STUDIES ON OCCURENCE AND DIAGNOSIS OF CANINE BRUCELLOSIS

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By

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CERTIFICATE

This is to certify that the thesis entitled ***"STUDIES ON OCCURENCE AND DIAGNOSIS OF CANINE BRUCELLOSIS"*** submitted by **Mr. BALANGOUDA MALLANAGOUDA PATIL., I.D. No. MVHK 1935** in partial fulfilment of the requirements for the award of **MASTERS OF VETERINARY SCIENCE in VETERINARY GYNAECOLOGY AND OBSTETRICS** of the Karnataka Veterinary, Animal and Fisheries Sciences University, Bidar is a record of bonafide research work carried out by him during the period of his study in the University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

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Dedicated to my beloved Family

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Bengaluru

Date : (**BALANGOUDA MALLANAGOUDA PATIL**)

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LIST OF ABBREVIATIONS

Ab	Antibodies
AGID	Agar Gel Immunodiffusion Assay
AGID cpa	Agar Gel Immunodiffusion Assay using Cytoplasmic Antigen
ALT	Alanine Amino Transferase
AMOS	*Abortus melitensis ovis suis*
ANOVA	Analysis of Variance
B	Basophils
BAPA	Buffered Acidified Plate Antigen
Bp	Base Pair
CFSPH	Centre for Food Security and Public Health
CFT	Complement Fixation Test
Cmm	Cubic Millimetre
CRT	Creatinine
DNA	Deoxyribonucleic Acid
Dot ELISA	Dot Enzyme Linked Immunosorbent Assay
E	Eosinophils
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
Fg	Femtogram
g/dL	Gram per Decilitre
Hb	Haemoglobin
HCT	Hematocrit
ICA	Immunochromatographic Assay

IFA	Immunofluorescence Assay
IHC	Immunohistochemistry
L	Ladder
L	Lymphocytes
M	Monocytes
MAT	Microscopic Agglutination Test
mg/dL	Milligram per Decilitre
N	Neutrophils
NC	Negative control
NCBI	National Centre for Biotechnology Information
Ng	Nanogram
ng/mL	Nanogram per milli litre
Ns	Non-significant
NTC	Non-template control
^{o}C	Degree Celsius
PC	Positive control
PCR	Polymerase Chain Reaction
PLT	Platelets
qPCR	Quantitative – Polymerase Chain Reaction
RBPT	Rose Bengal Plate Test
RNA	Ribonucleic Acid
Rpm	Rotations per minute
RSAT	Rapid Slide Agglutination Test
SAT	Slide Agglutination Test

SE	Standard error
TAT	Tube Agglutination Test
TLC	Total Leukocyte Count
TRC	Total Red Blood Cells
U/L	Units per Litre
USDAAHPIS	United States Department of Agriculture – Animal and Plant Health Inspection Service
UV	Ultra Violet
V/cm	Volt per Centimetre
viz.	Namely
µL	Micro litre
%	Per cent
<	Lesser than
>	Greater than
±	Plus or Minus

Introduction

I. INTRODUCTION

Dogs have been part of human life since time immemorial. They were one of the first domesticated animals and have been an integral part of history of human civilization. They have been hunting allies in hunter-gatherer societies and have served as bodyguards against predators. Dogs also served as herders and guardians of livestock when domestication started about 7,000 to 9,000 years ago. In present scenario, dogs are mainly used for social purposes and companionship. Hence also truly termed as "Man's best friend". They are also considered as symbol of loyalty. Today dogs are employed as guides for the blind and disabled or for police work. Dogs are even used in therapy in nursing homes and hospitals to encourage patients toward recovery.

Humans have bred a wide range of different dogs adapted to serve a variety of functions. This has been enhanced by improvements in veterinary care and animal husbandry. Breeding of dogs have been an important economic activity. A large number of people are involved in such activities as a source of their living, especially in the urban society.

Whenever we talk of breeding (reproduction), there arises a point of infertility. The causes of infertility can be broadly separated into infectious and non-infectious. Traumatic causes, congenital defects and maternal factors are among the non-infectious causes. Infectious causes of abortion in dogs can be broadly grouped into viral, bacterial, fungal, and protozoal diseases. The most common cause of viral abortion and neonatal death in dogs is herpes viral infection and other viral infections which may cause sporadic abortions include blue tongue virus, canine parvovirus-1 (canine minute virus), canine distemper

virus, and canine adenovirus-1. *Brucella canis* and Streptococcus spp are the two most common causes of bacterial abortion and neonatal death in dogs. Infection with other bacterial organisms, such as *Escherichia coli*, Campylobacter spp, Leptospira spp, and Salmonella spp can occur sporadically (Lamm and Njaa, 2012).

