STUDIES ON EPIDEMIOLOGY AND SEROPREVALENCE OF *Brucella canis* INFECTION IN DOGS IN AND AROUND KOLKATA

A Thesis
submitted to the
West Bengal University of Animal and Fishery Sciences
in partial fulfilment of the requirements for the Degree of
Master of Veterinary Science
in
VETERINARY EPIDEMIOLOGY AND PREVENTIVE MEDICINE

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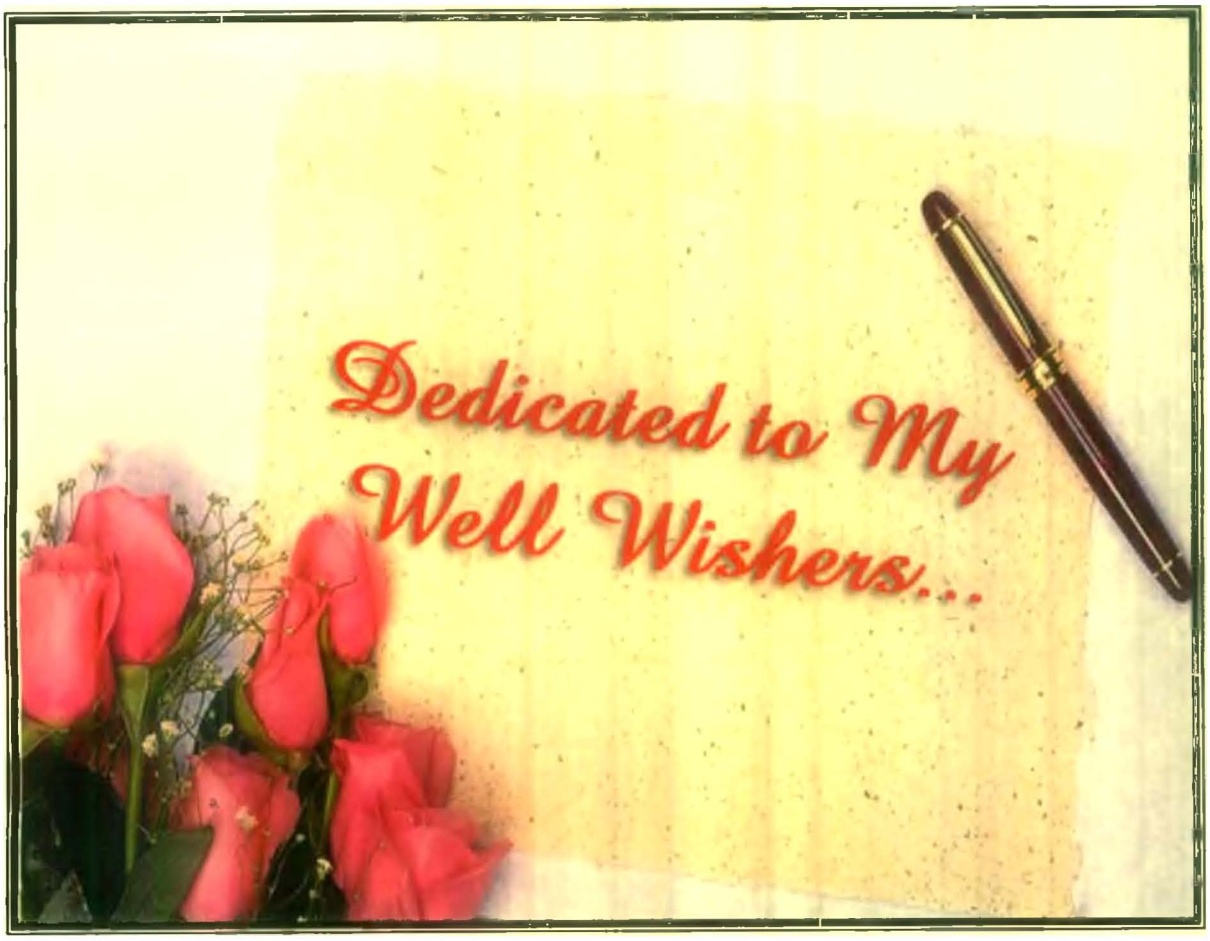
Dedicated to My
Well Wishers...

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Certificate

This is to certify that the work recorded in the thesis entitled **"STUDIES ON EPIDEMIOLOGY AND SEROPREVALENCE OF *Brucella canis* INFECTION IN DOGS IN AND AROUND KOLKATA "** submitted by **Dr. Bidhan Bandyopadhyay** in partial fulfilment of the requirements for the **Degree of Master of Veterinary Science in Veterinary Epidemiology and Preventive Medicine of the West Bengal University of Animal and Fishery Sciences**, is the faithful and bona-fide research work carried out under my personal supervision and guidance. The results of the investigation reported in the thesis have not so far been submitted for any other Degree or Diploma.

The assistance and help received during the course of investigation have been duly acknowledged.

Dated, Kolkata
The , 26th September, 2007

(Prof. C. Guha)
Chairman
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APPROVAL OF EXAMINERS FOR THE AWARD OF THE DEGREE OF MASTER OF VETERINARY SCIENCE IN VETERINARY EPIDEMIOLOGY AND PREVENTIVE MEDICINE

We, the undersigned, having been satisfied with the performance of Dr. Bidhan Bandyopadhyay, in the Viva-Voce Examination, conducted today, the 29th day of November, 2007, recommended that the thesis be accepted for the award of the Degree of Master of Veterinary Science in Veterinary Epidemiology and Preventive Medicine.

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:CONTENTS:

:LIST OF TABLE:

:LIST OF FIGURE:

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Bidhan Bandyopadhyay.

Dated, Kolkata **(DR. BIDHAN BANDYOPADHYAY)**

The 26th September, 2007

ABBREVIATIONS

ABC:	Animal Birth Control
cfu:	Colony forming unit
CSPCA:	Calcutta Society for Prevention of Cruelty to Animals
cm:	Centimetre
LPS:	Lipopolysaccharide
FA:	Fluorescent antibody
IVRI:	Indian Veterinary Research Institute
IAH & VB:	Institute of Animal Health and Veterinary Biologicals
µl:	Microlitre
Fig.:	Figure
gm(s):	Gram(s)
hr(s):	Hour(s)
Inc.:	Incorporated
mg:	Milligram
ml:	Millilitre
min(s):	Minute(s)
2-ME-TAT:	2-Mercapto ethanol tube agglutination test
No.:	Number

NGO:	Non Government Organisation
NSS:	Normal saline solution
PBS:	Phosphate buffered saline
pH:	Negative Logarithm of hydrogen-ion concentration
PI:	Post-infection
rpm:	Rotation per minute
RBPT:	Rose Bengal Plate test
RCF:	Relative centrifular force
STAT:	Standard tube agglutination test
SPBS:	Sorenson's phosphate-buffered
SAT:	Slide agglutination test
RSAT:	Rapid slide agglutination test
USA:	United States of America
USDA:	United States Department of Agriculture
^{0}C:	Degree Celsius
%:	Percent
WBUAFS:	West Bengal University of Animal and Fishery Sciences.

CHAPTER - 1

INTRODUCTION

INTRODUCTION

Dog has served as a faithful companion to man from ancient era. Probably, dog was the first animal to be domesticated on the earth, about 3400 years back, as king Yudhisthira, during Mahabharata era had a dog as pet. Dog as a pet is becoming more and more important in today's world.

The relationship between dog and man is not only confined / restricted to companionship, but was extensively used in hunting and rendering security as guard. In the present time they serve more important roles in providing security, crime investigation, dynamite tracking and also in defence services in most of the countries including India. In India, dog population was recorded 18.54, 17.95, 21.77, 25.28 and 27.59 million in the year 1982, 1987, 1992, 1997 and 2003 respectively, which reveals encouraging growth in each census except in the year 1987 (Anonymous, 2003).

Dog shows are being organized not only in different cities and state capitals in India; sub-divisional towns of most of the states are now organizing the same. Pet lovers are now increasing in the country, even in the rural areas. Increased demand of pups made the pet owners more interested to breed their stocks. At the same time pet product industry has been flourishing in the country, day by day. Moreover, in the changed scenario, Government of India has implemented some benefits to boost the pet-product industry which has been reflected in the last Union Budget for the first time in our country.

Brucellosis is one of the most important chronic contagious disease. It has a worldwide distribution with most wide public health and economic importance. It causes enormous economic loss to animal husbandry. Brucellosis is more common in countries that do not have effective public health and animal health programmes. The disease is caused by a small, Gram-negative coccobacillus, non-spore forming, non-motile, non-capsulated and facultative intracellular bacteria of the genus *Brucella* under the family Brucellaceae that can survive within a variety of

cells which is both phagocytic and non-phagocytic. Growth is somewhat slow and requires 2-3 days for colonies to mature.

Six species of *Brucella* have been reported viz. *B. abortus, B. melitensis, B. ovis, B. suis, B. neotomae and B. canis* affecting a wide range of vertebrates such as cattle, sheep, goats, pigs, dogs, as well as human beings. *B. neotomae* was isolated from the desert wood rat (*Neotoma lepida*) an animal inhabiting western parts of the USA. The first four species of *Brucella* described above occur normally in the smooth form. On the otherhand, *B. ovis* and *B. canis* have only been encountered in the rough form.

