as 33.

Acceptance of test sera data

Test sera having mean PP values equal to or greater than the calculated threshold PP values were considered as positive. Test sera having mean PP values less than calculated threshold PP values were considered as negative.

3.2.5 Analysis of Data

The epidemiological data were subjected to statistical analysis as per the procedures of Snedecor and Cochran (1994).

The diagnostic tests were evaluated as per the methods of Thrusfield (1997).

Results

4. RESULTS

A total of 1602 blood samples were collected from cattle and buffaloes of different districts of Kerala. They belonged to different age groups, sex, breed, lactation, gestation, health status and managemental practices. Of this 1602 sera samples, 1445 samples were collected from animals whose history were known, 157 samples were from animals brought for slaughter, 1535 samples belonged to cattle and 67 samples belonged to buffaloes. All the sera samples were subjected to Rose Bengal Plate Test, Standard Tube Agglutination Test, 2-Mercaptoethanol Test and Avidin-Biotin ELISA for the detection of *Brucella abortus* antibodies.

4.1 EPIDEMIOLOGY

4.1.1 Seroprevalence of Brucellosis in Cattle and Buffaloes

A total of 1535 cattle were studied. Of this 50 samples were found to be positive in RBPT (3.25 per cent), 40 samples scored positive in STAT (2.60 per cent), 28 samples scored positive in 2 MET (1.82 per cent) and 226 samples scored positive by ELISA (14.72 per cent).

Out of the total 67 samples collected from buffaloes six samples were positive by RBPT (8.95 per cent), eight samples were positive both by STAT and by 2 MET (11.94 per cent) and 19 samples were positive by ELISA (28.35 per cent). Of the total per cent positives a higher percentage was shown by buffaloes (28.35 per cent) than cattle (14.72 per cent) (Table 1, Fig. 1). Statistical analysis of the result using test for proportion revealed no significance between cattle and buffaloes in their susceptibility to brucellosis.

4.1.2 Sexwise Seroprevalence of Brucellosis in Cattle

Of the total 349 male cattle tested for brucellosis, 13 animals were positive by RBPT (3.72 per cent), 11 were positive by both STAT and 2MET (3.15 per cent) and 44 were positive by ELISA (12.60 per cent).

Table 1. Seroprevalence of brucellosis in cattle and buffaloes

Animal	Number of samples	Number of positives			
		RBPT	STAT	2MET	ELISA
Cattle	1535	50 (3.25)	40 (2.60)	28 (1.82)	226 ^{NS} (14.72)
Buffaloes	67	6 (8.95)	8 (11.94)	8 (11.94)	19 ^{NS} (28.35)
Total	1602	56	48	36	245

NS - Non Significant

Figures in parenthesis shows percentage

Table 2. Sexwise seroprevalence of brucellosis in cattle

Sex	Number of samples	Number of positives			
		RBPT	STAT	2MET	ELISA
Male	349	13 (3.72)	11 (3.15)	11 (3.15)	44^{NS} (12.60)
Female	1186	37 (3.11)	29 (2.44)	17 (1.43)	182 ^{NS} (15.34)
Total	1535	50	40	28	226

NS - Non Significant

Figures in parenthesis shows percentage

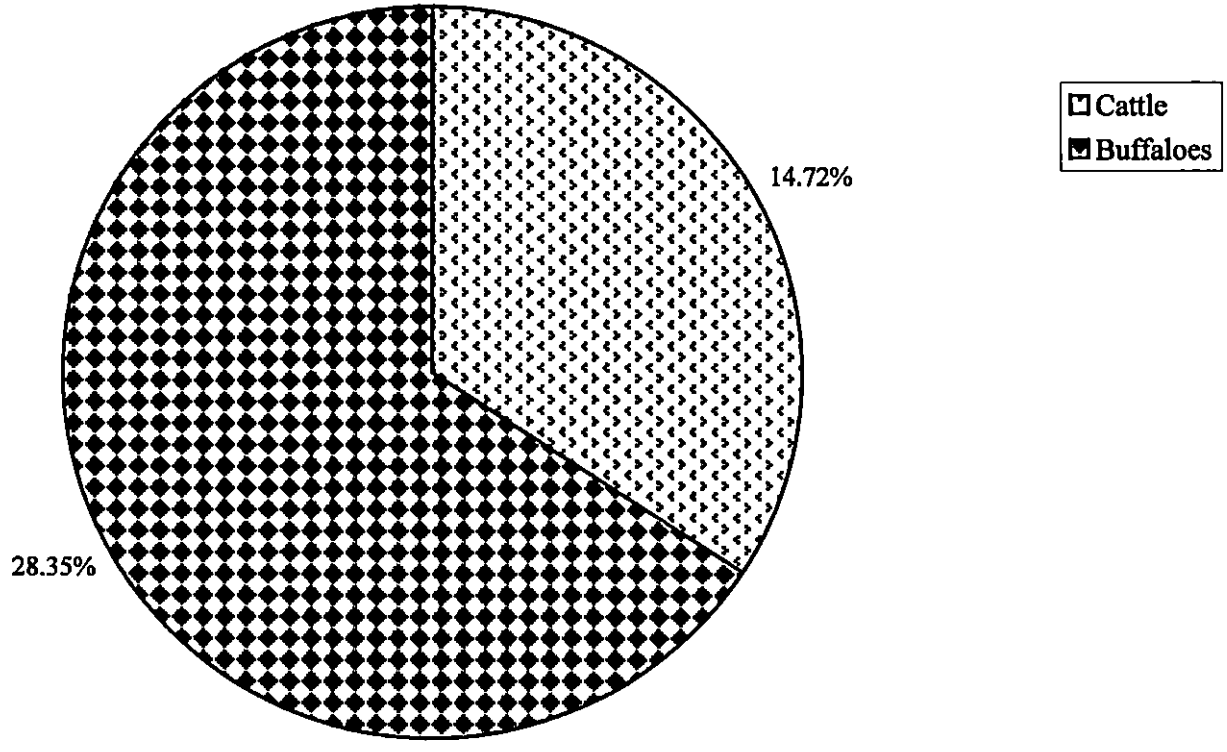

Fig.1.Seroprevalence of brucellosis in cattle and buffaloes by ELISA

Out of the 1186 female cattle tested, 37 scored positive by RBPT (3.11 per cent), 29 samples were positive by STAT (2.44 per cent), 17 samples were positive by 2MET (1.43 per cent) and 182 samples were positive by ELISA (15.34 per cent) (Table 2).

Of the total per cent positives, a higher percentage was shown by female cattle (15.34 per cent) than male cattle (12.60 per cent). Statistically no significant difference was noticed between male and female cattle in their seropositivity to brucellosis

4.1.3 Sexwise Seroprevalence of Brucellosis in Buffaloes

A total of 36 sera samples from male buffaloes were tested for the presence of antibodies to *Brucella abortus*, of which four samples were positive by RBPT (11.11 per cent), five samples were positive by STAT and 2MET (13.88 per cent) and 10 samples were positive by ELISA (27.77 per cent).

Of the total 31 sera samples obtained from female buffaloes, two samples were positive by RBPT (6.45 per cent), three samples each were positive by STAT and 2MET (9.67 per cent) and nine samples were positive by ELISA (29.03 per cent) (Table 3).

Of the total per cent positives a comparatively higher prevalence was noticed among female buffaloes (29.03 per cent) than the male buffaloes (27.77 per cent). Statistical analysis revealed that there is no significant difference between male and female buffaloes in the seroprevalence to brucellosis

Overall seroprevalence among male cattle, female cattle, male buffaloes and female buffaloes were 12.60, 15.34, 27.77 and 29.03 per cent respectively (Fig.2). Among this the female buffaloes had the highest seroprevalence. No statistical significance was noticed in female buffaloes in their seropositivity to brucellosis.

Table 3. Sexwise seroprevalence of brucellosis in buffaloes

Sex	Number of samples	Number of positives			
		RBPT	STAT	2MET	ELISA
Male	36	4 (11.11)	5 (13.88)	5 (13.88)	10 ^{NS} (27.77)
Female	31	2 (6.45)	3 (9.67)	3 (9.67)	9 ^{NS} (29.03)
Total	67	6	8	8	19

NS – Non Significant

Figures in parenthesis shows percentage

Table 4. Agewise seroprevalence of brucellosis in bulls

Age	Number of samples	Number of positives			
		RBPT	STAT	2MET	ELISA
Below 2 years	98	0	0	0	2 (2.04)
Between 2 and 5 years	132	3 (2.27)	2 (1.51)	2 (1.51)	11 (8.33)
Above 5 years	82	2 (2.43)	3 (3.65)	3 (3.65)	19* (23.17)
Total	312	5	5	5	32

* Significant ($P<0.05$)

Figures in parenthesis shows percentage

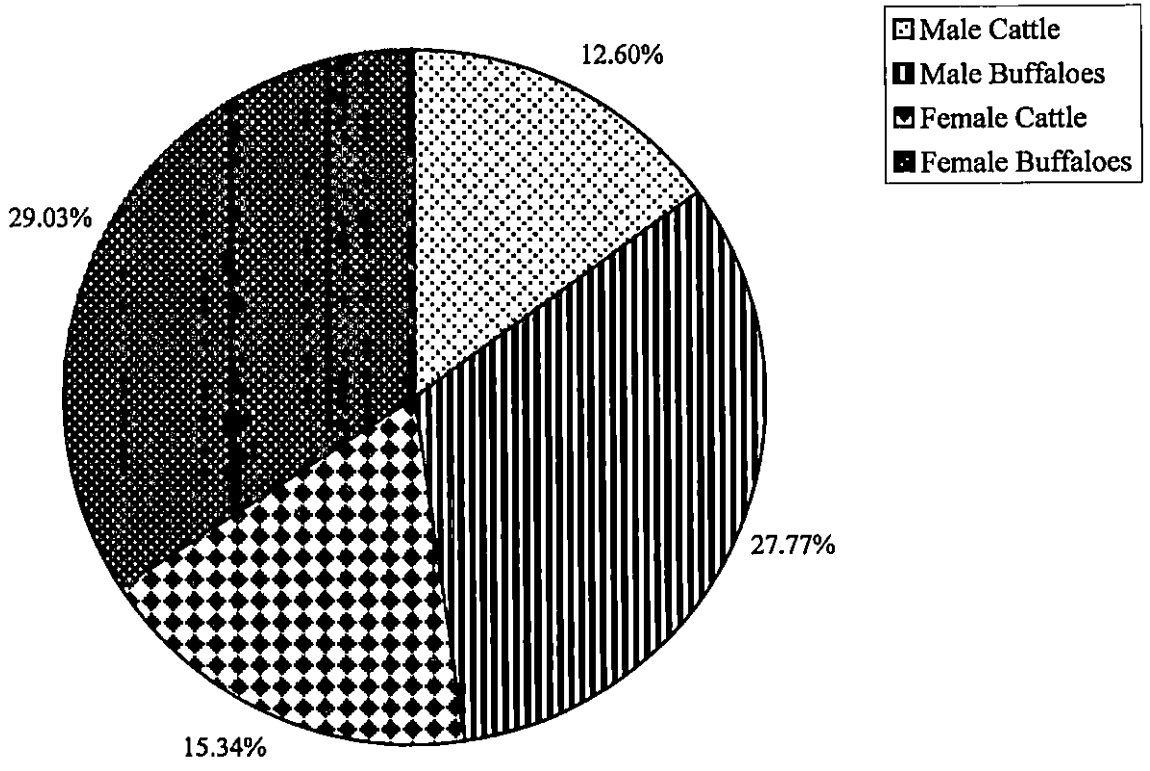

Fig. 2. Sexwise seroprevalence of brucellosis in cattle and buffaloes by ELISA

4.1.4 Agewise Seroprevalence of Brucellosis in Bulls

The agewise seroprevalence were studied in 312 bulls maintained in farms by categorizing them in to three age groups.

1. Below two years of age - 98 animals
2. Between two and five years - 132 animals
3. Above five years of age - 82 animals

Among the 98 bulls below two years of age, none of the samples were positive by RBPT, STAT and 2MET. Two samples (2.04 per cent) were positive by ELISA. Of the total 132 bulls screened between two to five years, three samples (2.27 per cent) was found positive by RBPT, two samples (1.51 per cent) each were positive by STAT and 2MET and 11 samples (8.33 per cent) were positive in ELISA. Among the bulls above 5 years of age, two bulls (2.43 per cent) showed positive reaction in RBPT. Three bulls each (3.65 per cent) showed a positive result in STAT and 2MET and 19 bulls (23.17 per cent) showed a positive reaction in ELISA (Table 4, Fig. 3).

Among the total per cent positives, bulls belonged to the third group showed a greater prevalence (23.17 per cent) than those bulls in the first and second group. Statistical analysis revealed that significant difference exists in the bulls more than five years of age in the seroprevalence of brucellosis ($P<0.05$).

Among the nine male buffaloes of age group two to five, all belong to farm and households and none were found to be positive by any of the tests.