Among many reasons of infertility, canine brucellosis stands as one of the major causes as it usually goes unnoticed due to poor knowledge, wide range of clinical symptoms and lack of proper diagnostic tools. Along with the economic losses it also poses a threat to public health as humans are in close contact with dogs considering the fact that they are considered a family member.

In dogs, canine brucellosis manifests different sings of infertility *viz*., abortion outbreaks, reproductive failure, enlargement of lymph nodes, and occasionally affects the osteo-articular system, although the occurrence of asymptomatic infections in dogs are not uncommon (Lopes *et al*., 2010; Carmichael and Greene, 2012). In humans, the disease is associated with a febrile syndrome, commonly with non-specific symptoms including splenomegaly, fatigue and weakness (Hensel *et al*., 2018; Santos *et al*., 2021).

Infection of dogs occurs mostly by the oronasal route when in contact with contaminated tissues such as aborted foetuses, semen, urine and vaginal secretions. In humans, contact with contaminated fluids from infected dogs is an important source of infection, and it is an occupational risk for veterinarians, breeders, laboratory workers, among other professionals who deal with infected animals or biological samples (Carmichael and Greene, 2006; Hollett, 2006; Makloski, 2011; Santos *et al*., 2021).

The diagnosis in dogs is largely based on serologic methods. However, serologic diagnosis of canine brucellosis remains very challenging due to the low accuracy of available tests. Immunofluorescent antibody tests (IFA), Rapid slide agglutination tests (RSAT) and Tube agglutination tests (TAT) are commonly used as initial screening tools to rule out brucella infection (Carmichael and Greene, 2006; Hollett, 2006; Keid *et al*., 2009). Both nonspecific and specific cross reactions with shared surface antigens on *Pseudomonas aeruginosa*, *Bordetella bronchiseptica*, *Actinobacillus equuli*, *Streptococcus sp.*, *Staphylococcus sp.*, Moraxella-type organisms and gram-negative bacteria, lead to false positive results (Carmichael and Greene, 2006; Hollett, 2006; Yu and Nielsen, 2010; CFSPH, 2017). Testing the dogs prior to seroconversion may yield false negative results and in some chronic cases of the infection due to low circulating antibody titers (Carmichael and Greene, 2006). Hence, in order to minimise the higher false positive or negative results inherited with the screening tests, a confirmatory test such as 2-mercaptoethanol RSAT (2ME-RSAT) or agar gel immunodiffusion assay using an internal cytoplasmic antigen (AGIDcpa) should be carried out (Carmichael and Greene, 2006; Hollett, 2006; Keid *et al*., 2009).

Molecular diagnostic methods like PCR have been increasingly used in the past few years. Therefore, development of novel and improved diagnostic methods is needed for an effective control of canine brucellosis and its associated zoonotic risk (Santos *et al*., 2021)

Battery of literature reveals paucity of information regarding occurrence of canine brucellosis following appropriate diagnostic procedures, to confirm the disease. With this background present study was designed with following objectives.

1) To study the occurence of brucellosis in female dogs.

2) To evaluate the relative efficacy of rapid one step immunochromatographic assay with PCR for diagnosis of canine brucellosis.

Review of Literature

II. REVIEW OF LITERATURE

2.1 Canine brucellosis

Brucella canis causes Brucellosis in dogs and is an important reproductive disease. *B. canis* is a zoonotic organism that can infect humans and is currently considered to be under reported in human medicine (NASPHV, 2012). In the recent years it has become evident that the incidence of *B. canis* infection within the dog breeding industry has been on the rise (Evans *et al*., 2005; Kauffman and Kinyon, 2006; Brower *et al*., 2007; Kauffman and Petersen, 2019). Bacteria in the genus, Brucella are nonmotile, nonencapsulated, non-spore-forming, facultatively intracellular gram-negative coccobacilli or short rods (Hollett, 2006; Carmichael and Greene, 2006). *B. canis, B. melitensis, B. suis and abortus* are classical brucella species known to cause disease in dogs and humans. The natural reservoirs for these organisms are dog, sheep and goats, pigs, and cattle, bison and buffalo, respectively. However, other two brucella species, *Brucella neotomae* (rodents, desert rats) and *Brucella ovis* (sheep) are not reported to be associated with brucellosis in dogs. Further, pathogenicity to dogs with terrestrial forms (*B. microti, B. inopinata*) and marine forms (*B. maris, B. pinnipediae, B. ceti*) is not yet elucidated (Hollett, 2006; Carmichael and Greene, 2006).