Human brucellosis usually occurs in association with animals or animal products and is uncommon in the developed countries. Undulant fever in man is caused by *B. melitensis, B. abortus and B. suis.* The potential of *Brucella* as a biological weapon is now widely used for bio-terrorism. The importance of the disease in humans is also an important justification for strengthening its eradication programme adopted in different countries.

B. canis causes a highly infectious form of brucellosis in dogs characterized by abortion in females and epididymitis, testicular atrophy and infertility in males. The disease is insidious and many dogs do not have prominent signs. Only dogs and wild canids have been found naturally susceptible to infection. Cats appear moderately susceptible and guinea pigs, mice, rats, non-human primates are susceptible to experimental infection, but, as in man, the infection is relatively mild. Rabbit is more susceptible than other laboratory animals. The three 'classical' *brucella* species (*B. abortus, B. suis and B. melitensis*) may also cause natural infection in dogs but occurrence is reported only sporadically and associated with close contact with infected herds (Morse, 1951; Deyoe, 1970; Palmer and Cheville, 1997).

B. canis was isolated and identified as an etiological agent of contagious abortion in dogs by (Carmichael, 1967). It is aerobic, growth is slow and requires about 48 hours to form mature colonies and it is inhibited by CO_2. The natural mucoidness of *B. canis* is a unique property. Within a few years of its initial identification, *B. canis* was recognized in different countries of the world. The organism was first isolated in India by Pilli *et al.* in 1991. Infection can start in any mucous membrane provided that

sufficient numbers of Brucellae are introduced. The organism is localized in the lymph nodes, spleen, bone marrow and reproductive tract of infected dogs. There is enlargement of lymph nodes, spleen and liver. Fever is uncommon due to lack of endotoxin.

In bitches, the principal sign is abortion. Abortion may occur at any time but generally takes place between 45-55th day of gestation although it may look healthy. Early embryonic deaths and abortion of infected conceptus have been observed, but they are often unnoticed and regarded as conception failures. Infections with *B. canis* do not interfere with normal estrous cycles, and most of the bitches may subsequently have normal litters. Some infected bitches do not abort and whelp both live and dead puppies within a single litter. Most live puppies die within a few hours or days, but those that survive have generalized enlargement of lymph nodes until they reach sexual maturity.

In males, the disease is characterized by poor reproductive performance and / or epididymitis. Other cardinal signs are atrophy of one or both testes and scrotal dermatitis. In infected males the volume of ejaculum is diminished remarkably with large numbers of abnormal sperm and inflammatory cells, especially within 3 months post infection. Chronically infected males may be azoospermic.

B. canis is infectious for humans but man is considered relatively resistant and the disease is mild as compared to infections caused by the three 'classical' *Brucella* species. Human infections have occurred as a result of laboratory accidents and of contact with infected dogs. The infected man shows a range of symptoms and may include malaise, sore throat, headaches, fever, chills and nausea.

Serological tests are important tools for the routine diagnosis of brucellosis though isolation and identification of organism is considered as the 'gold standard' test. However, it is not always possible to isolate the causal organisms from infected dogs. Review of different methods of serodiagnosis of canine brucellosis revealed that none of the procedure commonly used is adequate by itself. The modified mercaptoethanol tube agglutination test, the compliment fixation test, the gel-diffusion test are considered to provide satisfactory result in detecting infected dogs (Alton *et al.*, 1975).

In India, after the valued endeavour of Pilli and coworkers in 1991 a few studies have been made on this score till today. Hence, no systemic informations are available on prevalence of *B. canis* infection in dogs in India and Kolkata city too. Therefore the present study is undertaken with the following objectives –

i. Preparation of antigen of *B. canis* and its standardization.
ii. Raising of anti *Brucella canis* serum in dogs.
iii. Collection of sera samples from pet and stray dogs randomly under the study.
iv. Identification of seropositive dogs.
v. Isolation and identification of organisms will also be attempted from sero-positive dogs and dogs having the history of abortion, still birth, premature birth, infertility and other reproductive disorders.

CHAPTER - 2

REVIEW OF LITERATURE

Brucella canis causes a highly infectious form of brucellosis in dogs characterised by abortion in females and infertility, epididymitis, testicular atrophy in males. The disease is insidious, and characteristic of the disease is that it is not fatal but brings an end of its reproductive life. Besides *B. canis* infection, infection with the three 'classical' *Brucella* species (*B. abortus, B. suis* and *B. melitensis)* may also affect dogs. The infection in dogs caused by the three 'classical' species is self-limiting, and the organisms do not appear to persist or shed in the environment, (Nielsen and Duncan, 1990).

2.1. Seroprevalence of Canine Brucellosis:

2.1.1. Abroad:

In late 1960s several scientist reported the increased incidence of reproductive disorders in male and female dogs in the United States, particularly in large commercial breeding kennels and in field dogs, mainly beagles. *Brucella canis* was isolated and identified as an etiological agent of contagious abortion in dogs by Carmichael (1967). Within a few years of its identification, *B. canis* was recognized in Asian and African countries as Japan (Yamauchi *et al.,* 1974) and Madagascar (Verger *et al.,* 1975).

The disease was reported from different parts of the world and the causative organisms were isolated from different breeds kept in commercial kennel or roamed freely as strays.

Highest prevalence rates have been reported in Mexico and Peru (Englehardt, 1974).

In Tokyo, *B. canis* was found positive in 2 (2.89%) out of 69 stray dogs and in 2(4.65%) out of 43 dogs brought at the Tokyo Veterinary Clinic. *B. canis* was isolated from blood and prostrate gland (Ueda *et al.,* 1974).

Significant seroprevalence rates have been documented in Mexico, Central America and South America (Flores Castro and Segura, 1975; Englehardt 1974), especially where stray dogs are allowed to roam freely.

In a serological and bacteriological study of canine brucellosis in Mexico, 28% of 500 stray-dogs sera had agglutinin titers greater than 1:100. *Brucell canis* was isolated from the blood of 8 dogs (Flores Castro and Segura, 1975).

Seroprevalence of a population of Dogs in Georgia (USA) for *B. canis* infection using the slide agglutination test revealed that stray dogs had significantly higher titers than pet dogs (Brown *et al.*, 1976).

A serological survey was conducted in the German Federal Republic which revealed antibodies for *B. canis* at titres 1:50 – 1:1600 in 1190 (10.3%) clinically healthy beagles (Weber, 1976).

In another study of stray dogs in Mexico city documented a high rate (11.8%) of *B.canis* infection, 7 out of 59 stray dogs were culturally positive (Flores Castro *et al.*, 1977).

In France, serological tests on 250 dogs, including 180 samples from 4 kennels in Paris where infertility and abortion had occurred, of which 70 dogs with diseases of genital system, found negative (Menanteau, 1977).

In the first survey of brucellosis in dogs in the Philippines, serum samples were collected from 14 dogs in private kennels and 80 dogs examined at college of Veterinary Medicine, University of Philippines, 10 (10.63%) were found positive using tube agglutination test to *B. canis* infection (Baluyut and Dugui, 1977).

There was very limited information regarding the prevalence of *B. canis* infection in Sub-Saharan Africa. It has been reported in dogs owned by expatriates in Nigeria (Okoh *et al.*, 1978) and from South Africa (Schutte, 1977).

Nine hundred forty five dogs were tested for *B. canis* infection in Tokyo 27(2.9%) samples were positive using tube agglutination test, 11 being stray dogs and 16 non-strays (Saegusa *et al.*, 1978).

In another serological study on incidence of *B. canis* antibodies in pet dogs of German Federal Republic, 1000 random serum samples were examined, revealed only 2 (0.2%) positive cases (Weber and Schlisser, 1978).

B. canis infection has been documented in stray dogs in Moreno, Argentina. *B. canis* antibodies were detected in 30% of the 131 stray dogs using counter immuno elctrophoresis, gel-diffusion and mercapto

ethanol tube agglutination test, five of which yielded *B. canis* from the blood (Myers and Varela – Diaz, 1980).

Tsai and co-workers (1983) tested 1205 Serum samples for *B. canis* infection in 19 districts and 2 cities in Taiwan and found 132 (11%) were positive using tube agglutination test, and *B. canis* was isolated from 2 dogs.

Prevalence rates in the U.S. vary from approximately 1 to nearly 19%, with highest frequencies (~8%) observed in rural areas of the southern states (Carmichael, 1979; Pollock, 1979; Greene and George, 1984).

B. canis has not been reported in Australia and canine brucellosis was classified as an exotic disease (Medveczky and Crichton, 1986).

In Ankara, Turkey, 222 serum samples from 70 military service dogs, 64 stray dogs and 88 pets were examined. None of the military service dogs was positive to *B. canis* whereas 15.6% of stray dogs and 4.5% of pets were positive, using mercaptoethanol tube agglutination test (Diker *et al.*, 1987).

In Canada, 14 dogs obtained from 10 farms with brucellosis infected cattle herds, were found to be culturally positive for *B. abortus* suggesting a high rate of exposure and infection under field conditions (Forbes, 1990).

In Poland, antibodies against *B. abortus* and *B. ovis* were detected in 36 (9.57%) and 58 (15.42%), respectively, out of 376 canine sera collected from farm dogs and pets in urban areas (Iwaniak *et al.*, 1990).