4.1.5 Agewise Seroprevalence of Brucellosis between Cows and Heifers

A total of 1121 female cattle belonging to farms and households were categorized into three age groups as follows.

1. Below two years of age - 298 animals
2. Between two and five years - 560 animals
3. Above five years - 263 animals

Of the total 298 heifers aged below two years, five animals scored positive both in RBPT and STAT (1.67 per cent), three were positive in 2MET (1.00 per cent) and 29 were positive by ELISA (9.73 per cent). Of the total 560 cows aged between two to five years, three samples scored positive by RBPT (0.53 per cent), four samples each were positive by STAT and 2MET (0.71 per cent) and 38 samples were positive by ELISA (6.78 per cent).

A total of 263 samples belong to cows aged more than five years of age, of which nine samples each scored positive by RBPT and STAT (3.42 per cent), six sample (2.28 per cent) were positive by 2MET and 82 samples were positive by ELISA (31.17 per cent) (Table 5, Fig. 3).

Of the total per cent positives a higher percentage of positivity was noticed among cattle of more than five years of age (31.17 per cent) followed by heifers (9.73 per cent) and cattle aged between two and five years of age (6.78 per cent). Statistical analysis revealed that there is statistical significance among cows more than five years of age in the seroprevalence of brucellosis ($P<0.05$).

Of the three female buffaloes of age group two years which were presented in the hospital, all were negative by all the four tests.

4.1.6 Breedwise Seroprevalence of Brucellosis in Bulls

Bulls under study belonged to pure bred Jersey, Holstein Friesian (HF), Brown Swiss (BS) and Crossbreds.

Among 171 Jersey bulls, three bulls (1.75 per cent) gave a positive reaction in RBPT, STAT and 2MET and 18 animals (10.52 per cent) gave a positive result in ELISA. Among the 100 HF bulls, three bulls (3.0 per cent) showed a positive reaction in RBPT, two bulls (2.0 per cent) showed a positive result in STAT and 2 MET and 12 animals (12.0 per cent) were found to be positive in ELISA. Out of the 38 Brown Swiss bulls, two animals (5.26 per cent) showed positive reaction only in ELISA and all the other tests were negative (Table 6, Fig. 4).

None of the tests used could identify a positive reactor among three crossbred bulls. So the total per cent positivity revealed a higher seroprevalence of

Table 5. Agewise seroprevalence of brucellosis between cows and heifers

Age	Number of samples	Number of positives			
		RBPT	STAT	2MET	ELISA
Below 2 years	298	5 (1.67)	5 (1.67)	3 (1.00)	29 (9.73)
Between 2 and 5 years	560	3 (0.53)	4 (0.71)	4 (0.71)	38 (6.78)
Above 5 years	263	9 (3.42)	9 (3.42)	6 (2.28)	82* (31.17)
Total	1121	17	18	13	149

* Significant ($P<0.05$)

Figures in parenthesis shows percentage

Table 6. Breedwise seroprevalence of brucellosis in bulls

Breeds	Number of samples	Number of positives			
		RBPT	STAT	2MET	ELISA
Jersey	171	3 (1.75)	3 (1.75)	3 (1.75)	18^{NS} (10.52)
Holstein Friesian	100	3 (3.0)	2 (2.0)	2 (2.0)	12^{NS} (12.0)
Brown Swiss	38	0	0	0	2^{NS} (5.26)
CBHF	3	0	0	0	0
Total	312	6	5	5	32

NS - Non Significant

Figures in parenthesis shows percentage

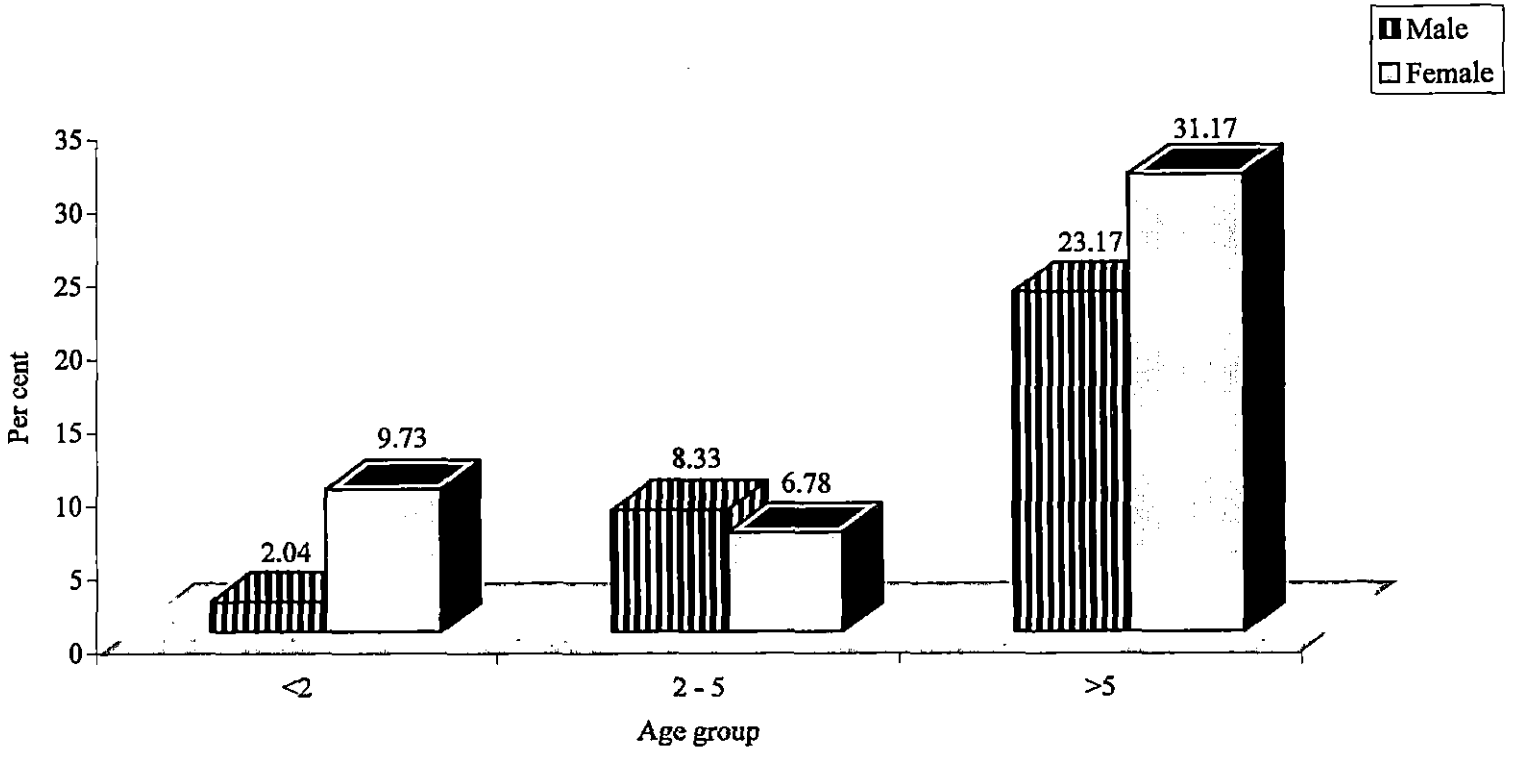

Fig. 3. Agewise seroprevalence of brucellosis in cattle by ELISA

brucellosis among HF bulls (12 per cent) followed by Jersey (10.52 per cent) and Brown Swiss (5.26 per cent).

Statistical analysis showed that there is no statistical significance in the susceptibility of different breeds to brucellosis ($P<0.05$).

Among the nine male buffaloes all belong to farm and households of Murrah breed and none were found to be positive by any of the tests.

4.1.7 Breedwise Seroprevalence of Brucellosis among Female Cattle

The breeds under study were crossbred Jersey, crossbred Holstein Friesian, crossbred Brown Swiss and non descript cattle.

Among 680 animals of Jersey crossbred, 10 (1.47 per cent) were positive by RBPT, 12 (1.76 per cent) animals were positive by STAT, eight (1.17 per cent) were positive by 2MET and 102 (15.0 per cent) were positive by ELISA. In 302 Holstein Friesian crossbreds, six (1.98 per cent) animals were positive by RBPT, five (1.65 per cent) animals were positive by STAT, three (0.99 per cent) animals by 2MET and 43 (14.23 per cent) animals were positive by ELISA. Among the 88 Brown Swiss crossbreds, one (1.13 per cent) animal was positive by RBPT, STAT and two (2.27 per cent) were positive by 2MET and three (3.40 per cent) animals were positive in ELISA. Among the 51 non descript cows none of the animals showed any positive reaction in RBPT, STAT and 2MET but one (1.96 per cent) showed a positive reaction in ELISA (Table 7, Fig. 4).

Among the total per cent positives crossbred Jersey showed a higher prevalence (15 per cent), followed by crossbred HF (14.23 per cent), crossbred Brown Swiss (3.4 per cent) and the non descripts (1.96 per cent).

Statistical analysis revealed that significant difference exists in the crossbred Jersey female cattle compared to other breeds in seroprevalence to brucellosis ($P<0.05$).

Among the three Murrah crossbred female buffaloes belonging to households, none were found to be positive by any of the tests.

Table 7. Breedwise seroprevalence of brucellosis among female cattle

Breed	Number of samples	Number of positives			
		RBPT	STAT	2MET	ELISA
CBJ	680	10 (1.47)	12 (1.76)	8 (1.17)	102* (15.0)
CBHF	302	6 (1.98)	5 (1.65)	3 (0.99)	43 (14.23)
CBBS	88	1 (1.13)	1 (1.13)	2 (2.27)	3 (3.40)
ND	51	0	0	0	1 (1.96)
Total	1121	17	18	13	149

* Significant (P<0.05)

Figures in parenthesis shows percentage

Table 8. Seroprevalence of brucellosis in pregnant and non pregnant cattle

Gestational status	Number of samples	Number of positives			
		RBPT	STAT	2MET	ELISA
Pregnant cattle	458	12 (2.62)	10 (2.18)	8 (1.74)	96* (20.96)
Non pregnant cattle	663	5 (0.75)	8 (1.20)	5 (0.75)	53 (7.99)
Total	1121	17	18	13	149

* Significant (P<0.05)

Figures in parenthesis shows percentage

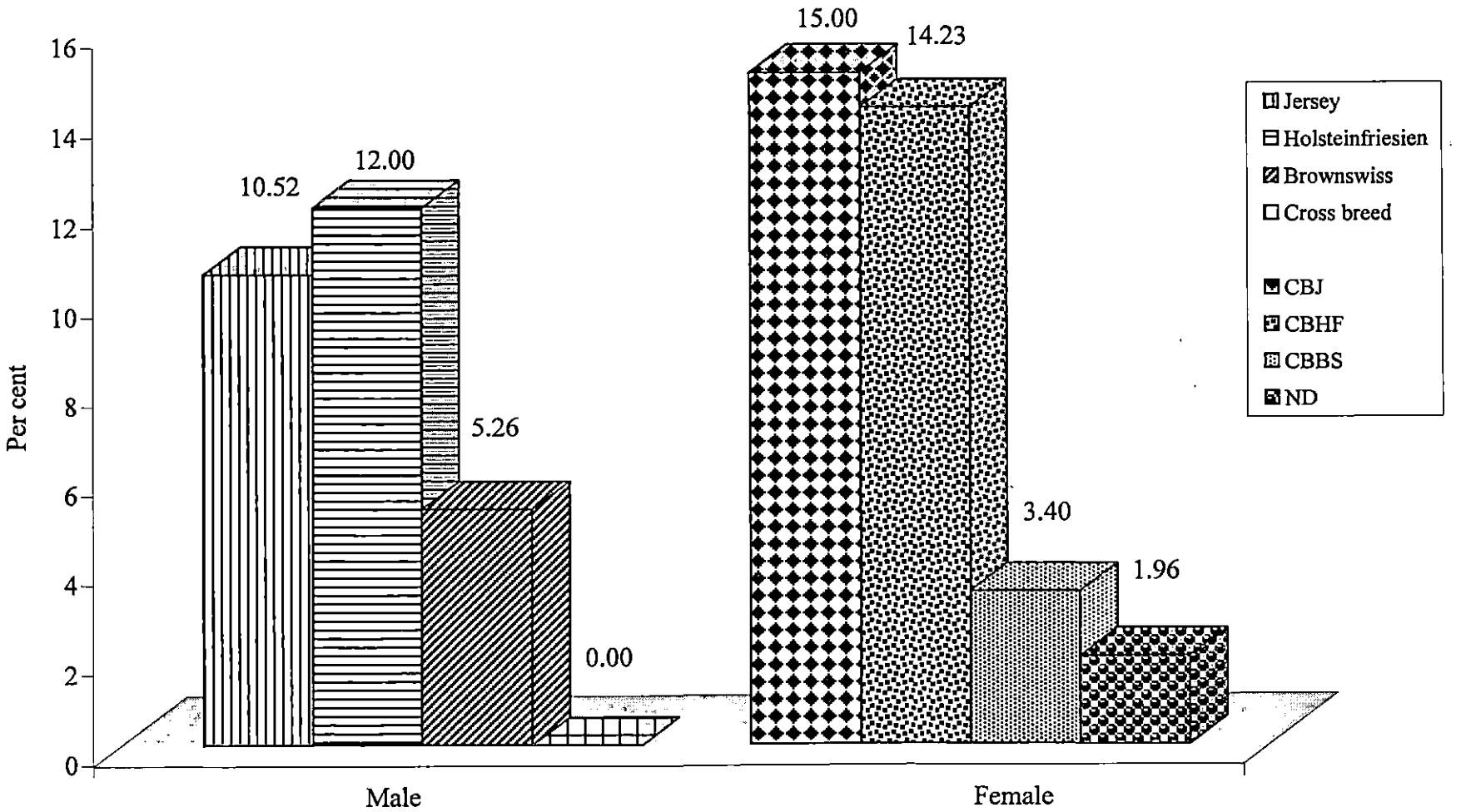

Fig.4 Breedwise seroprevalence of brucellosis in cattle by ELISA

4.1.8 Seroprevalence of Brucellosis in Pregnant and Non Pregnant Cattle

Among the 458 pregnant cattle, 12 (2.62 per cent) were positive by RBPT, 10 (2.18 per cent) by STAT, eight (1.74 per cent) by 2MET and 96 (20.96 per cent) were positive by ELISA.