During an investigation of abortion in beagles in 1966–1967, *Brucella canis* was discovered and isolated from aborted tissues and vaginal discharge (Carmichael, 1966; Moore and Bennet, 1967; Taul *et al*., 1967; Carmichael and Kenney, 1968). Initially, *B. canis* was thought to be a biotype of *Brucella suis* based on genotypic and phenotypic

similarities (Moreno *et al*., 2002) and therefore, it is challenging for differentiation between *B. canis* and *Brucella suis biovar* 3 (Brennan *et al*., 2008).

B. canis has host range predominantly for domestic dogs and also some wild canids like foxes and coyotes based on positive antibody titres of serologic studies. Carmichael and Greene (2006) experimentally tried to infect the cattle, swine and sheep involving conjunctival and oral inoculation and reported that these animal species are resistant to *B. canis* infection even though earlier there were two field reports of *B. canis* in cattle. Oral experimental infection of cats recorded mild and temporary bacteraemia only in about 21 per cent but without giving positive agglutinating antibody titers (Carmichael and Greene, 2006).

2.1.1 Transmission

Several authors reported that the major routes of transmission for *B. canis* are genital, conjunctival, and oronasal mucosae as it occurs during normal reproductive, social and grooming activities in dogs (Wanke, 2004; Carmichael and Greene, 2006; Hollett, 2006; Makloski, 2011). The main sources of transmission are vaginal discharges and semen contaminated with brucella organism. The aborted material and vaginal discharge were reported to have highest number of organisms. During estrus, the infected female dogs transmit the disease at breeding or after abortion through direct contact of vaginal discharges and aborted materials. The fetal, the placental and the vaginal tissues and fluids after abortion or stillbirth harbour highest number of organisms (Carmichael and Greene, 2006).

Carmichael and Greene (2006) and Hollett (2006) have reported that *Brucella canis* is shed in vulvar secretions for up to 6 weeks following abortion and during estrus. The male dogs continue to shed high concentrations of organisms for 6 to 8 weeks in semen following the infection and later in lower concentrations shedding intermittent for up to two years in semen remaining as potential carrier for other dogs. Both infected males and females may shed the organism in the urine for at least three months. Further, the organism can also be present in blood, milk, saliva, nasal and ocular secretions, and in the faeces (Carmichael and Greene, 2006).

It has been reported that the minimum infectious dose through the oral route is about 10^6 organisms/mL and 10^4 to 10^5 organisms/mL by the conjunctival route as reported by Carmichael and Greene (2006). Transmission in utero, through broken skin, blood transfusions, faeces, milk, and fomites like contaminated syringes, vaginoscopes and artificial insemination equipment are considered as minor routes. Hands and clothing, high pressure spraying and cleaning equipment and improper wash-down techniques can also facilitate the spread of the organism within a kennel. The puppies can become infected in utero, during birth, while nursing, by contact with contaminated surfaces, or by fomite spread (Wanke, 2004; Carmichael and Greene, 2006; Hollett, 2006; Makloski, 2011).

2.1.2 Pathogenesis

Brucella organisms adhere to mucous membranes, penetrate the epithelial layer and are phagocytosed by the mononuclear leucocytes and remain intracellular. The organisms inhibit bactericidal myeloperoxidase-peroxide-halide system through the release of 5-guanosine and adenine by utilizing virulence factors, VirB proteins which are part of the