In an epidemiological survey of *B. canis* infection of dogs in 259 dogs in the Towada area of Japan. Five (1.9%) samples were found positive with agglutination titres 1:160 or more and the causative organism was isolated from one stray dog, (katami *et al.*, 1991).

The high incidence of *B. canis* seropositivity recorded in Monte Gordo district, Bahia, Brazil. The results showed 40 (37%) positive out of 108 serum samples, using agar gel immunodiffusion test (Melo *et al.*, 1997).

Serological survey was conducted in between 1992 and 1993, and in 1996, in New Zealand. None of those samples was found positive, using ELISA (Gardner and Reichel, 1997).

Kiatisewee *et al.* (1997) reported presence of *B. canis* infection in 3 (1.68%) out of 178 dogs in Bangkok and Thailand, using 2-mercaptoethanol rapid slide agglutination test.

In Spain, Seroprevalence of *Brucella* infection in dogs was studied by rapid slide agglutination test and agar gel immunodiffusion test. In the regions of Catalogne 926 dogs and Castilla / Leon 308 dogs were tested for *B. canis* infection. *B. canis* had a seroprevalence of 4.5% in both regions. There was no difference in prevalence in rural and urban dogs (Mateu – de – Antonio *et al.*, 1998).

One hundred forty dogs, without clinical signs, were tested in Iran and 28 samples (20%) were positive for *B. abortus*, using Rose Bengal Plate test. The *B. abortus* serotype 4 was isolated from 12 dogs (Morshedi *et al.*, 1998).

In a serological survey in Ibadan, Nigeria between January and June, 1995, 3 (3.2%) out of 92 dogs were positive for *B. abortus* by the Rose Bengal Plate test and 4(4.35) by SAT. This is thought to be the first report of *B. abortus* infection in dogs in Nigeria, (Agunloye *et al.*, 1999).

Moon and co-workers (1999) reported presence of *B. canis* infection in Chonnam area of Korea in April 1994. Thirty three (53.25) out of 62 dogs were seropositive using 2-mercaptoethanol rapid slide agglutination test, agar gel immunodiffusion test and tube agglutination test.

Carvalho and co-workers (2000) investigated occurrence of *B. canis* and *B. abortus* in dogs of Para State, Brazil. Out of 236 samples of canine serum, 104 samples were collected from residential dogs, 40 samples from communal protection group, 62 samples from commercial kennels and 30 samples from six bovine breeding farms. It was observed that 45.34% of samples were positive for *B. canis* and 5.93% for *B. abortus*.

Blood samples from 200 randomly selected dogs in General Pico, Argentina revealed 14 samples were positive (7%) to *B. canis* infection using agar gel immunodiffusion test (Baruta *et al.*, 2001).

Park and associates (2001) reported 42.1% prevalence of canine brucellosis in dogs in Taegu city, Korea in 1999-2000, using tube agglutination test

The prevalence of *B. canis* infection in dog has also been recorded from Belo Horizonte, Minas Gerais, Brazil with 4.8% occurrence,

using agar gel immunodiffusion test. The prevalence was significantly higher in males compared with females (3.3% Vs. 1.6%). Sera from 151 Poodle dogs from 4 Kennels in Brazil were tested, revealed that the percentage of seropositive dogs in the 4 kennels varied from 4.6 to 57.1%. A high seroprevalence was associated with group housing and using stud dogs which also mated with nonkennel bitches, (Souza *et al.*, 2002).

Borie and co-workers (2002) have described reproductive traits of three *B. canis* seropositive dogs from Santiago, Chile.

Bulgaria is an area free from *Brucella* spp (*B. abortus*, *B. suis* and *B. melitensis)* but in some areas with a high density of population of wild pigs and stray dogs, *B. suis* biovar 2 and *B. canis* have been isolated (Taleski *et al.*, 2002).

In china, the infection rate of dogs with *B. canis* was reported to be 12% in South and Southeastern provinces and 4% in Northeastern and Northwestern provinces (Deqiu *et al.*, 2002).

In the year 2003, Nockler and co-workers isolated *B. canis* from ejaculate of a male dog after identifying of epididymitis and orchitis in Berlin, Germany.

Baek *et al.* (2003) in Korea reported that 3 dogs reared on a dairy farm with a high incidence for bovine brucellosis were positive for *B. abortus* infection.

2.1.2. India:

Presence of *B. canis* infection in dogs was first reported in India by Pillai *et al.* in 1991 from Chennai, Tamilnadu. A total of 640 sera samples (404 males and 236 females) were tested by 2-mercaptoethanol tube agglutination test with *B. cains* antigen revealed presence of infection in 14 samples (2.18%).

Srinivasan *et al.* (1992) reported the prevalence of canine brucellosis in urban and rural areas of Tamilnadu. Four hundred sixty serum samples from Dogs presented at Veterinary Hospital, Chennai and 261 samples from dogs in rural areas of Coimbatore, Madurai were taken in the study. In the urban area 9 (1.96%) and in the rural area 15 (5.75%) of the samples have positive, using 2-mercaptoethanol tube agglutination test (*B. canis*) and tube agglutination test (*B. abortus*) . In the urban areas the

samples reacted only to *B. canis* antigen, while in the rural area only 8 had *B. canis* infection and 7 were positive for *B. abortus*.

Studies on canine brucellosis in Punjab state and their public health significance were reported by Aulakh *et al.*, (1997).

Mrunalini and Ramasastry (1999) conducted a study between 1986 and 1996 in Andhra Pradesh, serum samples from 32 dogs were tested along with other domestic animals and none of the dogs was found positive for canine brucellosis.

Sharma (2004) conducted seroprevalence of *Brucella* infection in dogs, and also tried to find out the most suitable test to diagnose the disease.

2.2. Disease in Man:

Blood cultured from a 23 years old woman with fever, chills and pharyngitis yielded *B. canis* over a 7-day period. One of her dogs had positive blood cultures and was presumed to be the source of her infection. This was the first report of naturally acquired human infection. (Swenson *et al.*, 1972).

A study of 203 sera from hospital patients in Mexico City with undiagnosed febrile illness revealed a 13.3% rate of agglutination titers greater than 1:100. Virtually all patients had received antibiotic treatment, and *B. canis* could not be isolated from blood samples (Flores-Castro and Carmichael, 1986).

A study was conducted to determine the prevalence of *B. canis* antibodies in different human population groups based on their exposure to dogs, 5.7% of the newborn infants had shown maternal antibodies and 67.8% of persons with an average exposure to dogs had *B. canis* antibodies, with 62.1% prevalence in males and 72.4% prevalence in females respectively. Veterinarians had a much higher rate of infection (72.6%) than male blood donors (59.9%). Patients with pyrexia of unknown origin had significantly higher antibody titres to *B. canis* than all other patients (Monroe *et al.*, 1975).

In USA, House *et al.* (1975) observed 10, out of 415 human sera were positive serologically and also by cultural isolation. Godoy and coworkers (1979) reported infection of a research worker with *B. canis* in the laboratory. A serum agglutination titre of 1:100 gradually fell and finally

disappeared as the patient responded to treatment with Streptomycin and sulfadiazine.

In Argentina, *B. canis* was isolated (using haemoculture) from a veterinarian while palpating the uterus of a bitch, which had aborted, had a exposure through his teared glove. After 25 days the veterinarian suffered acute brucellosis with characteristic symptoms. A relapse occurred after 30 days of tetracycline treatment but ampicillin treatment for a further 21 days was followed by complete cure. It was also the first report of isolation of *B. canis* from man in Argentina (Ramacciotti, F., 1980).

In USA, *B. canis* was isolated from an aortic blood clot of an individual who died 3 years later due to some unrelated causes (Anonymous, 1974).

Moreover, *B. canis* infection in human was also reported by Wintermantel (1980) in Germany, Ramacciotti (1978) in Argentina, Fox and Kaufinann (1977) in USA.

2.3. Clinicopathologic findings:

Henderson *et al.* (1974) reported diskospondylitis in three dogs infected with *B. canis*. Similarly, Kerwin and co-workers (1992) performed a retrospective study of 135 dogs with diskospondylitis showed 14 dogs with concurrent *B. canis* infection in United States. Follow-up evaluation of 13 out of the 14 dogs showed complete remission of clinical signs in 9 of them. Radio-graphical follow-up evaluation in 6 dogs revealed active lesions despite complete remission of clinical abnormalities.

In a kennel of 45 Airedale Terriers diskospondylitis was detected by spinal radiography in 17 male and 14 female dogs in United States. However, serological evidence of *B. canis* infection was not detected in those dogs (Turnwald *et al.*, 1986).

Saegusa and associates (1977) reported ocular lesions in experimental canine brucellosis. During work on *Brucella canis* infection on dogs, 2-3 experimentally infected animals developed corneal opacity aqueous fluid from the anterior chamber was strongly positive to the agglutination test for *B. canis*. The organism was also recovered from the aqueous humour of one of the dogs. All were strongly positive to the blood agglutination test and the organism was recovered from the blood of 2 dogs.

2.4. Serological tests:

Several serological methods have been utilized for the diagnosis of canine brucellosis, but standard procedures that enjoy widespread acceptance have not been established as yet (Zoha and Carmichael, 1982; Carmichael *et al.*, 1984).