Out of the 663 non pregnant cattle, five (0.75 per cent) were positive in RBPT, eight (1.20 per cent) were positive in STAT and five (0.75 per cent) in 2MET and 53 (7.99 per cent) in ELISA (Table 8).

Among the total per cent positives, a higher prevalence rate (20.96 per cent) was shown by pregnant cattle than non pregnant cattle (7.99 per cent). Statistical analysis of the results showed a significant difference ($P<0.05$) exist in pregnant cattle in their seropositivity to brucellosis.

4.1.9 Seroprevalence of Brucellosis in Female Cattle According to Stage of Gestation

Pregnant cows and heifers were categorized into three groups.

1. Early (upto four months) – 230 animals
2. Mid (four to seven months) – 170 animals
3. Late (seven months and above) – 58 animals

Among the 230 animals in early gestation, four (1.73 per cent) animals were positive by RBPT, one (0.43 per cent) by STAT and 35 (15.21 per cent) animals by ELISA. None of the animals were positive by 2MET.

Out of the 170 animals in the mid gestation, (second group) four (2.35 per cent) animals tested were positive by RBPT, two (1.17 per cent) by STAT and by 2MET and 26 (15.29 per cent) animals revealed positive by ELISA.

Among the 58 animals which were in the late gestation period showed that 4 (6.89 per cent), 7 (12.06 per cent), 6 (10.34 per cent) and 35 (60.34 per cent) animals were positive by RBPT, STAT, 2MET and ELISA respectively (Table 9).

Among the total per cent positives, highest seroprevalence was noticed in cows and heifers in their late gestation(60.34 per cent) than animals in the middle (15.29 per cent) and early gestation (15.21 per cent).

Statistical analysis revealed a significant difference exist in cows in late gestation period in their seroprevalence to brucellosis ($P<0.05$).

4.1.10 Seroprevalence of Brucellosis in Cows According to Stage of Lactation

The cows were categorized into three categories.

1. Non pregnant lactating - 403 animals
2. Pregnant lactating - 372 animals
3. Pregnant dry - 48 animals

Out of the 403 non pregnant lactating cows, two (0.49 per cent) animals were positive by RBPT, three (0.74 per cent) animals by STAT, two (0.49 per cent) by MET and 29 (7.19 per cent) animals revealed positive by ELISA.

Three hundred and seventy two cows belonged to pregnant lactating group of which three (0.80 per cent) were positive by RBPT, one (0.26 per cent) each by STAT and 2MET and 62 cows (16.66 per cent) were positive by ELISA. Among the 48 pregnant dry cows, two (4.16 per cent) were positive by RBPT, STAT and 2MET and 29 (60.41 per cent) animals were positive by ELISA (Table 10).

Among the total per cent positives highest prevalence was noticed in pregnant dry cows (60.41 per cent) followed by pregnant lactating (16.66 per cent) and non pregnant lactating (7.19 per cent).

Statistical analysis revealed that there is significance in the susceptibility of pregnant dry cows to brucellosis ($P<0.05$).

4.1.11 Seroprevalence of Brucellosis in Female Cattle with Reproductive Disorders

Out of the total 159 animals with reproductive disorders, 51 animals were positive (32.07 per cent) for brucella antibodies. Twenty six animals were having

Table 9. Seroprevalence of brucellosis in female cattle according to stage of gestation

Stage of gestation	Number of samples	Number of positives			
		RBPT	STAT	2MET	ELISA
Early	230	4 (1.73)	1 (0.43)	0	35 (15.21)
Middle	170	4 (2.35)	2 (1.17)	2 (1.17)	26 (15.29)
Late	58	4 (6.89)	7 (12.06)	6 (10.34)	35* (60.34)
Total	458	12	10	8	96

* Significant (P<0.05)

Figures in parenthesis shows percentage

Table 10. Seroprevalence of brucellosis in cows according to stage of lactation

Stage of lactation	Number of samples	Number of positives			
		RBPT	STAT	2MET	ELISA
Non pregnant lactating	403	2 (0.49)	3 (0.74)	2 (0.49)	29 (7.19)
Pregnant lactating	372	3 (0.80)	1 (0.26)	1 (0.26)	62 (16.66)
Pregnant dry	48	2 (4.16)	2 (4.16)	2 (4.16)	29* (60.41)
Total	823	5	4	3	120

* Significant (P<0.05)

Figures in parenthesis shows percentage

history of abortion in late gestation of which four animals (15.38 per cent) were positive by RBPT and 2MET, five animals (19.23 per cent) by STAT and six animals (23.07 per cent) were positive by ELISA. Fifteen and nine animals had the history of abortion in mid gestation and early gestation respectively and none of the animals were positive in any of tests. Out of the 16 animals with history of abortion and subsequent repeat breeding, three animals (18.75 per cent) were positive by RBPT and STAT, two animals (12.50 per cent) by 2MET and four animals (25.0 per cent) were positive by ELISA. Six animals had the history of abortion and retention of placenta, and one animal (16.66 per cent) was found positive by RBPT and 2 MET and two animals (33.33 per cent) were positive by STAT and ELISA.

Eighty animals were with the history of repeat breeding, anoestrum, suboestrum and three animals (3.75 per cent) scored positive in RBPT, four animals (5.00 per cent) were positive in STAT, two animals (2.50 per cent) were positive in 2MET and 38 animals (47.50 per cent) scored positive in ELISA. Of the five animals having the history of retention of placenta one (20.0 per cent) showed positive reaction in RBPT, STAT and ELISA. Two animals which had the history of metritis, none of the animals gave positive reaction in any of the four tests (Table 11).

Among the total per cent positives, highest percentage was shown by repeat breeders (47.50 per cent), followed by those with abortion and retention of placenta (33.33 per cent) abortion and repeat breeding (25.0 per cent) and abortion in late gestation (23.07 per cent). Statistical analysis revealed that significant difference exists in the repeat breeders in their seropositivity to brucellosis ($P<0.05$).

4.1.12 Seroprevalence of Brucellosis among Cattle and Buffaloes Maintained under Different Farming Systems

Of the total 1445 samples, 1076 samples belonged to animals managed by organised system of management. Of this 15 samples (1.39 per cent) scored positive by RBPT, 12 samples (1.11 per cent) by STAT, 10 samples (0.92 per ent)

Table 11. Seroprevalence of brucellosis in female cattle with reproductive disorders

Reproductive disorders	Number of samples	Number of positives			
		RBPT	STAT	2MET	ELISA
Early abortion	9	0	0	0	0
Mid abortion	15	0	0	0	0
Late abortion	26	4 (15.38)	5 (19.23)	4 (15.38)	6 (23.07)
Abortion and repeat breeding	16	3 (18.75)	3 (18.75)	2 (12.50)	4 (25.0)
Abortion and ROP	6	1 (16.66)	2 (33.33)	1 (16.66)	2 (33.33)
Repeat breeding	80	3 (3.75)	4 (5.0)	2 (2.5)	38* (47.50)
ROP	5	1 (20.0)	1 (20.0)	0	1 (20.0)
Metritis	2	0	0	0	0
Total	159	12	15	9	51

* Significant (P<0.05)

Figures in parenthesis shows percentage

Table 12. Seroprevalence of brucellosis in cattle and buffaloes maintained under different farming systems

Farming systems	Number of samples	Number of positives			
		RBPT	STAT	2MET	ELISA
Organised	1076	15 (1.39)	12 (1.11)	10 (0.92)	113 (10.50)
Unorganised	369	10 (2.71)	6 (1.62)	7 (1.89)	68* (18.42)
Total	1445	25	18	17	181

* Significant (P<0.05)

Figures in parenthesis shows percentage

by 2MET and 113 samples (10.50 per cent) by ELISA. Out of the 369 samples from unorganised farming system, 10 samples (2.71 per cent) were positive by RBPT, 6 samples (1.62 per cent) by STAT, 7 (1.89 per cent) by 2MET and 68 samples (18.42 per cent) by ELISA (Table 12).

Out of the total per cent positives, a higher percentage was noticed in animals under unorganised system of management (18.42 per cent) compared to (10.50 per cent) in animals maintained in organised sector. Statistical analysis revealed that a significant difference exists in the animals managed under unorganised farming system ($P<0.05$).

4.1.13 Seroprevalence of Brucellosis in Animals in different Agroecological Regions

Out of the total 1445 animals, 1041 animals belonged to type I agroecological region. Of this 11 animals (1.05 per cent) scored positive by RBPT, 8 animals (0.76 per cent) were positive by STAT, 6 animals (0.57 per cent) by 2MET and 118 animals (11.33 per cent) were positive by ELISA. Of the 404 animals in the type II region, 14 samples (3.46 per cent) were positive by RBPT, 10 samples (2.47 per cent) by STAT, 11 samples (2.72 per cent) by 2 MET and 63 samples (15.59 per cent) by ELISA (Table 13).

A comparatively higher seropositivity was noticed in the animals in the high altitude region (type II) that is 15.59 per cent than the animals maintained in low altitude region (type I) which is 11.33 per cent. No statistical significance was noticed between animals belonging to two regions.

4.1.14 Seroprevalence of Brucellosis in Cattle and Buffaloes in different Zones

Of the total 165 sera samples from the animals belonged to south zone none of the samples were positive by RBPT, STAT and 2MET. Four samples (2.42 per cent) revealed positive by ELISA. Of the 1067 sera samples from the central zone 19 samples (1.78 per cent) scored positive by RBPT, 14 samples (1.31 per cent) scored positive by STAT, 15 samples (1.40 per cent) by 2MET and 125 samples

Table 13. Seroprevalence of brucellosis in cattle and buffaloes in different agroecological regions

Regions	Number of samples	Number of positives			
		RBPT	STAT	2MET	ELISA
Type I	1041	11 (1.05)	8 (0.76)	6 (0.57)	118 (11.33)
Type II	404	14 (3.46)	10 (2.47)	11 (2.72	63* (15.59)
Total	1445	25	18	17	181

* Significant (P<0.05)
Figures in parenthesis shows percentage

Table 14. Seroprevalence of brucellosis in cattle and buffaloes in different zones

Zones	Number of samples	Number of positives			
		RBPT	STAT	2MET	ELISA
South zone	165	0	0	0	4 (2.42)
Central zone	1067	19 (1.78)	14 (1.31)	15 (1.40)	125 (11.71)
North zone	213	6 (2.81)	4 (1.87)	2 (0.93)	52* (24.41)
Total	1445	25	18	17	181

* Significant (P<0.05)
Figures in parenthesis shows percentage

(11.71 per cent) scored positive by ELISA. A total of 213 samples were collected from animals belonging to north zone and a positive reaction was shown by six animals (2.81 per cent) by RBPT, four (1.87 per cent) by STAT, two (0.93 per cent) by 2MET and 52 (24.41 per cent) by ELISA (Table 14).

Of the total per cent positivity highest seroprevalence was shown by animals belonging to north zone (24.41 per cent) compared to the other two zones. Statistical analysis revealed that there is statistical significance in the animals belonging to north zone in the susceptibility to brucellosis ($P<0.05$).

4.1.15 *Districtwise Seroprevalence of Brucellosis in Kerala*

Number of samples collected from each district and the number of seropositive animals by each tests were given in Table (Table 15, Fig. 5).

Of the 58 sera samples from Thiruvananthapuram district none of the samples were positive by RBPT, STAT and 2MET. Two sera samples (3.44 per cent) were positive by ELISA. Samples collected from Kollam district was 90 in number, and two samples (2.22 per cent) were positive by ELISA and all the other tests were negative. Seventeen sera samples were collected from Kottayam district and all the samples were negative by all the four tests. Out of the 342 sera samples collected from Idukki district, 14 samples (4.09 per cent) revealed positive by RBPT, 10 samples (2.92 per cent) by STAT, 11 samples (3.21 per cent) by 2 MET and 61 samples (17.83 per cent) by ELISA. From Ernakulam district, 40 sera samples were collected and only one sample each (2.50 per cent) was positive by RBPT and ELISA. The other two tests were negative. A total of 383 sera samples were collected from Thrissur district and one sample each (2.61 per cent) were positive by RBPT, STAT and 2MET and 21 samples (5.48 per cent) by ELISA.