type IV secretory system (Hollett, 2006, Davidson and Sykes, 2014; Chacon-Diaz *et al.*, 2015; Pollak *et al*. 2015). These intracellular organisms travel to local lymph nodes *viz*., retropharyngeal, inguinal, superficial iliac and to liver, spleen and bone marrow via the reticuloendothelial system. About one to four weeks later, the organisms enter into the blood to result in intermittent bacteremia. The main organs of predilection are steroid-dependent reproductive tissues such as the gravid uterus and placenta in females and prostate, testicles, and epididymis in males (Hollett, 2006). Brennan *et al* (2008) found that progestational, non-gravid uterus acted as a reservoir of Brucellae while analysing an outbreak in a Saskatchewan kennel. Lymphocytes, plasmacytes and histiocytes were found and opined that it was due to mixed inflammatory response in the reproductive tissues (Brennan *et al*., 2008; Carmichael and Greene, 2006; Hollett, 2006). Further, in the aborted placenta, they observed the chorionic villi with focal coagulative necrosis, necrotizing arteritis and plenty of bacteria in trophoblastic epithelial cells (Hollett, 2006; Brennan *et al*., 2008).

The bacteraemia spreads organisms along with antibody-antigen complexes that are formed in response to infection in organ systems other than the reproductive systems like intervertebral disk and the eye (Carmichael and Greene, 2006, Hollett, 2006).

Recently the brucella organism are classified as smooth and rough forms based on presence or absence of the external antigen, O-polysaccharide of bacterial cell wall where the loss of O-polysaccharide results from the spontaneous excision of the wbkA glycosyl transferase gene (Carmichael and Greene, 2006; Mancilla *et al*., 2012; Mancilla, 2016). The smooth (*B. melitensis, B. abortus*, and *B. suis*) and rough (*B. canis*, and *B. ovis*) forms

have been traditionally classified. Further, some strains of *B. canis* like a less mucoid (M-) laboratory strain, usually maintained for serological studies, is avirulent in dogs but has potential to infect humans like wild-type *Brucella canis* (Wallach *et al*., 2004).

2.1.3 Course of infection

Hollett (2006) recorded the bacteraemia episodes in dogs that lasted for more than five years in experimentally infected dogs indicated by positive blood cultures. The seroconversion in dogs has been reported to occur from two to 12 weeks following infection, the intensity of bacteraemia declines thereafter but the bacteria persist in the blood or/and infected tissues (Carmichael and Greene, 2006; Bramlage *et al.*,2015).

The *B. canis* infection is believed to get eliminated through cell-mediated immune responses within three years but humoral immune responses do not eliminate the organism and thus, results in potential carrier dogs (Carmichael and Greene, 2006).

Carmichael and Greene (2006) observed the experimentally infected dogs that were allowed to recover without any antimicrobial treatment become immune up to four years for subsequent oral or intravenous re-inoculation. Other dogs which did not eliminate the bacteria naturally were susceptible to oronasal inoculation three months after antimicrobial treatment.

2.1.4 Clinical signs

In dogs, clinical signs depend on the sex and age of the affected dog and are far-flung and not consistent. The disease may not manifest obvious clinical signs hence, may pose challenge to the owner or/and veterinarian to identify or sometimes can be a storm of

abortion. Therefore, it is always suggested to first rule out *B. canis* infection while diagnosing any reproductive problems as it considerably contributes to infertility in breeding dogs. Besides reproductive abnormalities, a range of non-reproductive issues can also be manifested. The clinical signs of canine brucellosis can be similar to many other diseases and therefore, the United States in their best practices described the infection as *the Great Imposter* (Bramlage *et al.*,2015).

In female dogs, a high morbidity and a low mortality in this infection have been reported. Death is usually observed in utero, neonates or animals with severe illness. Early embryonic death at 2 to 3 weeks after transmission in venereally infected bitches and spontaneous abortion at late stage (45 to 55 days) occurs most commonly when the pregnancy progresses (Carmichael and Greene, 2006; Lamm and Makloski, 2012). Infected female dogs may show vaginal discharge of mucoid, serosanguinous, or grey-green colour up to 42 days post abortion with endometritis. Sometimes, infected bitches can deliver litters that clinically appear normal but such puppies are born infected and disease symptoms can be expressed in later part of the life. Thus, conception failures, late term abortion with prolonged vaginal discharge, stillbirths, decreased litter size and decreased puppy survivability can be noticed months after infection (Lopes *et al*., 2010; Carmichael and Greene, 2012).