A unique characteristic of *B. canis* is that it is mucoid in nature and does not share the surface antigen of smooth brucellae. Therefore, the antigen used in serological testing for *B. abortus, B. suis and B. melitensis* will not detect antibodies against *B. canis* (Alton *et al.*, 1975). The lipopolysaccharide (LPS) endotoxin of smooth brucella organisms associated with agglutination is reduced, or lacking, in *B. canis* (Jones *et al.*, 1968; Diaz *et al.*, 1968). However, according to Carmichael and Bruner (1968) there is only slight cross-reaction between *B. canis* and *B. abortus* or *B. melitensis.*

The serodiagnosis of canine brucellosis presents difficulties for several reasons, but the principal one is extensive cross-reactivity between the rough cell envelope antigen of *B. canis* and hetero specific antibodies in the sera of normal, healthy dogs (Carmichael, 1968; Flores-Castro and Carmichael, 1981; Zoha and Carmichael, 1977; Nicoletti and Chase, 1987). False positive reactions with *B. canis* whole cell or envelope antigens may also occur with normal sera from other species. For example, agglutinins and precipitins to *Actinobacillus equuli* raised in rabbits (Carmichael, 1968) was found in normal equine sera (Nicoletti *et al.*, 1982) react with both homologous and *B. canis* antigens.

The advantage of close antigenic similarity between *B. canis* and *B. ovis* has been used in serological tests for *B. canis* (Myers *et al.*, 1972; George and Carmichael, 1978). In addition, using a less-mucoid (M-) variant/mutant of *B. canis* as antigen in serodiagnosis to reduce the percentage of false positive reactors has been documented (Carmichael and Joubert, 1978). Latter, different scientist used *B. ovis, B. canis* (M- Strain RM 6/66) cells as antigen for serodiagnosis of *B. canis* infection in dogs to reduce the false positive reactors (Melo *et al.*, 1997; Necsulescu *et al.*, 1995; Azevedo *et al.*, 2004).

2.4.1. Agglutination Tests:

2.4.1.1. Rose Bengal Plate Test (RBPT):

It is a plate agglutination test in which sample sera are examined at a single dilution. The test was introduced by U.S.D.A. and accepted as the official screening test for brucellosis in animals in most of the countries. FAO/WHO expert committee on brucellosis (1986) recommended RBPT as a screening test with positive sera tested by CFT for confirmation in bovine brucellosis. Nicoletti (1967) described the test as a better indicator of infection than STAT, under field condition. Moreover, results of RBPT have been found to correlate well with the results of CFT (Morgan *et al.,* 1969). Necsulescu *et al.,* (1995) also recommended RBPT for serodiagnosis of *B. canis* infection. The preparation and standardization of a Rose Bengal plate-test antigen for the rapid detection of *B. canis* antibodies is described by George and Carmichael (1978).

2.4.1.2. Standard Tube Agglutination Test (STAT):

Standard tube agglutination test was used as the first serological test for brucellosis (Wright and Smith, 1897) and since then it has been used extensively in most of the countries for diagnosis of brucellosis in man and animals. Though it has been reported as the useful serological test for diagnosis of *B. canis* infection. The IgM isotype of antibody is the most active agglutinin at a neutral or slightly acidic pH (Rice and Boyes, 1971; Corbel, 1972; Nielson *et al.,* `984). Therefore the tube agglutination test is susceptible to false positive reaction by cross-reacting antibody. To overcome the problem, modifications were made to destroy or inactivate IgM agglutinins.

2.4.1.3. 2-Mercaptoethanol-Tube Agglutination Test (2-ME-TAT):

2-Mercaptoethanol (Anderson *et al.,* 1964; Rose and Roepke, 1964) is the reducing agent that reduces disulfide bridges. It reduces IgM into monomeric units thereby reducing its ability to agglutinate. 2-ME is added to serum diluent before mixing it with working antigen.

2-ME-TAT provides satisfactory results in detecting infected dogs (Alton *et al.,* 1975). Nicoletti (1969) also described the test as confirmatory test for serodiagnosis of *B. canis* infection.

The reaction is classified 'negative' at 1:50 titre, 'Suspicious' if incomplete or complete reaction at 1:50 or 1:100 dilution or incomplete at

1:200 dilution and 'positive' when complete agglutination occurs at 1:200 dilution or higher (Alton *et al.,* 1975). Nicoletti and Chase (1987) described 2ME-TAT as a valuable test to evaluate responses to antibiotic therapy.

2.4.1.4. 2-Mercaptoethanol Rapid Slide Agglutination Test (2-ME-RSAT):

Since existence of differences in the sensitivity and specificity between 2-ME-TAT and slide test results (Carmichael *et al.,* 1984; Nicoletti and Chase, 1987), the 2-ME-RSAT is considered the preferred test for initial screening. Some modification of the test (2-ME-RSAT) has further reduced the rate of false positive reactions (Carmichael and Joubert, 1987).

The conventional methods (Carmichael *et al.,* 1989) used for the serodiagnosis of canine brucellosis are the 2-ME-RSAT using either *B. ovis* (George and Carmichael, 1978; Badakhsh *et al.,* 1982) or a *B. canis* (M-) mutant (Carmichael and Joubert, 1987) as antigen and the TAT, with or without the addition of 2ME (Alton *et al.,* 1975).

2.5. Isolation and Identification:

Isolation and identification is the only method establishing a definitive diagnosis. *B. canis* has been isolated from vaginal discharges, aborted foetuses, milk, urine, semen and various tissues (Alton *et al.,* 1975). Vaginal discharges may contain up to 10^{10} organisms per milliliter (Carmichael and Joubert, 1988) and shedding by this route may continue for several weeks following an abortion (Nielsen and Duncan, 1990). Seminal fluid and urine from dogs that harbour the organisms in the prostate glands and epididymides have been determined as important sources of infection (Serikawa and Muraguchi, 1979; George *et al.,* 1979; Moore and Kakuk, 1969). The rate of *B. canis* isolation from the semen of infected males is high for the initial 6-8 weeks of infection, but intermittent shedding of the organism in low numbers has been observed up to 60 weeks PI (George *et al.,* 1979). Concentration of 10^3 to10^6 organisms per milliliter of urine have been observed in males, with lesser numbers of bacteria in females (Serikawa and Muraguchi, 1979).

However, blood is the bacteriological specimen of choice as infected dogs are bacteraemic for long periods of time, though infected dogs remain serologically positive for several months after becoming abacteraemic (Alton *et al.,* 1975).

CHAPTER - 3

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1. Collection of samples for serodiagnosis:

The epidemiological study was undertaken on the serum samples of dogs collected from canine population in and around Kolkata from different sources.

The samples were collected from pet dogs brought to Dog Ward, Faculty of Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata; Referral Veterinary Polyclinic, Govt. of West Bengal, Barasat, 24-Parganas (North); Veterinary Clinics of South Kolkata. Some serum samples were also collected from dogs owned by a few dog breeders of Kolkata.

Serum samples were collected from stray dogs fetched by the NGOs from different wards of the Kolkata Municipal Corporation, the Howrah Municipal Corporation and rural areas of nearby districts of Kolkata. These dogs were kept at their kennels usually for 5-6 days for Animal Birth Control (ABC) programme. During the period of their stay, the samples were collected randomly irrespective of their age, and reproductive disorders.

About 3 ml of blood was collected slowly from the radial vein in a sterile syringe with hypodermic needle of medium gauge (No. 21G) in order to avoid haemolysis and transferred into a sterile test tube. After collection, the blood samples were kept in slanted position for two hours at room temperature. The samples were then kept overnight at 4^0C in a refrigerator.

3.1.1. Separation of Sera:

The serum samples were separated in sterile microcentrifuge tubes under aseptic condition. Then the serum samples were subjected to centrifugation at 3000 rpm for 15 minutes at room temperature and the supernatant was further transferred in sterile microcentrifuge tubes under aseptic condition.

3.1.2. Inactivation of sera and preservation:

The sera samples were inactivated by heating for 30 minutes at 56^0C in a water bath. Finally the sera samples were preserved without adding any preservative at -20^0C until used.

3.2. Collection of samples for isolation and identification of B. canis:

Blood samples from seropositive dogs and other specimens from suspected clinical cases were collected using procedures as described by Alton *et al.* (1975).

3.3. Designing proforma:

In order to acquire the required information from dog owners, a proforma was prepared taking care that necessary information could be gathered (annexeure-III).

3.4. Laboratory materials:

3.4.1. Biological Materials:

3.4.1.1. Bacterial culture:

Brucella canis MEX 51 Strain was procured from Division of Standardisation, IVRI, Izatnagar (India).

Brucella abortus S99 Strain was used during the study at I.A.H. & V.B., Kolkata, maintained by them. This strain was used for comparative study during purity test for biochemical characters of the culture *(Brucella canis* MEX 51).

3.4.1.2. RBPT (*B. abortus)* antigen:

The RBPT (*B. abortus)* antigen was procured from I.A.H. & V.B., Kolkata (India).

3.4.1.3. *Brucella canis* positive control serum:

The *Brucella canis* positive control serum was procured from VMRD, USA.

3.4.1.4. *Brucella abortus* Positive serum:

The *B. abortus* positive serum was procured from Division of Biological Products, IVRI, Izatnagar (INDIA).