Three samples (0.99 per cent) each were positive by RBPT, STAT and 2MET and 42 samples (13.90 per cent) were positive by ELISA, out of the total 302 sera samples from Palakkad district. All of the 26 sera samples collected from Malappuram district were negative in all the four tests. Sixty sera samples were collected from Kozhikkode district, which revealed three samples (5.00 per cent) positive by RBPT, 2 samples (3.33 per cent) by STAT, 1 sample (1.66 per cent) by

Table 15. Districtwise seroprevalence of brucellosis in Kerala

Districts	Number of samples	Number of positives			
		RBPT	STAT	2MET	ELISA
Thiruvananthapuram (TVM)	58	0	0	0	2 (3.44)
Kollam (KLM)	90	0	0	0	2 (2.22)
Kottayam (KTM)	17	0	0	0	0
Idukki (IDK)	342	14 (4.09)	10 (2.92)	11 (3.21)	61 (17.83)
Ernakulam (EKM)	40	1 (2.50)	0	0	1 (2.50)
Thrissur (TCR)	383	1 (2.61)	1 (2.61)	1 (2.61)	21 (5.48)
Palakkad (PKD)	302	3 (0.99)	3 (0.99)	3 (0.99)	42 (13.90)
Malappuram (MPM)	26	0	0	0	0
Kozhikkode (KZD)	60	3 (5.00)	2 (3.33)	1 (1.66)	25* (41.66)
Wayanad (WYD)	62	0	0	0	3 (4.83)
Kannur (KNR)	65	3 (4.61)	2 (3.07)	1 (1.53)	24 (36.92)
Total	1445	25	18	17	181

* Significant ($P<0.05$)
Figures in parenthesis shows percentage

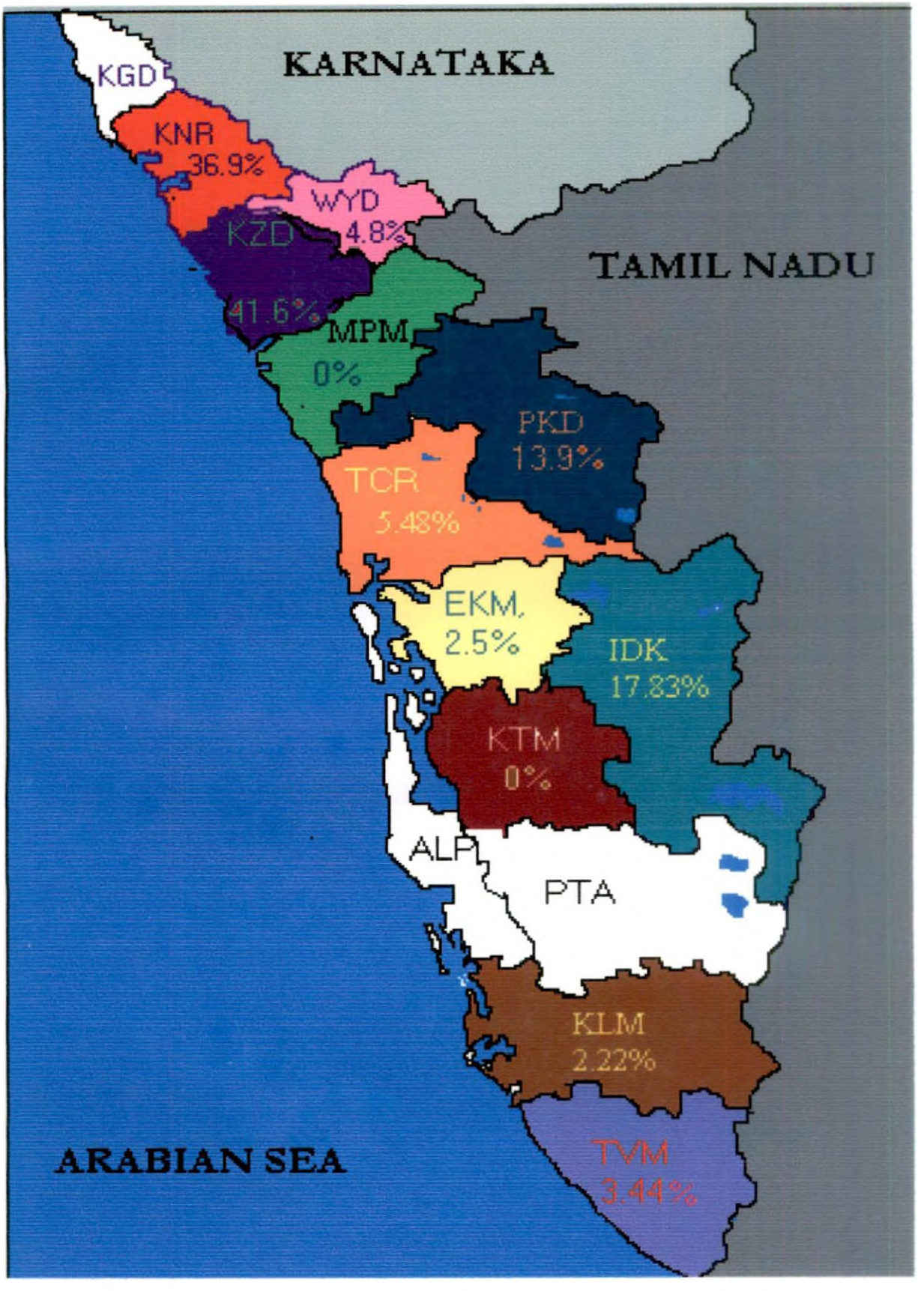

Fig. 5. Districtwise seroprevalence of brucellosis in Kerala

2 MET and 25 samples (41.66 per cent) by ELISA. A total of 62 sera samples were collected from Wayanad district, of which three samples (4.83 per cent) were positive by ELISA only. All the other three tests were negative. Out of the 65 samples collected from Kannur district, three samples (4.61 per cent) scored positive by RBPT, two samples (3.07 per cent) by STAT, one sample (1.53 per cent) by 2 MET and 24 samples (36.92 per cent) by ELISA.

Highest seropositiveness was noticed in Kozhikkode (41.66 per cent) followed by Kannur (36.92 per cent), Idukki (17.83 per cent), Palakkad (13.90 per cent), Thrissur (5.48 per cent), Wayanad (4.83 per cent), Thiruvananthapuram (3.44 per cent), Ernakulam (2.50 per cent) and Kollam (2.22 per cent). None of the sera samples collected from Malappuram and Kottayam showed positive reaction in any of the tests.

Of the total per cent positives, highest seroprevalence to brucellosis was shown by animals in the Kozhikkode district (41.66 per cent). A statistically significant difference exists in the animals belonging to Kozhikkode district in their seropositivity to brucellosis ($P<0.05$).

4.2 DIAGNOSTIC TESTS

All the 1602 sera samples were subjected to RBPT, STAT, 2MET and ELISA and the results were detailed below.

4.2.1 Rose Bengal Plate Test

Among sera samples tested, 26 samples (1.62 per cent) showed a recording sign (+), 21 (1.31 per cent) showed (++) and nine (0.56 per cent) samples showed (+++). (Table 16, Fig. 6 and Plate 1).

Thirteen samples (0.81 per cent) were found to be doubtful and 1533 (95.69 per cent) were negative.

Table 16. Rose Bengal Plate Test

Grading	Suggested meaning	Number of reactors
+	Weak positive	26
++	Moderate positive	21
+++	Strong positive	9
±	Doubtful	13
-	Negative	1533

Table 17. Standard tube Agglutination test

Serum dilution	Titre	Number of Samples
1 in 10	20 IU	10
1 in 20	40 IU	21
1 in 40	80 IU	26
1 in 80	160 IU	9
1 in 160	320 IU	4
1 in 320	640 IU	1

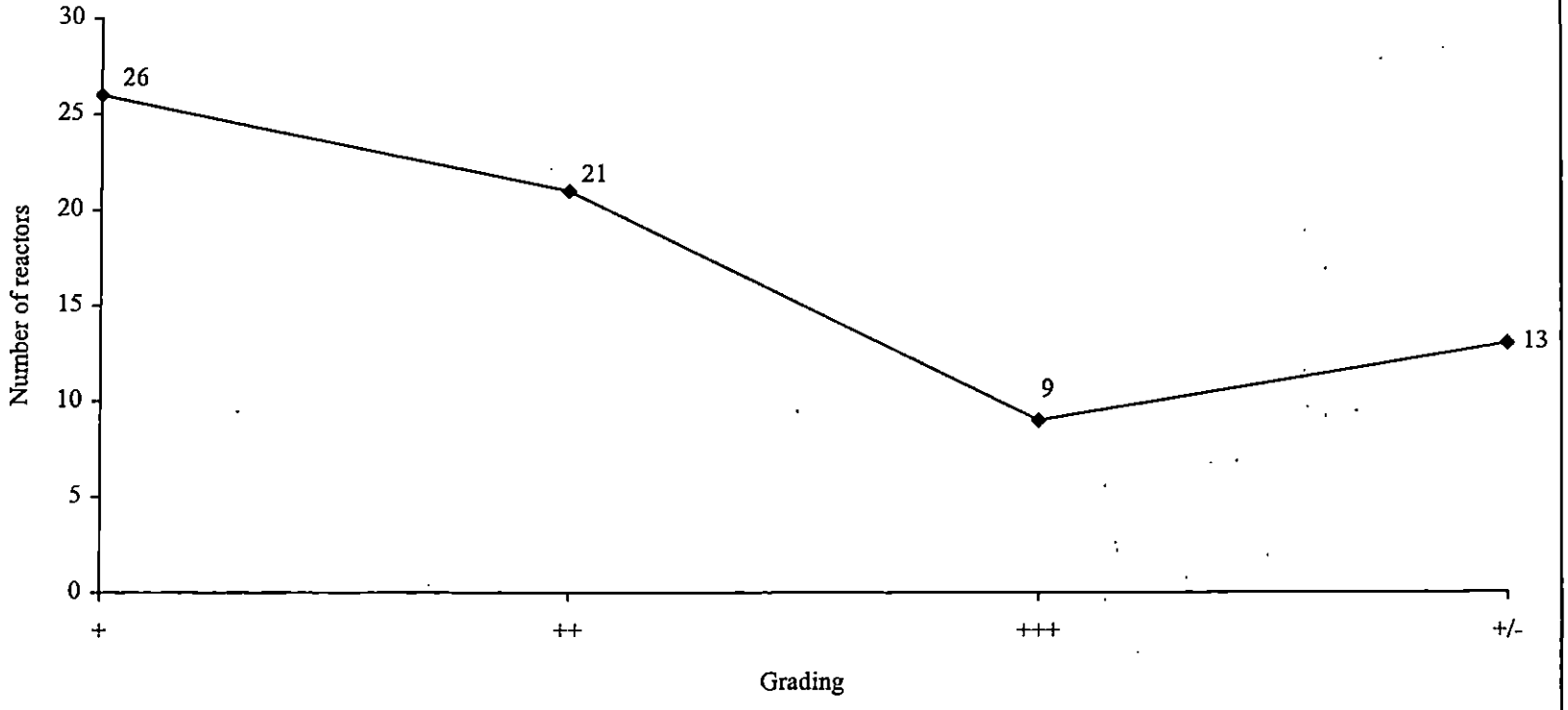

Fig. 6. Rosebengal plate test

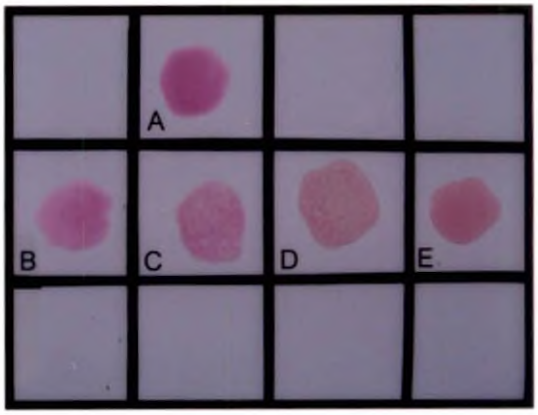

Plate 1. Rose Bengal Plate Test

A-Negative
B-Doubtful
C-Moderate Positive
D- Strong Positive
E-Weak Positive

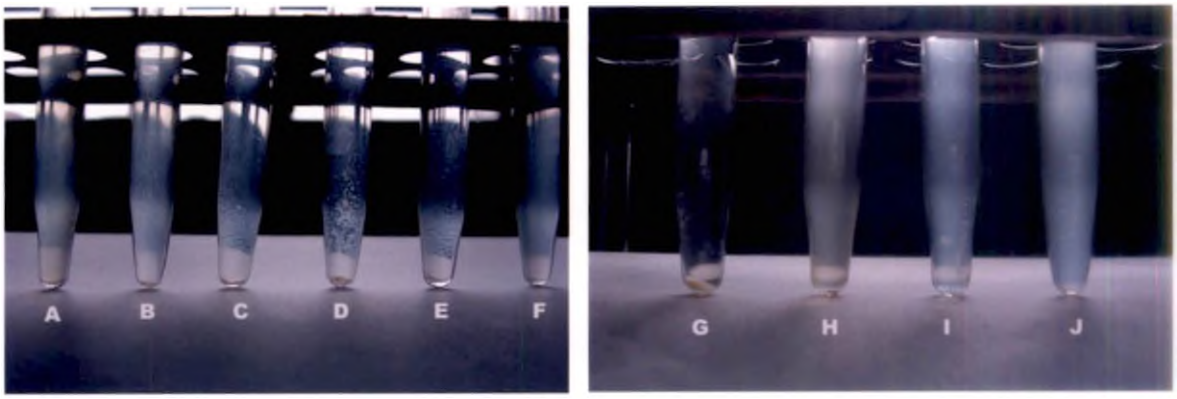

Plate 2. Standard Tube Agglutination Test

A-F -Test sera sampies, Titre = 10 IU to 320 IU

G - Positive control
H - Negative control
I - 50 Per cent control
J - Antigen control