3.4.2. Media, Buffers and Reagents:

The composition of the media, buffers and reagents used in the present study has been discussed in the annexeure-I.

3.4.3. Freund's incomplete adjuvant:

The freund's incomplete adjuvant for raising of anti *B. canis* serum in dogs was collected from Sigma-Aldrich, Inc.

3.4.4. Chemicals:

All the chemicals used in the present study were of analytical reagent (AR) or molecular biology grade and were procured from sigma, Glaxo, Himedia, Merck, SRL.

3.5. Purity test for biochemical characters of the culture (*B. canis* MEX 51 Strain):

Before use, the culture was tested for purity and biochemical characters.

3.5.1. Gram Staining:

The organisms were found small gram-negative coccobacillus like other brucellae.

3.5.2. Colony Character:

Translucent, mucoid colonies (1-1.5 mm. in diameter) were observed on tryptose agar plates after incubation at 37^0 C for 48 hours without carbon dioxide (FIG.-1).

3.5.3. Production of hydrogen sulfide:

No change of colour of the lead acetate paper (annexure-I) was observed in the tube on liver agar slopes after inoculation and incubation for the stipulated period of time without carbon dioxide (FIG-2).

3.5.4. Urease activity:

The slopes (using Christensen's medium) were inoculated with a loopful of culture and kept at room temperature. Within 2-3 minutes yellow coloured medium became purple-pink (FIG-3).

3.5.5. Growth in presence of dyes:

Tryptose agar plates were used with different concentration of the thionin and basic fuchsin (annexure-I) as per Alton *et al.,* (1975). In case of thionin growth was observed (after incubation at 37^0 C for 48hrs. without carbon dioxide) in all concentrations i.e. 1:25,000, 1:50,000 and 1:1,00,000 whereas in presence of basic fuchsin growth was detected only in 1:1,00,000 concertration but not in 1:50,000 (1:25,000 concentration was not tried, as per Alton *et al.1975.*

3.5.6. Agglutination of sera used as positive control:

No agglutination was observed with *Brucella abortus* positive serum on slide test. A negligible agglutination was observed with *Brucella canis* positive control serum (VMRD).

3.6. Preparation of antigen:

After conducting the purity test, the *B. canis* Mex 51 strain was used for antigen preparation.

3.6.1. RBPT (*B. canis*) antigen:

The RBPT antigen using *B. canis* cells was prepared with a slight modification of the method described by Alton *et al.*, (1975).

The seed culture was prepared from *B. canis* Mex 51 strain on tryptose agar slope, harvested by adding 2.5ml. of sterile phosphate buffered saline. The tube was rolled between the palms of the hand until the brucellae were all suspended. The suspension in each tube was transferred aseptically to each Roux flask of tryptose agar. Each Roux flask was placed horizontally with the medium towards the bottom for 2 hrs. and tilted backwards and forwards so that the suspension was uniformly distributed over the surface of the media. The flasks were then incubated at 37^0 C for 24 hrs (without carbon dioxide) in an inverted position.

After incubation, the flasks were examined individually and placed vertically. The fluid contained in each flask was aspirated aseptically in a beaker containing disinfectant. For harvesting 10ml of phenol-saline was added to each flask aseptically and flasks were left for about 1 hour horizontally with the culture wash towards the bottom. After which each flask was gently agitated until the mucoid brucellae were suspended. The harvests from the flasks were pooled and filtered aseptically through sterile absorbent cotton covered by a piece of fine cloth. The brew was then centrifuged at 10000g for 30 minutes at 4^0 C after heat inactivation at 70^0 C for 1 hour in a water bath and tested for sterility. The supernatant was discarded and phenol-saline (annexure-I) was added to bring the volume to 22.5 ml per gram of packed cell weight. One millitre of Rose Bengal solution was added per 35ml of suspension and then put a magnetic stirrer for 2 hours. The stained brucellae were then separated from the suspension by centrifugation in a refrigerated centrifuge at 10,000 g for 30 minutes at 4^0 C. Seven millilitre of buffered diluent was added to 1gm of stained brucellae were suspended and mixed by shaking vigorously and pH was adjusted to 3.65±0.05, and kept in vials and stored at 4^{0}C.

3.6.2. Antigen for 2-ME-TAT:

The stock reagents and working reagents for the modified mercaptoethanol tube agglutination test were prepared as described by Alton *et al.*, (1975).

3.6.2.1. Preparation of stock reagents:

Preparation of stock solution of formalized saline, stock solution of sodium chloride, sorenson's phosphate-buffered saline have been described in the annexure II.

Stock antigen was prepared (with a slight modification) with the seed material of *B. canis* Mex 51 strain on tryptose agar slope and harvested with 2.5 ml of sterile 0.85% saline solution. The Roux flasks of tryptose agar were inoculated, incubated as described earlier. Ten millilitre of sorenson's phosphate-buffered saline containing 0.06% of formalin (annexure-II) was added to each flask for harvesting and finally the harvests from the flasks were pooled, filtered, heat inactivated (70^{0}C for 1 hour) and centrifuged (10,000g for 30 minutes at 4^{0}C) as described earlier after sterility test. The supernatant was discarded and the pellet was resuspended in sorenson's phosphate-buffered saline containing 0.5% formalin (annexure-II). The antigen was standardized to a density of 4.5% of cells by using the packed cell volume method in graduated tubes. The stock antigen was kept in vial and stored at 4^{0}C.

3.6.2.2. Preparation of working reagents:

Working antigen and test diluent were prepared as described by Alton *et al.*, (1975) and are given in the annexure-II.

3.7. Anti *Brucella Canis* serum was raised in dogs:

Anti *B. canis* serum was raised in dogs as described by Sharma (2004) with slight modification.

3.7.1. Preparation of Inoculum:

After propagation of seed material in Roux flasks, the harvests were pooled, filtered and heat inactivated at 70^0 C for 1 hour and then passed for sterility test. Sonication was performed in Ultrasonic homogeniser (Labsonic® U, Biotech International) using a titanium probe operated at 150 watt and 0^{0}C temperature (keeping in ice pack) repeatedly (for 15 cycles) for 1 min with an interval of 30 seconds in each cycle. Day-wise schedule of inoculation and collection of serum were as follows.

3.7.2. Inoculation schedule and collection of blood for raising of anti *B. canis* serum in dogs:

On 0 day two healthy mongrel dogs were taken and 2ml of blood was collected from radial vein and after separation of serum, plate agglutination test was performed with the RBPT antigen (B. abortus) and RBPT B. canis antigen. Dogs were inoculated subcutaneously with inactivated and sonicated 1ml mass containing 4.5% cells plus 1ml Freund's incomplete adjuvant (Sigma-Aldrich, USA).

On 14th day booster was given to each dog with the same inoculum and same route of inoculation.

On 21st day 2ml of blood was collected from each dog and were tested for desired titer.

On 22nd day after observing the satisfactory titer, 10ml of blood was collected from each dog and sera were separated.

3.8. Agglutination test of the prepared antigen:

The RBPT antigen (*B. canis*) and antigen (*B. canis*) for the modified mercaptoethanol tube agglutination test were tested with *B. canis* positive control serum (VMRD, USA).

3.9. Testing protocol of the sera sample:

3.9.1. Rose Bengal Plate Test (RBPT):

All sera samples were tested with two RBPT antigens (*B. canis* and *B. abortus*). 30µl of serum from each sample was taken and placed on glass testing plate of the plate agglutination box. Equal quantity of antigen (*B. canis*) was placed on the serum and both were mixed with a sterile toothpick separately. After 4 minutes the results were read. Similar test procedure was followed with *B. abortus* RBPT antigen.

3.9.2. 2-Mercaptoethanol Tube Agglutination Test (2-ME-TAT):

All sera samples were tested and the test was performed as described by Alton *at al.*, (1975).

- Three test tubes were taken for each sample and were arranged in a rack.
- 40µl (1:50 dilution), 20µl (1:100 dilution) and 10µl (1:200 dilution) serum was taken in respective test tubes.
- 1ml of test diluent was added to each test tube.

- Then 1ml of working antigen was added to each test tube.
- Tubes were shaken for properly and incubated at 37°C for 48 hours.
- The reading was accomplished by determining the degree of clearing of the supernatant.
- Interpretation –
  a. Negative – negative reaction at the 1:50 dilution
  b. Suspicious – incomplete or complete reaction at the 1:50 Or 1:100 dilutions or incomplete reaction at the 1:200 dilution
  c. Positive – Complete reactions at the 1:200 dilution

3.10. Isolation and identification:

3.10.1. Haemoculture from seropositive dogs:

Isolation of the causative organisms have been attempted from a few seropositive dogs subject to availability and feasibility for further collection of blood sample for the same and were followed with slight modification as described by Alton *et al.*, (1975). About 8ml of blood was collected from radial vein with a sterile syringe and 3ml was put into a sterile test tube containing heparin. The remaining 5ml (without anticoagulant) was put into a culture tube contaning 10ml of tryptose broth and was mixed and then frozen overnight at -20°C.

3.10.1.1. Direct culturing of blood:

This method provides results in a shorter time (in 4-7 days), but the method of subculturing from broth is more sensitive.

0.1ml of heparinized blood was delivered with a 1.0ml sterile pipette on to each tryptose agar plate and spread with a spreader and incubated at 37°C (without carbon dioxide) for 4-7 days. The plates were examined for presence of typical colony. Selected colonies were tested for characterization as described by Alton *et al.*, (1975).

3.10.1.2. Plating from broth:

After the overnight freezing, 10ml broth containing 5ml of blood was incubated at 37°C (without carbon dioxide) for 6-7 days. If *B. canis* was not isolated by direct culturing of blood, 0.1ml of blood-broth mixture was inoculated on to plates of tryptose agar medium. The inoculated plates were incubated and examined as earlier.

3.10.2. Samples attempted for culture other than blood:

This was performed as per the procedures with slight modification as described by Alton *et al.,* (1975). Selections of specimens were restricted within vaginal swab.

The vaginal swab was collected aseptically with sterile swab stick and was carried to the laboratory in a ice pack. The swabs were rubbed directly or diluted in small amount of PBS and inoculated into the medium containing tryptose agar under sterile condition.

The inoculated plates were incubated and examined as done earlier.

Fig – 1: Significant colonies of *Brucella canis* on tryptose agar plate.

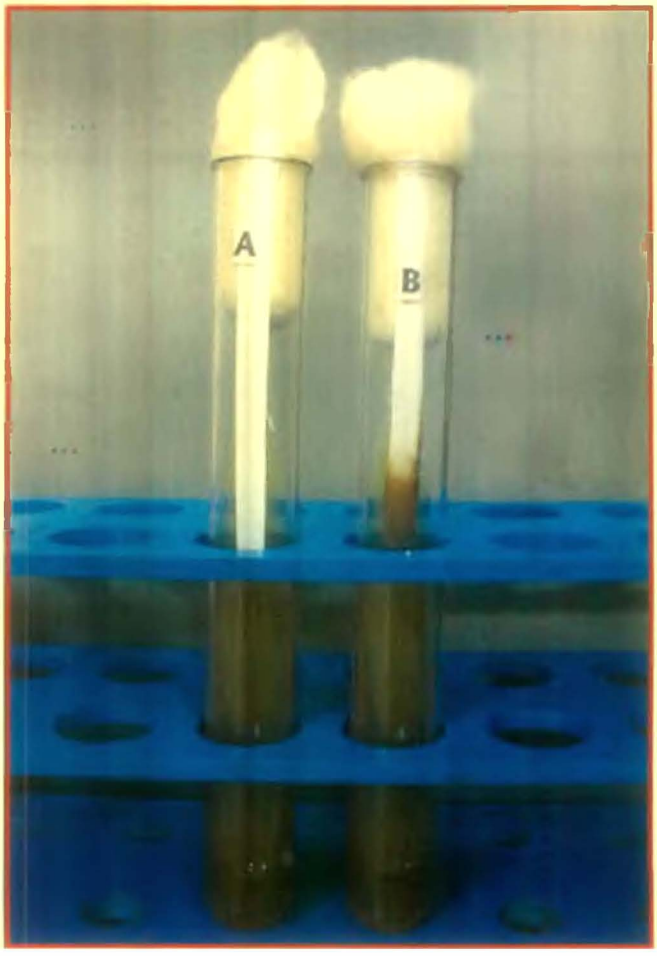

Fig – 2: Hydrogen sulfide production test-
Tube A showing no change of colour of the lead acetate paper, characteristic of *Brucella canis*.
Tube B Showing change of colour of the lead acetate paper, characteristic of *Brucella abortus*.

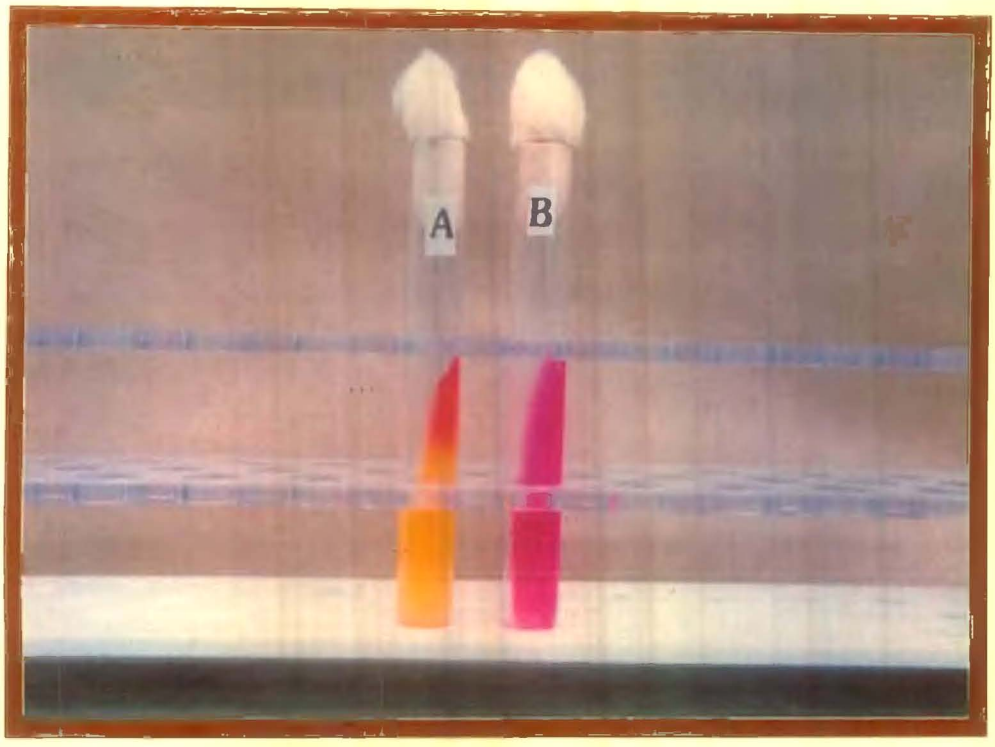

Fig – 3: Urease activity, Tube A showing change of colour after 2 minutes, Tube B showing the same after 30 minutes.

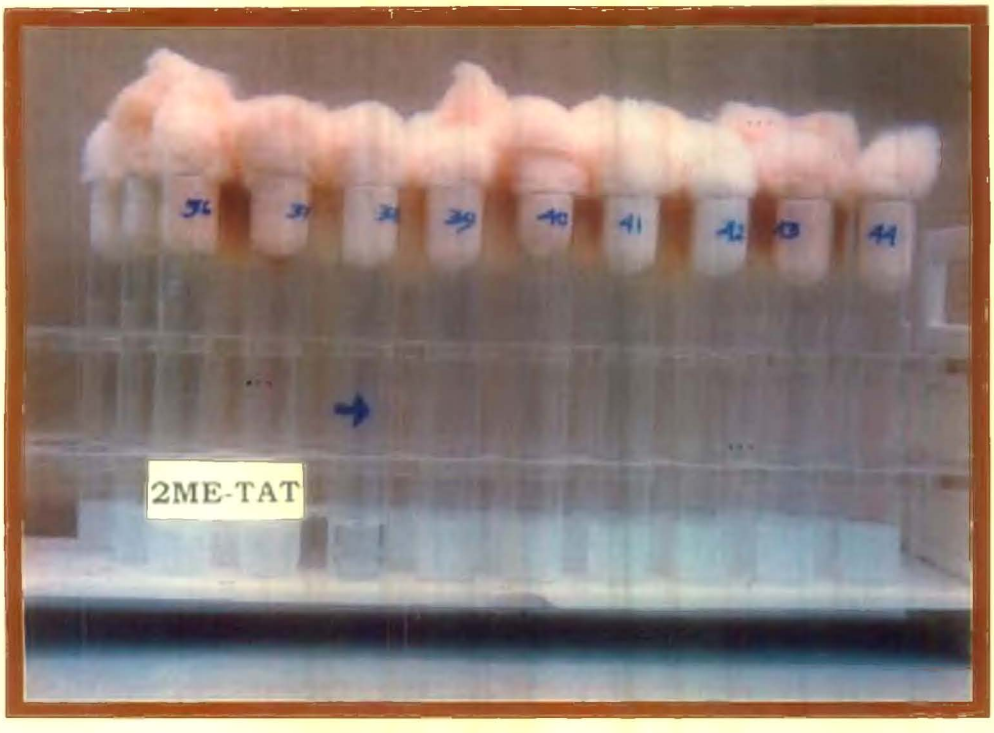

Fig – 4: 2-Mercaptoethanol Tube Agglutination Test, arrow mark indicates positive reaction.

CHAPTER - 4

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

4.1. Agglutination test performed with the known positive sera with the antigens prepared from *B. canis* :

4.1.1. *B. canis* positive control serum (VMRD, USA):

The RBPT antigen (*B. canis*) and antigen (*B.canis*) for the modified mercaptoethanol tube agglutination test were tested with *B. canis* positive control serum (VMRD, USA) showed negligible, suspicious reaction respectively.

4.1.2. Antisera raised against *B. canis* in dogs:

Both the antigens prepared showed positive reaction with the raised anti *B. canis* serum in dogs.

4.1.3. With *B. abortus* positive serum:

The antigens prepared (for RBPT and 2-ME-TAT) showed negative reaction with *B. abortus* positive serum procured from IVRI, which is in accordance with Alton *et al.,* (1975).

4.2. Distribution of serum samples according to source, breed, age, sex and reproductive disorders:

The present study was conducted among pet and stray dogs in and around Kolkata.