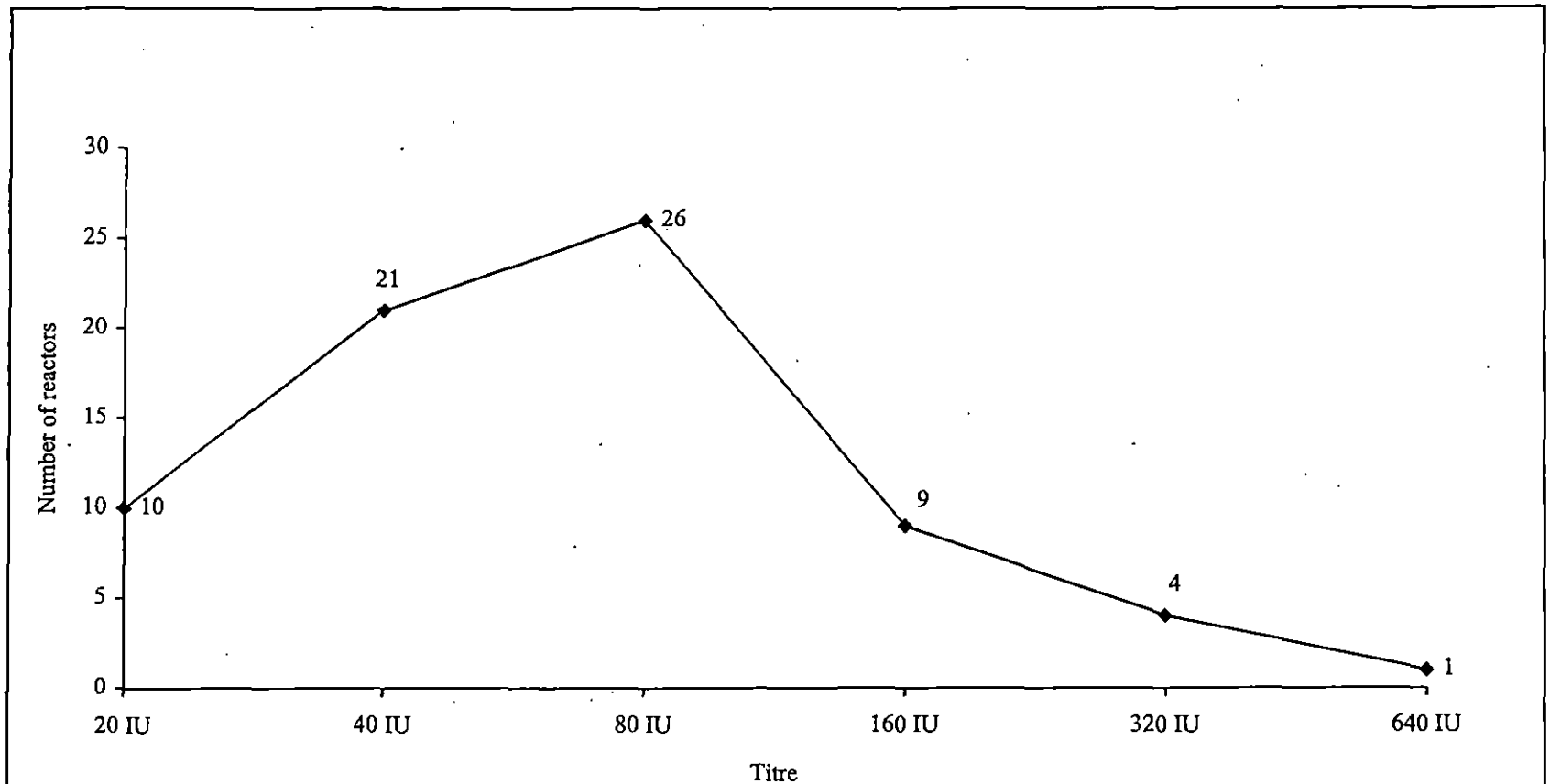

Fig. 7. Standard tube agglutination test

Table 18. 2-Mercaptoethanol test

Serum dilution	Titre	Number of Samples
1 in 10	20 IU	12
1 in 20	40 IU	24
1 in 40	80 IU	28
1 in 80	160 IU	6
1 in 160	320 IU	6
1 in 320	640 IU	1

Table 19. Evaluation of diagnostic tests using ELISA as 'gold standard'

		ELISA		Sensitivity (%)	Specificity (%)	Predictive value of		Accuracy
		Positive	Negative			+ve test	-ve test	
RBPT	Positive	56	0	22.85	100	100	87.77	88.20
	Negative	189	1357					
STAT	Positive	48	0	19.59	100	100	87.32	87.70
	Negative	197	1357					
2ME T	Positive	36	0	14.69	100	100	86.54	86.95
	Negative	209	1357					

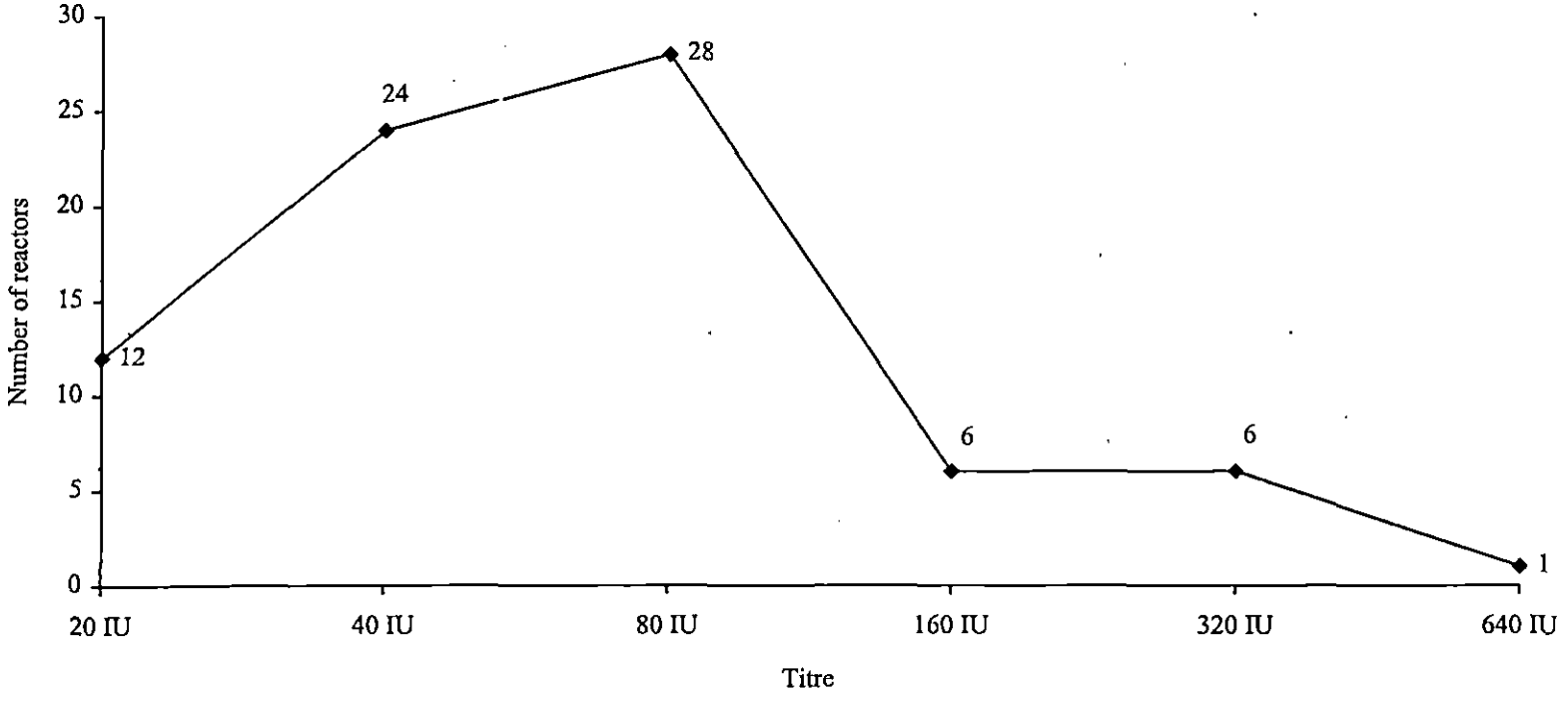

Fig. 8. 2 Mercaptoethanol test

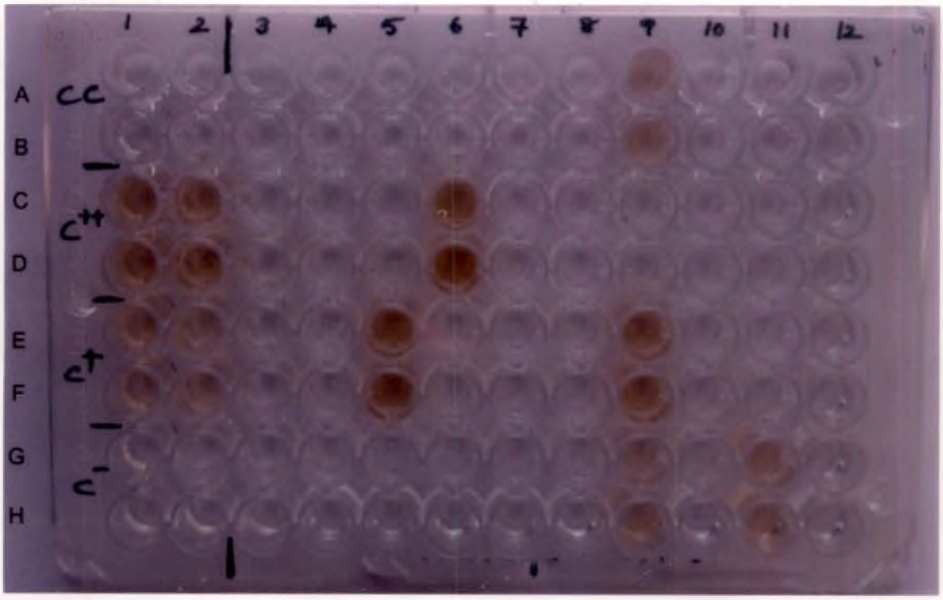

Plate 3. Avidin - Biotin Enzyme Linked Immunosorbent Assay (AB-ELISA) - Test Plate

Conjugate Control (Cc)	- A1, A2, B1 and B2
Strong Positive Control (C++)	- C1, C2, D1 and D2
Weak Positive Control (C+)	- E1, E2, F1 and f2
Negative Control (C-)	- G1, G2, H1 and H2
Test Sera Samples	- A3 to H12
Positive Sera Samples	- 5 (E5 and F5) - 6 (C6 and D6) - 9 (E9 and F9) - 9 (G9 and H9) - 11 (G11 and H11)

4.2.2 Standard Tube Agglutination Test

Of the 1602 sera samples subjected to STAT, 10 (0.62 per cent) samples showed a titre of 20 IU, 21 samples (1.31 per cent) showed a titre of 40 IU, 26 (1.62 per cent) samples had an agglutination titre of 80 IU, nine (0.56 per cent) samples were positive with a titre of 160 IU, four samples (0.24 per cent) showed a titre of 320 IU and one sample (0.06 per cent) showed a titre of 640 IU (Table 17, Fig. 7 and Plate 2).

4.2.3 2-Mercaptoethanol Test

A total of 12 (0.74 per cent) samples showed a titre of 20 IU, 24 (1.49 per cent) samples showed a titre of 40 IU, 28 samples (1.74 per cent) showed a titre of 80 IU, six samples (0.37 per cent) showed a titre of 160 and 320 IU each and serum sample from one animal (0.06 per cent) showed a titre of 640 IU (Table 18, Fig. 8).