4.2.1. Blood Samples collected from different sources:

Samples collected from different sources have been divided as pet dogs, of different breeds and age, sex, reproductive disorders and stray dogs, male and female and rural and urban are presented in tables (1-6).

Table –1: Source-wise distribution of serum samples from pet dogs:

SL No.	Source	Number		
		Urban	Rural	Total
1.	Dog Ward, Faculty of Veterinary and Animal Sciences, West Bengal University of Animal and Fishery Sciences, Kolkata	61	2	63
2.	Referral Veterinary Polyclinic, Barasat, 24 Pgs(N)	15	19	34
3.	Veterinary Clinic – 'A', Kolkata	18	-	18
4.	Veterinary Clinic – 'B', Kolkata	21	-	21
5.	Dog Breeders, Kolkata	22	-	22
	TOTAL	**137**	**21**	**158**

Total 158 serum samples from pet dogs were collected. The data was presented on the basis of information on breeds, age group, sex, reproductive disorder and clinical findings (Table – 2 to 4).

Table – 2: Distribution of serum samples from pet dogs according to the breed and age:

Breeds	Age (years)						Samples tested
	<2	2-4	4-6	6-8	8-10	>10	
Spitz	4	7	5	3	5	4	28
Labrador	2	3	1	2	4	2	14
GSD	3	4	3	2	3	4	**19 (5.26%)**
Pomeranian	1	0	3	2	2	1	09
Doberman	3	2	2	1	2	1	11
Daschound	2	2	1	2	0	1	08
Golden Retriever	1	3	2	0	1	0	07
Lassaapso	3	2	1	4	1	1	**12 (8.33%)**
Cocker sp.	2	3	2	2	2	3	14
Boxer	0	1	0	0	1	0	02
Bhutia	1	2	0	2	1	1	07
Bull dog	0	1	1	0	1	0	03
Mongrel	3	3	6	4	3	5	**24 (4.16%)**
TOTAL	**25**	**33**	**27**	**24 (4.16%)**	**26 (7.69%)**	**23**	**158 (1.90%)**

Figure in parenthesis represents respective seroprevalence.

Table – 3: Distribution of serum samples from pet dogs according to the breed and sex:

Breed	Males	Females	Samples Tested
Spitz	11	17	28
Labrador	06	08	14
GSD	08	11	19
Pomeranian	05	04	09
Doberman	06	05	11
Daschound	03	05	08
Golden Retriever	02	05	07
Lassaapso	07	05	12
Cocker sp.	08	06	14
Boxer	01	01	02
Bhutia	02	05	07
Bull dog	02	01	03
Mongrel	11	13	24
TOTAL	**72**	**86**	**158**

Table – 4: Distribution of serum samples from pet dogs according to reproductive disorders:

Breeds	Abortion	Stillbirth	Pyometra	Premature Birth	Testicular atrophy	Scrotal dermatitis	Infertility	Epididymitis	Orchitis	Samples Tested
Spitz	4	2	8	1	2	3	4	2	2	28
Labrador	1	1	3	1	1	1	3	1	2	14
GSD	3	1	4	2	2	2	2	1	2	19
Pomeranian	1	0	2	0	1	2	2	0	1	09
Doberman	1	0	2	1	2	2	1	1	1	11
Daschound	1	0	2	1	0	1	1	1	1	08
Golden Retriever	0	1	2	1	0	0	2	0	1	07
Lassaapso	2	1	1	0	1	2	2	1	2	12
Cocker sp.	1	0	3	1	2	3	3	1	0	14
Boxer	0	0	1	0	0	0	1	0	0	02
Bhutia	0	1	2	0	0	1	2	0	1	07
Bull dog	0	1	0	0	0	1	1	0	0	03
Mongrel	2	1	6	2	1	2	3	3	4	24
TOTAL	**16**	**9**	**36 (2.7%)**	**10**	**12**	**20 (5%)**	**27**	**11**	**17 (5.8%)**	**158 (1.90%)**

Figure in parenthesis represents respective seroprevalence.

Two hundred sixty eight serum samples were collected. The data was presented on the basis of source, sex (table 5-6)

Table – 5: Source-wise distribution of serum samples from stray dogs:

SL No.	Source	Number		
		Urban	Rural	Total
1.	CSPCA, B.B. Ganguly Street, Kolkata	147	-	147
2.	Love and Care, Jagannathpur, South 24 Parganas	79	-	79
3.	Animal and Bird Welfare Society, Udaynarayanpu, Howrah.	-	42	42
	TOTAL	**226**	**42**	**268 (2.23%)**

Figure in parenthesis represents respective seroprevalence

Table – 6: Distribution of serum samples from stray dogs according to the breed and sex:

Breed	Males	Females	Samples Tested
Mongrel	126	135	261
Others	2	5	7
TOTAL	**128**	**140**	**268**

4.3. Seroprevalence of Canine Brucellosis:

All the sera samples (426) were subjected to Rose Bangal Plate Test (RBPT) with two RBPT antigens (*B. canis* and *B. abortus*) and 2-Mercaptoethanol Tube Agglutination Test (2-ME-TAT) with *B. canis* antigen.

Twelve sera samples showed positive result with RBPT antigen (*B. canis*) and negative with RBPT antigen (*B. abortus*). Nine out of 12 samples showed positive result with 2-ME-TAT (FIG.-4).

Only one sample (rural source) showed positive result with RBPT (*B. abortus*) and negative with the other two tests. The result indicated 0.23% seroprevalence of *B. abortus* infection in dogs and 1.58% in rural area. However Srinivasan and associates (1992) reported seroprevalence of *B. abortus* infection 2.68% in rural dogs in Tamilnadu (India).

Out of the total 12 samples which were found positive to RBPT (*B. canis*), only 9 were found positive to 2-ME-TAT. The possible explanation of this finding might be that cross reactivity of *B. canis* antigen with hetero specific antibodies in the sera of normal, healthy dogs, which was in accordance with the observations of Carmichael (1968); Flores-Castro and Carmichael (1981); Zoha and Carmichael (1977); Nicoletti and Chase (1987).

In the present study, the samples were found positive to *B. canis* did not show any agglutination with *B. abortus* and vice versa. Similar observation had also been reported by Alton *et al.*, (1975).

In the present study 2-ME-TAT found 2.11% positive to *B. canis* infection, which was in accordance with the findings of Pillai *et al.*, (1991) and Srinivasan *et al.*, (1992), who reported a seroprevalence of 2.18% and 1.96%, respectively in Chennai city, India.

4.3.1. Seroprevalence of *B. canis* infection among pet dogs:

Three samples from pet dogs were found positive to 2-ME-TAT, which reflects a seroprevalence of 1.90%.

Breedwise distribution of seroprevalence reveals 5.26%, 8.33% and 4.16% among GSD, Lassaapso and Mongrel, respectively.

Agewise seroprevalence study with 158 sera samples of pet dogs with 2-ME-TAT and found that 7.69% and 4.16% samples were positive in the age group of 8-10 and 6-8 years, respectively. This finding reveals that the seroprevalence rate increased along in the aged dogs, which is in agreement with the findings of Sharma (2004). Moon and co-workers (1999) and Azevedo (2002) however reported no significant difference in breed and age of dogs positive for canine brucellosis in Chonnam area of Korea and Sau Paulo, Brazil, respectively.

Canine brucellosis in respect to reproductive disorders i.e. orchitis, scrotal dermatitis, pyometra was observed as 5.8%, 5%, 2.7%, respectively. This is also in agreement with the findings of Sharma (2004).

4.3.2. Seroprevalence of *B. canis* infection among stray dogs:

In the present study out of 268 sera samples collected from stray dogs, 6 (2.23%) samples were found positive to 2-ME-TAT, which is in accordance with the findings (2.89%) of Ueda and associates (1974).

4.3.3. Seroprevalence of *B. canis* infection according to sex of total samples:

A total of 426 samples were collected from 200 males and 226 females and found 4(2%) males and 5(2.2%) females were positive by 2-ME-TAT, which revealed that there is no relation of sex in respect to occurrence of infection of canine brucellosis. The findings of the present study suggest that there was hardly any variation in positivity in relation to sex. This is in agreement with the findings of Srinivasan *et al.*, (1992), Moon *et al.*, (1999) and Azevedo (2002).

4.3.4. Seroprevalence of *B. canis* infection in rural and urban samples:

Out of total 426 samples, 63 and 363 samples were collected from rural and urban sources, respectively. Two samples (3.17%) from rural area and 7 (1.92%) samples from urban area were found positive by 2-ME-TAT. This is in accordance with the findings of Srinivasan *et al.*, (1992).

4.4. Isolation and identification of the *B. canis* organism:

4.4.1. Haemoculture from sero-positive animals:

Attempt was made for isolation of organism from the dogs which were found positive to 2-ME-TAT. For this purpose haemoculture was attempted but none of the samples yielded *B.canis* by cultural isolation. Further haemoculture was attempted from 46 stray dogs which showed some clinical signs viz. vaginal discharge in females, orchitis in males, but here also *B.canis* organism could not be isolated from any of the samples.

4.4.2. Culture from vaginal swab:

Twenty seven vaginal swabs were taken from the pet dogs having the history of abortion, still birth, premature birth, infertility and clinical disorders as per proforma (annexure – III) for cultural examination and isolation, none of the sample was found positive.

CHAPTER - 5

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

Brucellosis is one of the most important contagious and infectious disease. It has a worldwide distribution with most wide public health and economic importance. *B. canis* causes a highly infectious form of brucellosis in dogs characterized by abortion in females and epididymitis, testicular atrophy and infertility in males.

Brucella canis MEX 51 strain was collected from Division of standardization, IVRI, Izatnagar (India). Before use, the culture was tested for purity and biochemical characters through a battery of tests. RBPT antigen (*B. canis*) and 2-ME-TAT antigen (*B. canis*) were prepared from the said culture.

Anti *B. canis* serum was prepared in two dogs by subcutaneous inoculation of inactivated and sonicated *B. canis* suspension.

The anti *B. canis* serum which was raised in dogs was tested and found positive with the known laboratory antigens which was inoculated to laboratory dogs for raising anti *B. canis* serum.

Altogether four hundred twenty six blood samples were collected from pet dogs and stray dogs. Twelve samples were found positive with RBPT antigen (*B. canis*) and negative with RBPT antigen (*B. abortus*). Out of 12 samples, 9 were positive to 2-ME-TAT (*B.canis*). Therefore seroprevalence of *B. canis* infection was 2.11%.

Out of 9 seropositives 3 (1.90%) and 6 (2.23%) were pet and stray dogs, respectively.

Among pet dogs, breed wise distribution of seroprevalence revealed 5.26%, 8.33% and 4.16% among GSD, Lassaapso and Mongrel, respectively.

In the present study maximum seroreactors to *B. canis* infection was found in the age group 8-10 years (7.69%) followed by in the age group of 6-8 years (4.16%).

Distribution of canine brucellosis with respect to reproductive disorders i.e. orchitis, scrotal dermatitis and pyometra among pet dogs were 5.8%, 5% and 2.7%, respectively.

In the present study out of 200 males and 226 females, 4 (2%) males and 5 (2.2%) females were positive. So it is revealed that sex does not have any significance in the occurrence of canine brucellosis.

In the present study seroprevalence of *B. canis* infection in relation to rural and urban areas were found 3.17% and 1.92%, respectively.

An attempt was made to isolate *B. canis* organism from the animals which were found positive to 2-ME-TAT (*B. canis*), but no organism could be isolated from any of the samples.

CHAPTER - 6

FUTURE SCOPE OF RESEARCH

FUTURE SCOPE OF RESEARCH

An extensive attempt may be made for isolation *B. canis* organism from aborted foetus, placenta and vaginal excretion from bitches with history of abortion. Simultaneously haemoculture of such bitches may also be conducted.

Attempt may be made to develop suitable, sensitive, cheap and easy to conduct test so that it can be performed before mating.

Public health importance of the disease should be studied, especially with in contact persons.

Seroprevalence of the farm dogs and dog population nearby a slaughterhouse or abattoir should be studied especially of *Brucell abortus* and *Brucella suis* infection in dogs.

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ANNEXURE

Annexure – I

Normal Saline Solution (NSS):

Measured 8.5gm of sodium chloride and dissolved in 1000ml of double distilled water. This was sterilized by autoclaving at 15 lbs for 15 minutes.

Phenol Saline:

Phenol saline was prepared with the solution containing 0.85% of sodium chloride and 0.5% of phenol in double distilled water. This was sterilized by autoclaving at 15 lbs for 15 minutes.

Rose Bengal Solution:

Four gm of Rose Bengal was added to 386ml of sterile distilled water and mixed.

Buffered diluents:

One hundred twenty gm of sodium hydroxide was added to 2 liters of sterile 0.85% sodium chloride solution containing 0.5% phenol and mixed. Then added 540ml of concentrated lactic acid and mixed. Added sterile 0.85% sodium chloride solution containing 0.5% phenol to make 6 litres.

Phosphate Buffer Saline (PBS, pH 7.2):

Sodium Chloride	8.000gm
Potassium Chloride	0.200gm
Disodium hydrogen Phosphate	2.312gm
Potassium Hydrogen Phosphate	0.200gm
Distilled water to	1000ml

Autoclaved at 15 lbs for 15 minutes.

Tryptose agar:

Ingredients	Grams/litre
Trytose	20.0
Dextrose	1.0
Sodium Choride	5.0
Agar	15.0

Forty-one gm was suspended in 1000ml-distilled water and dissolved properly by application of heat. Sterilized by autoclaving at 15 lbs for 15 minutes.

Liver agar:

One hundred gram minced fresh goat liver was taken in a beaker, 200ml distilled water was added and allowed to free steam for 3 hours. The infusion was subjected to filtration with a thin film of absorbent cotton covered with a piece of fine cloth. The pH of the filtrate was adjusted to 7.4. An amount of 2.8 gm of nutrient agar was suspended in 100ml of filtrate and dissolved properly by application of heat. Sterilized by autoclaving at 15 lbs for 15 minutes.

Nutrient agar:

Ingredients	Grams/litre
Peptic digest of animal tissue	5.00
Beef extract	1.00
Yeast extract	2.00
Sodium chloride	5.00
Agar	15.00

Twenty eight in 1000ml distilled water. Sterilization by at 15 lbs for 15 minutes.

Christensen's Medium:

Peptone	1gm
Sodium Chloride	5gm
Dipotassium hydrogen phosphate (K_2HPO_4)	2gm
Phenol red (1:500 aqueous solution)	6ml
Dextrose	1gm
Agar	20gm
Distilled Water	1000ml

After proper boiling to dissolve the medium is distributed in 5ml amounts in test tubes and autoclaved at 15 lbs for 15 minutes. Then the tubes were cooled to 50° C, 0.5ml of a 20% solution of urea (Sterilized by syringe filter) was added to give a final concentration of 2% of urea. The medium was allowed to solidify in a sloped position and become yellow when cooled.

Tryptose Broth:

Ingredients	Grams/litre
Tryptose	20.0
Dextrose	1.0
Sodium Chloride	5.0

Twenty-six gm was suspended in 1000ml-distilled water and boiled to dissolve properly. Sterilized by autoclaving at 15 lbs for 15 minutes.

Preparation of lead acetate papers:

One filter paper was taken and divided into strips of about 10cm x 1cm. Strips were autoclaved at 15 lbs for 15 minutes. A 10% suspension of neutral lead acetate (Merck) was prepared in distilled water and sterilized by syringe filter. The strips were dipped in the suspension and allowed to dry in air under sterile condition.

Incorporation of dyes into base media:

The dyes basic fuchsin and thionin were incorporated in different concentration as described by Alton *et al.*, (1975) into the melted base media (Tryptose agar, Trypticase-soy agar etc.). A 0.1% stock solution of each dye was made in distilled water (100 ml stock solution was made with 100mg dye) and subjected to tyndallization. The required amount of stock dye solution was added (4ml dye was added to 100 ml melted medium for 1:25000 concentration, similarly 2 ml and 1 ml dye was added to 100 ml melted medium for 1:50000, 1:100000 concentrations, respectively) to the melted Tryptose (autoclaved at 15 lbs for 15 minutes) base medium which, after thorough mixing, was poured into Petri plates under sterile condition.

Annexure – II

The modified mercaptoethanol tube agglutination test:

Preparation of stock reagents

a. Stock solution of formalized saline:

Add 10ml of formaldehyde solution (37-40%) to 90ml of 0.85% sodium chloride solution.

b. Stock Solution of sodium Chloride (3.5% w/v):

Add 3.5g of sodium chloride to 100ml distilled water.

c. Sorenson's Phosphate-buffered saline (pH 7.0):

Prepared by dissolving 1.33g of potassium dihydrogen phosphate and 5.23g of anhydrous disodium hydrogen phosphate in 1 litre of distilled water.

The above reagents were autoclaved at 15 lbs for 15 min.

Preparation of working reagents:

a. Working antigen (3.5% sodium chloride, 0.06% formalin, 0.2% cells)
Prepared the diluent by adding 0.6ml of stock solution of formalized saline to 99.4ml of the stock solution of sodium chloride. Add 4.4ml of stock antigen to 95.6ml of this diluent and stored at 4^0 C until used.

b. Test diluent (3.5% sodium chloride, 0.06% formalin, 0.1 mol/litre 2-mercaptoethanol)
Prepared by adding 0.6ml of the stock solution of formalized saline to 99.4ml of the stock solution of sodium chloride and then add 0.7ml of 2-mercaptoethanol to 99.3ml of this solution and stored at 4^0C until used.

Preparation of other reagents:

a. Sorenson's phosphate-buffered saline containing 0.06% of formalin
Prepared by adding 0.6ml of the stock solution of formalized saline to 99.4ml of Sorenson's phosphate-buffered saline.

b. Sorenson's phosphate-buffered saline containing 0.5% of formalin
Prepared by adding 5.0ml of stock solution of formalized saline to 95.0ml of Sorenson's phosphate-buffered saline.

Annexure – III

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Proforma for Collection of information on canine brucellosis

SL No.	Sample No.	Date of Collection	Name of the owner	Address	Age of the dog	Sex	Breed	Gestation No.	Litter size	Abortion	Still birth	Pyometra	Premature birth	Testicular atrophy	Scrotal dermatitis	Infertility	Epididymitis	Orchitis	Remark