4.2.4 Avidin-Biotin ELISA

Avidin-Biotin ELISA detected 245 positive reactors (15.29 per cent), out of 1602 samples (Plate 3).

4.2.5 Evaluation of Diagnostic Tests

Diagnostic tests were evaluated in terms of

(a) Sensitivity

(b) Specificity

(c) Predictive values and

(d) Accuracy

In this study Avidin-Biotin ELISA was used as the 'gold-standard' and other 3 tests were evaluated accordingly (Table 19).

Discussion

5. DISCUSSION

Brucellosis is a disease of worldwide occurrence in domestic as well as game animals which causes severe economic losses to the intensive and extensive animal production systems in the humid tropics. Hence reporting of animal and human brucellosis is of paramount importance in formulating strategies in the control of this zoonotic disease, which adds to the national brucellosis eradication programme. The prevalence of infection varies between herds and areas and the serosurvey spread over different geographical as well as agroclimatic areas would help in understanding the epidemiology and magnitude of the infections. The epidemiology of brucella species is complex and influenced by several technical and non-technical factors. Density of animal populations, herd size, type and breed of animal, the type of husbandry systems and other environmental factors are important determinants of infection dynamics. Serological tests form an important component of the programmes designed to eradicate bovine brucellosis. They are used to detect infected cattle in herds so that these animal may be culled and slaughtered. Eventhough voluminous literatures are available on various aspects of the disease in different species, the study on the epidemiology of bovine brucellosis in Kerala is scanty.

In the present study an attempt was made to document and classify the informations presently available on the epidemiology of bovine brucellosis for use as a foundation component of path analysis which can be recommended for the eradication programme. The overall seroprevalence of brucellosis among cattle and buffaloes among various districts of Kerala were assessed using RBPT, STAT, 2MET and ELISA and was found to be 15.29 per cent. The studies conducted by Baby and Paily (1979) and Vinod (1999) revealed a seroprevalence of 2.2 and 3.9 per cent respectively. A comparatively higher prevalence figures reported in the present study could be attributed to large area for which samples were collected, inclusion of significant number of samples from animals having reproductive problems and the use of a highly sensitive and specific serologic

test, ELISA. The present study concurs with findings of Dohoo *et al.* (1986), Bachh *et al.* (1988), Chandramohan *et al.* (1992) and Guarino *et al.* (2001).

5.1. EPIDEMIOLOGY

5.1.1 Seroprevalence of Brucellosis in Cattle and Buffaloes

Brucella abortus antibodies were detected from both cattle and buffaloes. Of this buffaloes showed a higher seroprevalence (28.35 per cent) than the cattle (14.72 per cent). Statistical analysis of the result using test for proportion revealed that there is no statistical significance between cattle and buffaloes in their seroprevalence to brucellosis. The results of the present study were found to be in agreement with the observations of earlier workers like Mathur *et al.* (1979), Suresh *et al.* (1993) and Mrunalini and Ramasastry (1999). Many workers like Kulkarni *et al.* (1991) and Sandhu *et al.* (2001) had reported that the seroprevalence was found to be more in cattle than buffaloes. But Rampal and Dwivedi (1992) reported that both cattle and buffaloes are equally susceptible to brucellosis.

A significantly higher prevalence rate (28.35 per cent) of brucellosis in buffaloes in this study can be attributed to the relatively smaller sample size (n = 67) compared to cattle (n = 1535). So also more than 80 per cent of the buffalo samples were collected from the slaughter house. A higher seroprevalence among animals brought for slaughter were observed by many workers (Baby and Paily 1979; Khire *et al.* 1998; Vinod 1999). Mostly old and unproductive animals are brought for slaughter and these animals were brought from neighbouring states, endemic for brucellosis without any quarantine measures (Baby and Paily 1979). These might be the contributing factors for the higher seroprevalence among buffaloes.

5.1.2 Sexwise Seroprevalance of Brucellosis in Cattle

Of the 349 male animals tested 44 animals were positive to *Brucella abortus* antibodies and out of the 1186 females tested 182 were serologically positive. In the present study higher seroprevalence was noticed in females. This is in agreement with the findings of Mathur *et al.* (1979), Suresh *et al.* (1993), Ghani *et al.* (1998), Silva *et al.* (2000). But this result are not in agreement with the findings of Bandey *et al.* (1989) who found that the incidence rate was higher in males than females. Zarfass and Friszscbe (1954) recorded a similar observation in sheep, where the infection rate is much higher in rams than ewes. However Baby and Paily (1979) observed that both sexes are equally affected by brucellosis. Stableforth (1959) stated that it is not possible to reach any satisfactory conclusion regarding relative sex susceptibility in bovines because males and females are kept under different managemental conditions.

Higher prevalence rate in females in this study can be due to the large number of samples from females and inclusion of significant number of samples from females with reproductive complaints. Higher prevalence rate in females can also be attributed to the increased susceptibility of brucella to the female genital tract.

5.1.3 Sexwise Seroprevalence of Brucellosis in Buffaloes

In buffaloes, the seroprevalence of brucellosis was comparatively higher in females (29.03 per cent) than males (27.77 per cent). Statistical analysis showed that there is significant difference between female and male buffaloes ($P<0.05$) in their susceptibility to brucellosis. Similar findings have been reported by Mathur *et al.* (1979); Suresh *et al.* (1993), Ghani *et al.* (1998), and Silva *et al.* (2000). Bandey *et al.* (1989) has reported a comparatively higher prevalence rate in males than females. Higher occurrence in females could be due to the fact that susceptible females may be artificially inseminated with semen from brucella infected bulls (Suresh *et al.*, 1993).

5.1.4 Agewise Seroprevalence of Brucellosis in Bulls

In the present study, the seroprevalence of brucellosis was studied among the bulls of three different age groups. The results indicated that the seroprevalence in bulls aged more than five years was significantly higher ($P<0.05$) than bulls less than two years and between two and five years of age. This observation concurs with findings of Mathur, *et al.* (1979) Kapoor *et al.* (1985), Masoumi, *et al.* (1992), Suresh *et al.* (1993), Ghani *et al.* (1998), Silva *et al.* (2000).

As expected the seropositivity is higher in bulls more than five years of age, because brucellosis is essentially a disease of mature animals (Silva *et al.*, 2000). Mathur *et al.*, (1979) opined, a lower prevalence rate in younger stock may be due to the less exposure to antigenic stimuli in younger stock. However, these results do not agree with the findings of Gangulee *et al.* (1967), Rampal and Dwivedi (1992) and Bandey *et al.* (1989), who reported that, there is no significant variation in prevalence among different age groups.

In this study a higher rate of brucellosis (23.17 per cent) was noticed in older bulls. As the bulls of this age group are used for breeding purpose, such bulls can act as potential spreaders of disease if they are used for artificial insemination (Radostitis, 2000). In younger bulls congenital transmission can also occur, as a result of *inutero* infection and the infection may persist, but may be serologically negative till they become sexually mature (Crawford *et al.*, 1986, Maiti *et al.*, 1999). However calves up to the time of breeding age are relatively less susceptible to the disease (Huddleson, 1943).

The diagnosis of the disease in bulls usually proves difficult due to the failures encountered during the isolation of the organism even from the seropositive bulls (Thapliyal, 1999). Artificial insemination with infected semen, may therefore cause enormous loss of health and productivity in a dairy herd.

5.1.5 Agewise Seroprevalence of Brucellosis between Cows and Heifers

The prevalence of brucellosis among cattle below two years, between 2 and five years and more than five years was 9.73 per cent, 6.78 per cent and 31.17 per cent respectively. Statistical analysis revealed a significant difference in cattle aged more than five years ($P<0.05$). A significantly higher seroprevalence was noticed among cattle of age group more than five years. Bachh *et al.* (1988) found out that the prevalence of brucellosis was found higher (53.49 per cent) in cattle above two years than in young stock below two years and they opined that the variation could be partly due to the relative number of animals in different age groups and the fact that young animals had not attained the puberty. Another reason that can be attributed may be because some animals might have been in incubation period which reveals themselves as seronegative (Radositis *et al.*, 2000).

Silva *et al.* (2000) reported that seroprevalence of bovids more than three years was twice as high as the younger age group because brucellosis is essentially a disease of sexually mature animals.

In the present study the seroprevalence of about (9.73 per cent) was noticed in cattle below two years, slightly higher than the cattle of two to five years. This may be due to the increased sample size of cattle of the age group two to five years.

5.1.6 Breedwise Seroprevalence of Brucellosis in Bulls

Breedwise seroprevalence of brucellosis among bulls were studied and it was found that pure bred Holstein Friesian bulls had higher seroprevalence of brucellosis than Jersey, Brown Swiss and Crossbreds.

No significant difference was observed among pure bred Jersey, Holstein Friesian, Brownswiss and crossbred bulls in their susceptibility to brucellosis. Suresh *et al.* (1993) also reported similar finding that Holstein Friesian was found

to be having higher incidence rate than native cattle. These findings do not agree with the finding of Kumar *et al.* (1974) who reported a higher prevalence rate in non-descript bulls than exotic breeding bulls and Omer *et al.* (2000) found that exotic pure breds are less susceptible to brucellosis.

Variation in seroprevalence to brucellosis among sheep was also reported by Bandey *et al.* (1989). In this study, the number of Brownswiss bulls and crossbreds were very less compared to Jersey and Holstein Friesian and the prevalence was also found to be lesser.

5.1.7 Breedwise Seroprevalence of Brucellosis among Female Cattle

The present study revealed that seroprevalence of brucellosis was more in crossbred Jersey females. Crossbreds were found to be more susceptible to brucellosis than non-descript cattle.

Crossbreds Jersey had higher seroprevalence (15 per cent) than crossbred HF, Brown-Swiss and non-descripts. This findings does not concur with that of Suresh *et al.* (1993) who also reported that crossbred HF had a significantly higher seropositivity than crossbred Jersey and native female cattle.

Statistical analysis of the result revealed that significant difference exists in crossbred Jersey female cattle in their seroprevealence to brucellosis ($P<0.05$). Lesser seroprevalence noticed in crossbred Brown-Swiss and non-descripts cows could be due to lesser sample size.

5.1.8 Seroprevalence of Brucellosis in Pregnant and Non-Pregnant Cattle

Brucella abortus antibodies were detected from pregnant and non-pregnant cattle and the seroprevalence was found to be higher in pregnant cattle (20.96 per cent). Ribiero *et al.* (1990) isolated *Brucella melitensis* biotype I from a pregnant doe. So the organism has special prediliction for the embryonic tissues of the maternal and fetal placenta as well as the foetus. When the animal becomes pregnant, the organisms invade the uterus from the mammary glands

during one of the bacteremic phases and multiply in the epithelium of chorionic villi. Erythritol, a carbohydrate produced by the foetus is capable of stimulating the growth of *Brucella abortus,* which occurs naturally in greatest concentration in the placental and foetal fluids and is responsible for localization of the infection in these tissues. A higher seroprevalence noticed in pregnant cattle has to be viewed seriously because congenital infection can occur in calves as reported by Fensterbank (1978), Crawford *et al.* (1986) and Radostitis *et al.* (2000). As a result of *inutero* infection, the infection may persist in a small proportion of calves which may also be serologically negative until after their first parturition or abortion.

The percentage seropositivity recorded by non-pregnant cattle in this study was (7.99 per cent). Non pregnant cattle can also become infected, but loose their humoral antibody to the organism much more quickly than cattle infected while pregnant. In the adult non-pregnant cows, localization occurs in the udder and uterus, if it becomes gravid, is infected from periodic bacteremic phases originating in the udder (Radostitis *et al.*, 2000).

Statistical analysis showed that there is significant difference in the susceptibility of pregnant cattle to brucellosis ($P<0.05$).

5.1.9 Seroprevalence of Brucellosis in Female Cattle According to Stage of Gestation

From the percentage of seroprevalence it was clear that as the stage of gestation advances, percentage of seropositivity also increases, and it was found to be statistically significant. Radostitis *et al.* (2000) and Silva *et al.* (2000) also reported similar findings.

The host mechanisms responsible for increased susceptibility to infection as the pregnancy advances are not known, but they may be related to the differential susceptibility of placental trophoblasts during the early, middle and late stages of pregnancy (Thoen *et al.*, 1986). Here the percentage seropositivity

during late gestation was significantly higher (60.34 per cent) because of the smaller sample size compared to the number of samples from animals in early and middle gestation.

5.1.10 Seroprevalence of Brucellosis in Cows According to Stage of Lactation

Of the three categories of animals like non-pregnant lactating, pregnant lactating and pregnant dry, a significantly higher percentage seropositivity (60.41 per cent) was found in pregnant dry animals. Statistical analysis revealed a significant difference in pregnant dry cows in their seroprevalence to brucellosis ($P<0.05$). This finding agrees with the observations of Radostitis *et al.* (2000) and Silva *et al.* (2000) who reported that the seropositivity was higher in pregnant dry animals. Pregnant dry cow, means the animal which in the late stage of gestation where there may be increased susceptibility for brucella organism to the placental trophoblast and also due to increased concentration of erythritol, which favours the growth of the bacteria. However this result does not agrees with the findings of Omer *et al.* (2000) that the seroprevalence is higher in pregnant lactating cows and non-pregnant lactating cows. In these two groups, there will be loss of humoral antibody to the organism much more quickly. The higher seroprevalence recorded by pregnant dry cows were due to the smaller sample size compared to other two groups.

5.1.11 Seroprevalence of Brucellosis in Female Cattle with Reproductive Disorders

Out of the total 159 animals with reproductive disorders, 51 animals were positive. The seroprevalence of brucellosis was observed in animals with history of late abortion, abortion and retention of placenta, abortion and repeat breeding, retention of placenta and repeat breeding alone. Result reveals statistical significance among animals with various reproductive disorders. Association of *Brucella abortus* with such reproductive disorders was also described by earlier

workers (Roberts, 1986; Bachh *et al.*, 1988; Das and Paranjappe, 1988; Chandramohan *et al.*, 1992 and Radostitis *et al.*, 2000).

Abortion occur in pregnant cows due to brucella infection especially in the third trimester of gestation. The host mechanisms responsible for increased susceptibility to infection as pregnancy advances are not clearly known, but they may be related to the differential susceptibility of placental trophoblasts during the middle and late stages of pregnancy (Thoen *et al.*, 1986). During pregnancy adjacent chorioallentoic trophoblasts become infected and support massive growth of bacteria. The presence of elevated amounts of erythritol in uterine tissues of cattle is another reason for the enhanced growth of bacteria within the uterus. A severe ulcerative endometritis of the intercotyledonary spaces occurs. The villi undergoes fatty degeneration and later fibrinopurulent exudates gradually detaches the connection of the villi with maternal placental cells. The bacilli may reach the foetus either by way of blood stream or through swallowing of infected amniotic fluid by the foetus and lesions are produced in the foetus. The detachment of foetal membranes from the maternal caruncle results in the gradual separation resulting in stoppage of blood supply to foetus which inturn dies. A dead foetus is a foreign body and is so expelled (Shastri, 1983).

One of the sequalae for brucellosis infection is retention of placenta. This occurs in cases where the disease is slowly progressing or in animals which have had an earlier abortion, the placenta is not shed because the connective tissue of the placenta proliferates and adhesion between the foetal and maternal placenta occurs.

Brucella abortus also has a role in causing infertility in a herd (Roberts, 1986). Brucella positive cows averaged 2.8 services per conception following normal calvings and 3.6 services per conception following abortion. One of the typical sign which leads to significant reduction in herd fertility is repeat breeding (Seifert, 1999). After recovery from an apparent or inapparent abortion, females may develop immunity. But because of permanent lesions in

reproductive system, as a consequence of metritis, this animal may remain as sterile. The immunity developed after an acute infection may remain as an unsterile immunity in females and thus the animals may become life long carriers.

5.1.12 Seroprevalence of Brucellosis among Cattle and Buffaloes Maintained Under Different Farming Systems

In the present study serological evidence of brucellosis was observed both in organised herds as well as in animals maintained by rural farmers (unorganised farming).

The percentage of seroprevalence was more in animals reared under rural farming system (18.42 per cent) than animals of organised farms (10.50 per cent). This is contradictory to earlier finding by several workers who has reported that seroprevalence was higher in organised farms (Omer *et al.*, 2000; Radostits *et al.*, 2000). On statistical analysis a significant difference was noticed in animals in the unorganised sector in their seroprevalence to brucellosis ($P<0.05$). However, Baby and Paily (1979) and Bandey *et al.* (1989) has reported that the prevalence of brucellosis was higher among the animals maintained in field condition.

The lower prevalence rate of the brucellosis under organised farming system may be due to efficient management practices and good sanitary conditions. Salman and Meyer (1984) opined the style of management as one of the factor influencing the epidemiology of brucella infections. The regular screening of animals and the segregation of positive reactors, quarantine measures, culling of unproductive animals, practice of artificial insemination, prompt veterinary care, use of maternity pens for calving can be considered as some of the factors that has contributed to lesser prevalence rate in organised farms.

In the current study, the inclusion of a significant number of samples from repeat breeder, aborted cows, and animals with various other reproductive

complaints from the field samples can also be one of the reason for a comparatively higher prevalence among the unorganised group and the higher prevalence rate of brucellosis among such animals was reported by Rampal and Dwivedi (1982).

5.1.13 Seroprevalence of Brucellosis in Cattle and Buffaloes in different Agroecological Regions

Seroprevalence of brucellosis was studied among animals of the two agro-ecological regions based on altitude. The results showed that animals belonged to high altitude region showed a comparatively higher prevalence rate (15.59 per cent) than the animals from type I region (11.33 per cent).

Statistical analysis showed that there is no significant difference in animals in high altitude region in their seroprevalence to brucellosis ($P>0.05$).

Animals grouped in Type II belonged to the animals from the districts of Idukki and Wayanad, >1000 m in altitude from the mean sea level.

This findings are in close agreement with the statements made by Polding (1950) and Sreenivasan (1972) that humidity, rainfall and lack of sunlight play an important role in transmission of brucellosis. In the highrange areas, there is lack of proper sunlight. The brucella organism are highly susceptible to sunlight. So the lack of proper sunlight may be one of the reason for the increased seroprevalence of brucellosis in cattle in the highrange areas.

Another reason can be due to contact of the cattle in the high altitude region with the game animals like bison, stags etc which are potential reservoirs of brucellosis (Radostits, 2000). These cattle also has chances of grazing in the common pastures with the game animals. Sharing of common pasture land and increased interaction with the game animals were cited as risk factors for brucellosis by former workers (Nicoletti, 1980; Ridler *et al.*, 2000; Kabagambe *et al.*, 2001).

5.1.14 Seroprevalence of Brucellosis in Cattle and Buffaloes in Different Zones

The serological evidence of brucellosis was recorded from south zone, central zone and north zones of Kerala. Based on the location, Kerala is divided to three zones of which north zone includes the districts like Malappuram, Kozhikode, Wayanad, Kannur and Kasargode. Idukki, Ernakulam, Thrissur and Palakkad districts come under central zone and south zone is composed of Thiruvananthapuram, Kollam, Pathanamthitta, Alappuzha and Kottayam. A high seropositivity was noticed in cattle and buffaloes from the north zone (24.41 per cent) among the 213 animals tested. All the samples collected from north zone were from the animals maintained by rural farmers and the seroprevalence was found higher in rural farming system (Bandey *et al.*, 1989), also many of the animals showed reproductive disorders like abortion, retention of placenta, repeat breeding. Such findings were also reported by Rampal and Dwivedi (1992). In the central zone most of the samples belonged to the animals maintained under organised farms and the sample size was comparatively higher (n = 1067). All these factors contributed to the moderate prevalence rate (11.71 per cent).

Eventhough the sample size was lower in south zone (n = 165), prevalence was also found to be lower (2.42 per cent), because the samples collected belonged to the animals maintained in organised farms with a smaller herd size. Similar findings were also reported by Omer *et al.* (2000) that prevalence of brucellosis will be higher when the herd size increases.

5.1.15 Districtwise Seroprevalence of Brucellosis in Kerala

Seroprevalence of brucellosis was recorded from 11 districts of Kerala. Seroprevalence ranged from zero per cent in Kottyam and Malappuram to 41.66 per cent in Kozhikkode with average value of 11.62 per cent. Number of samples collected from Kottayam and Malappuram was very low and the samples were from one organised farm. Good hygienic conditions and the efficient

management practices being followed in those farms may be the reasons for zero prevalence rate.

In Kozhikkode (41.66 per cent) and Kannur (36.92 per cent) all the samples belonged to the cattle maintained by rural farmers. A higher seroprevalence among cattle under rural farming system was reported by Baby and Paily (1979) and Bandey *et al.* (1989). In addition a significant number of samples were from those animals having reproductive abnormalities. Similar finding was also reported by Rampal and Dwivedi (1992).

In Idukki district the seroprevalence was recorded as 17.83 per cent. The Idukki district comes under the high range zone and reduced sunlight is one of the risk factor for brucellosis in that place. Polding (1950) also reported similar findings. In Palakkad district the seropositivity was 13.90 per cent. Eventhough the number samples collected from Idukki and Palakkad districts were high (n = 342 and 302 respectively) prevalence was moderately high. So also all the samples collected were from organised farms. In those farms the herd size and the density of animal population was higher, which can be stated as one of risk factor for transmission. This finding concurs with Thapliyal (1999) who reported that large sized herds and higher densities of animal population greatly facilitate the transmission of brucellosis. The animals in the farms located at Idukki district had more chances of coming in contact with game animals while sharing the pastures. This is in agreement with the findings of Nicoletti (1980), Ridler *et al.* (2000) and Kabagambe *et al.* (2001) who reported sharing of pastures and increased interaction with game animals as the risk factors for transmission. In districts like Thiruvananthapuram, Kollam, Ernakulam, Thrissur the prevalences were recorded as 3.44, 2.22, 2.50 and 5.48 per cent respectively. Here most of the samples were collected from animals belonging to organised farms. The efficient management systems and good hygienic practices may be one of the reason for lower prevalence rates in these districts. Eventhough the Wayanad district belonged to the highrange zone, the seroprevalence was comparatively less (4.83 per cent) as against the expectation.

Statistical analysis showed a significant difference exists in animals in Kozhikkode district in their seroprevalence to brucellosis ($P<0.05$).

5.2 DIAGNOSTIC TESTS

5.2.1 Rose Bengal Plate Test

In the present study 56 samples were detected positive by RBPT giving a sero positivity of 3.49 per cent. Similar findings were reported by Mrunalini and Ramasastry (1999). However this seropositivity is comparatively lesser than what reported by the former workers like Stephen *et al.* (1978), Tabaida and Abeledo (1979), Sevalgi *et al.* (1987), Suresh *et al.* (1993) and Hussain *et al.* (2000). The Rose Bengal Plate test antigen consists of *Brucella abortus* S 99 or 1119-3 cells stained with Rose Bengal and suspended in acidic buffer having a pH 3.65.

Successful control programmes rely on the use of simple screening tests of high sensitivity such as the RBPT followed by confirmatory serological test of high sensitivity and specificity such as ELISA. In the present study a high concordance was found between the high RBPT scores and ELISA. All the serum samples with RBPT score (+++) and (++), (+) were positive in ELISA too.

Here in this study, RBPT showed more number of positive reactors than STAT and 2MET but less than ELISA. Sutherland (1980) reported that this could be attributed to the acidified buffer used in Rose bengal antigen inhibits immunologically non-specific agglutinins and is a reliable test. However Khire *et al.* (1998) found that all the samples which were positive in RBPT were also positive in STAT. In a study conducted, using bovine sera samples, it was found that when RBPT positive sera were subjected to STAT and considering 80 IU as diagnostic STAT titre, all the sera samples tested by RBPT which scored ++ reaction was negative by STAT. Out of the 16 sera samples showing +++ RBPT reaction, 3 were found positive by STAT such variations in results of both of these tests can attributed to the molecular and functional difference of

immunoglobulins (Ali, *et al.*, 1985). Chung *et al.* (1980) opined that RBPT could detect both the IgM and IgG antibodies following vaccination. RBPT can be described as a simple, quick agglutination test which gives fewer false negative reactions and is good for diagnosing early stages of infection. (Seifert 1999, Kalorey *et al.*, 2000).

5.2.2 Standard Tube Agglutination Test

Standard tube agglutination test detected 48 samples (2.92 per cent) as positive out of the 1602 sera samples. Among the 1535 cattle sera samples, 40 samples were positive by STAT (2.60 per cent). Of the total 349 male cattle, 11 were positive by STAT (3.15 per cent). A titre of 40 IU was taken as positive in breeding bulls and 20 IU as doubtful. Here 4 samples out of the 11 belonged to breeding bulls and since the titre was 40 IU they were regarded as positive. Two of the breeding bulls tested came under doubtful reactor group with a titre of 20 IU.

A total number of 1186 female cattle were tested by STAT of which 29 samples were positive (2.44 per cent). Seventeen (1.01 per cent) were doubtful reactor and the rest were found to be negative to brucellosis since their serum titres were below 40 IU per ml.

Among 67 buffaloes, five samples from males (13.88 per cent) and three samples from females (9.67 per cent) were positive by STAT. None of the animals showed doubtful reaction. Here, in this study none of the males were showing any clinical symptom of brucellosis. But among the females, there were animals, which had previous histories of abortion, retention of placenta and repeat breeding. The highest titre shown by males and females was 1:40 and 1:320 respectively.

STAT has been more widely used than any other test in both man and animals and it was in fact, in the search for a more reliable and accurate diagnostic test for brucellosis that STAT was discovered (Stableforth, 1959). It is

still the method of choice for cattle and in all species a positive result is of value. Some times in species like sheep and goat even though positive results were shown in CFT, RBPT etc., STAT gives a negative result. The factors responsible for this are qualitative and are quite distinct from the quantitative differences in titres which exist owing to the use of different antigens and methods. The factors which influence the result of an agglutination test quantitatively includes, the bacterial content of the antigen, the sensitivity of the strain used, any roughness leading to increased titres and the nonspecific reaction, the presence of dissolved agar which increases sensitivity, temperature and duration of incubation, the presence of haemoglobin in the serum which causes non-specific reactions. As in any other serological tests for infectious diseases non-specific reactions are observed in the case of brucellosis which limit the accuracy of STAT. Vaccination of cattle with haemorrhagic septicaemia vaccine may increase the serum titre against *Brucella abortus* antigen (Pandey *et al.*, 1999). Also the cross reactions have been observed between smooth brucella species, and *Escherichia coli* 0:116, *Vibrio cholerae*, yersinia, salmonella and *Pseudomonas maltophilia* (Corbel, 1985). Other drawbacks with respect to STAT are the presence of prozone phenomena and producing doubtful or negative result with chronic infection. Even with these limitations STAT remains the most commonly employed diagnostic tool for bovine brucellosis.

5.2.3 2-Mercaptoethanol Test

The 2 MET employed in the present study detected sera samples from 36 animals as positive out of the 1602 samples (2.24 per cent). Among the 1535 cattle sera samples, 28 were positive by 2MET (1.8 per cent) and 8 samples out of the 67 buffalo samples were positive (2.60 per cent). Two samples which showed a titre of 40 IU in STAT showed a titre of 20 IU in 2MET, and sample which showed a titre of 160 IU showed a titre of 40 IU in 2 MET, and 1 sample which revealed negative in STAT showed a tire of 40 IU in 2MET. Two samples which showed a STAT titre of 160 IU reduced to 80 IU in 2 MET. Two samples

which was found to be negative in STAT showed a titre of 80 IU in 2 MET and 2 samples which was negative by STAT showed a titre of 320 IU by 2 MET.

The mercapto ethanol test is based on the observation that the activity of IgM antibodies is destroyed after the serum has been treated with 2-mercaptoethanol while the activity of IgG is not so affected. The reduction in agglutination titre of the samples in MET could be due to the treatment of serum with 2 MET, a sulph hydryl reducing agent, which dissociates IgM pentamer and reduces its agglutinating activity without affecting the IgG isotypes. Test gives fewer false positive reactions compared to STAT and RBPT. The test differentiates antibodies resulting from vaccination and those from infection because in vaccination the IgG antibodies appear later, reach lower values and disappear sooner than IgM antibodies and in infection IgG antibodies will persist longer and at high titres than IgM. 2 MET also detects chronic carrier animal since, although STAT titre may be low, the serum will contain predominantly or exclusively IgG antibodies.

5.2.4 Avidin-Biotin ELISA (A-B ELISA)

The Avidin-Biotin ELISA detected 245 positive reactors (15.29 per cent) out of the total 1602 sera samples tested. Of this 226 samples belong to cattle (14.7 per cent) and 19 samples belong to buffaloes (28.35 per cent).

Enzyme linked immunosorbent assay form the back bone of the battery of tests used in the study of infectious diseases and are often used to diagnose the causative agents and assess the extent and nature of diseases in the population. They help to provide highly sensitive and precise methods for the estimation of biological parameters with the added advantage of handling and analysis of large number of samples through automation. Indirect ELISA are mainly used for antibody detection. The Avidin-Biotin ELISA is another form of indirect ELISA and is considered more sensitive and specific because of the following reasons like, a single biotinylated antiglobulin molecule binds with three molecules of

Avidin-HRP thereby increases the sensitivity of the test, property of specific binding of avidin to biotin renders. A-B ELISA a highly specific assay, conjugation of HRP to avidin rather than to anti-globulin eliminates non-specific binding and very high dilutions of immunoconjugates (1:20,000) make the assay more economical. The overall specificity and sensitivity of A-B ELISA was reported to be 98 and 98.2 per cent respectively by Renukaradhya *et al.* (2001). The diagnostic specificity of indirect ELISA was found to be 99.6 per cent in brucellosis free buffalo herds and 68.33 per cent for samples from brucella infected herds as reported by Guarino *et al.* (2001).

The Lipopolysaccharide antigen is mainly used for indirect ELISAs. Shringi *et al.* (2002) reported that LPS antigen had better sensitivity in assaying brucella antibodies than protein antigen. The ELISA test has the advantage of giving clear cut results with anti-complementary and haemolysed sera and also gives a quantitative estimate of antibody concentration from a single dilution of serum. This test is not affected by prozone effects. Cho and Niilo (1987) found that ELISA could detect both IgG, and IgG2 subclasses of antibody and to a lesser extent other classes of immunoglobulins. Many workers like Lee *et al.* (1985), Cho and Niilo (1987), Chand *et al.* (1989), Saravi *et al.* (1995), Agarwal and Bhatra (1999), Shringi *et al.* (2002) reported ELISA as a test of high sensitivity and specificity compared to agglutination assays and CFT. In short the application of ELISA is simple and convenient but the requirement of good quality ELISA plates and ELISA reader has restricted its use to the laboratories having such facilities.

5.2.5 Evaluation of Diagnostic Tests

The diagnostic tests like RBPT, STAT and 2MET were evaluated using A-B ELISA as 'Gold standard' for sensitivity, specificity, predictive values and accuracy. Different workers like Philpott and Auko (1972), Tabaida and Abeledo (1979), Sutherland (1980), Stemshorn *et al.* (1984), Ghani (1995), Agarwal and Batra (1999), Amin *et al.* (2001) and Shringi *et al.* (2002) has commented on the

sensitivity and specificity of RBPT, STAT and 2MET. In the present study a high correspondence was found between the high RBPT scores and ELISA, all the serum samples with RBPT score +, ++, and +++ were positive in ELISA too. Thus RBPT showed more number of positive reactors than STAT and 2 MET. But all the samples positive in STAT, 2MET was also positive in RBPT. On analyzing the results, RBPT can be considered as a test of higher sensitivity (22.85) when compared with STAT and 2MET (19.59 and 14.69 per cent) respectively. But the specificity was found to be 100 per cent in all the three tests compared to ELISA. Stemshorn *et al.* (1984) had reported a specificity of 98.9 per cent for RBPT. This is in close agreement with the results of the present study. However, workers like Sutherland (1980), Das and Paranjape (1987) and Amin *et al.* (2001) opined that RBPT is a test of high sensitivity, eventhough the sensitivity of RBPT in the current study was 22.85 per cent. Regarding the predictive values and accuracy also RBPT ranked more than STAT and 2 MET. Hence from the present study it was concluded that RBPT can be used as a preliminary screening test and ELISA as a confirmatory diagnostic test.

Summary

6. SUMMARY

Brucellosis, caused by the members of the genus brucella is one of the economically important infectious diseases resulting in abortions and infertility in sexually mature animals. Seroprevalence studies showed that the disease is prevalent throughout the country.

The present study was envisaged to assess the seroprevalence of bovine brucellosis in Kerala and the comparative evaluation of different serological tests for screening large number of sera samples for the diagnosis of brucellosis. Samples were collected both from the organised farms and households, from the 11 districts of Kerala and also from the bovines slaughtered at Municipal slaughter house, Thrissur. Among the total 1602 sera samples, 1535 samples were from cattle and 67 samples were from buffaloes. All the 1602 samples were subjected to Rose bengal plate test, Standard tube agglutination test, 2 Mercaptoethanol test and Avidin-Biotin ELISA for detecting brucella antibodies. The epidemiological data regarding the sex, age, breed, gestation, lactation, reproductive complaints, system of management and location were also collected. The results were subjected to statistical analysis. The overall seroprevalence of brucellosis among cattle and buffaloes in Kerala was recorded as 15.29 per cent.

Out of the 1535 cattle, there were 349 male cattle and 1186 female cattle. Of the 67 buffaloes, 36 sera samples belonged to males and 31 were from females. The results of the present investigation showed that buffaloes had higher seroprevalence (28.35 per cent) than cattle (14.72 per cent). Among cattle and buffaloes, females showed higher seroprevalence than males. For the agewise analysis of the seroprevalence of brucellosis, cattle were categorized to three age groups viz. below two years, between two and five years and above five years of which bulls and female cattle above five years showed the highest seroprevalence, 23.17 and 31.17 per cent respectively and it was found to be statistically significant ($P<0.05$). Breeds included in the study were pure bred

Jersey, Holstein Friesian and Brownswiss and crossbreds among bulls and pure Holstein Friesian bulls showed a higher seroprevalence. Among females the breeds included were crossbreds of Jersey, Holstein Friesian and Brownswiss and the non-descript cattle. Crossbred Jersey cows and heifers showed the highest seroprevalence.

Pregnant cattle showed a higher seropositivity than non-pregnant cattle. As the stage of gestation advances, seropositivity also increased and the animals in the advanced stage of gestation had the highest seroprevalence (60.34 per cent) and a statistical significance was noticed ($P<0.05$). Animals were classified based on the stage of lactation, and the pregnant dry animals showed a significantly higher, prevalence rate (60.41 per cent) and was statistically significant ($P<0.05$). A total of 159 samples were collected from animals with various reproductive disorders like abortion, repeat breeding, metritis and retention of placenta. A high seroprevalence was detected in animals with the history of repeat breeding (47.5 per cent) and was found to be statistically significant ($P<0.05$).

Serological evidence of brucellosis was observed in organised herds as well as in animals maintained by rural farmers (unorganised farming). A significant difference was noticed between animals maintained under different management systems and the animals reared under unorganised system had a high seroprevalence of brucellosis compared to those maintained under organised farms. Based on the agroecological region, animals reared in high altitude region (including Idukki and Wayanad districts) showed higher seroprevalence of brucellosis (15.59 per cent) and it was found to be statistically significant ($P<0.05$). Zonewise analysis was also conducted classifying Thiruvananthapuram, Kollam, Pathanamthitta, Alappuzha and Kottayam districts as south zone, Idukki, Ernakulam, Thrissur and Palakkad districts as central zone and Malappuram, Kozhikode, Wayanad, Kannur and Kasargode districts under north zone. Animals belonged to north zone had a higher seroprevalence (24.41 per cent) sera samples collected from Kozhikkode district had a significantly higher seroprevalence (41.66 per cent) and was found to be statistically

significant ($P<0.05$). The overall seroprevalence of brucellosis among cattle and buffaloes was found to be 15.29 per cent.

The four diagnostic tests used for the study were RBPT, STAT and 2 MET and ELISA. The tests like RBPT, STAT and 2 MET were evaluated for sensitivity, specificity, predictive values and accuracy taking A-B ELISA as the 'gold standard'. It was found that all the three tests are having 100 per cent specificity and predictive value of positive test with that of A-B ELISA. RBPT was found to have highest sensitivity (22.85 per cent), predictive value of a negative test (87.77 per cent) and accuracy (88.20 per cent) than the STAT and 2MET. Hence from the present study it was concluded that RBPT can be used as a preliminary screening test and ELISA as a confirmatory diagnostic test.

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SEROEPIDEMIOLOGY OF BOVINE BRUCELLOSIS

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ABSTRACT

An investigation was carried out on 1602 cattle and buffaloes of Kerala including 1535 cattle and 67 buffaloes to assess the seroprevalence of brucellosis using serological tests like Rose Bengal plate test, Standard tube agglutination test, 2-Mercaptoethanol test and Avidin-Biotin ELISA. Sera samples were collected from bovines belonging to different farms, hospitals and households of Kerala covering eleven districts. Samples were collected randomly from animals of different sex, age, breed, gestation, lactation, reproductive performance, management practices and also from slaughter house. Out of the 1602 sera samples tested 15.29 per cent gave positive result for brucella antibodies. Of this cattle showed a seropositivity of 14.72 per cent and buffaloes showed 28.35 per cent seropositivity. Female cattle and female buffaloes showed higher seroprevalence than male cattle and male buffaloes. Bulls and cows aged more than five years showed the highest seropositivity. Among the breeds pure bred Holstein Friesian bulls and crossbred Jersey female cattle showed the highest seroprevalence. Seroprevalence of brucellosis was found higher in pregnant cattle, in late stage of gestation, in pregnant dry animals and also in cows and heifers with the history of repeat breeding. Animals maintained under unorganised farming system, animals from high altitude agroecological region showed more number of positive reactors. Northern zone of Kerala showed more number of positive reactors. Districtwise analysis revealed that animals belonging to Kozhikode district having highest seropositivity and animals from Kottayam and Malappuram districts as zero positives. RBPT, STAT and 2 MET was evaluated for the sensitivity, specificity, predictive values and accuracy using, A-B ELISA as the 'gold standard'. RBPT was found to have higher sensitivity, predictive value of a negative test and accuracy than STAT and 2 MET and all the three tests found to have 100 per cent specificity and predictive value of a positive test with that of ELISA. It was concluded that RBPT can be used as a preliminary screening test and ELISA as a confirmatory diagnostic test.