In male dogs, epididymitis, orchitis, testicular swelling or atrophy, sperm abnormalities, uveitis, meningoencephalitis, spinal arthritis, weight loss, poor hair coat, listlessness, swollen lymph nodes, and behavioural changes may develop as the disease progresses. Chronically infected dogs may be totally asymptomatic carriers and act as a

potential source of infection. During the acute stage, venereally infected male dogs develop epididymitis and scrotal oedema. Orchitis is not so common but can lead to scrotal dermatitis due irritation caused by licking. Chronic stages are characterized by either unilateral or bilateral testicular atrophy, chronic epididymitis and infertility due to anti-sperm agglutinating antibodies as a result of delayed-type hypersensitivity reactions against the spermatozoa which results in spermatogenic arrest (Forbes and Pantekoek, 1988; Carmichael and Greene, 2012).

Sperm abnormalities are usually noticed from fifth week post infection and more marked by eight weeks in animals with chronic epididymitis. Immature sperm, deformed acrosomes, swollen mid pieces, and retained protoplasmic droplets are few abnormalities noticed in early stages. Four months after infection bent tails, detached heads, and head-to-head agglutinations may be visualized and later abnormal sperms up to 90 per cent may be evident (Bramlage *et al.*,2015). Some proportion of dogs acts as a persistent carrier through seminal fluid of affected prostate without developing either sperm abnormalities or infertility (Carmichael and Greene, 2012).

Carmichael and Greene (2012) have reported most commonly noticed clinical signs including chronic uveitis, endophthalmitis and disco-spondylitis involving other systems than genital system in canine brucellosis. Ocular manifestations include blepharospasm, aqueous flare, constricted pupils, synechiae, hypopyon, and hyphema. Stiffness, back pain, lameness, exercise intolerance, paresis and paralysis due to spinal compression are few signs indicating disco-spondylitis. Lymphadenitis, pyogranulomatous dermatitis, endocarditis, appendicular osteomyelitis, and meningoencephalitis are few other clinical

signs. Nonspecific symptoms rarely noticed are fever, lethargy/fatigue, exercise intolerance, decreased appetite, weight loss, and behavioural anomalies such as loss of alertness and poor performance of tasks.

The *B. canis* has zoonotic relevance and the clinical manifestations of the infection in humans commonly noticed are periodic and nocturnal fever, fatigue, headache, weakness, malaise, chills, sweats, weight loss, hepatomegaly, splenomegaly, and lymphadenopathy. In few human cases with *B. canis* infection, serious manifestations including septic arthritis, aortic valve vegetations, calvarial osteomyelitis, epidural abscess, pleural effusion, oral lesions, lower extremity aneurysms, and culture negative endocarditis have been reported though the organism is known to cause milder disease manifestations compared to other brucellae (Bramlage *et al.*,2015; Hensel *et al*., 2018).

2.1.5 Occurrence

Brucella canis infection has significant role in reproductive failures in dogs across the globe and is considered as endemic in Southern USA, in central and South America and in Mexico (Flores-Castro *et al*., 1977; Brower *et al*., 2007; Kuster *et al*., 2013; Krueger *et al*., 2014; Keid *et al*., 2017). It has been frequently reported about dogs with infections with *B. canis* also from Asia (China, Japan, India) (Yoak *et al*., 2014) and Africa (Nigeria, Zimbabwe) (Brower *et al*., 2007; Cadmus *et al*., 2011; Di *et al*., 2014). In Oceania, it is exotic in Australia and does not occur in New Zealand (Gardner and Reichel, 1997; Mor *et al*., 2016; Rovid Spickler, 2018). In Germany, *B. canis* was reported in 1976 in a colony of Beagle dogs (Von Kruedener, 1976) and 2003 in one male dog with epididymitis and orchitis (Nöckler *et al*., 2003). Rarely cases were also reported from other European

countries, such as Sweden (Holst *et al*., 2012; Kaden *et al*., 2014), the United Kingdom (Taylor, 1980; Morgan *et al*., 2017), Austria (Hofer *et al*., 2012), Italy (Corrente *et al*., 2010), and Hungary (Gyuranecz *et al*., 2011).

The countries with a large stray dog population have presumably a higher prevalence of the disease, since they act as potential carriers for spreading the organisms (Brown *et al*., 1976; Reynes *et al*., 2012; Dreer *et al*., 2013; Yoak *et al*., 2014). Bramlage *et al*. (2015) stated that there is always an increased risk of maintaining the infection if a dog gets brucella infected in the breeding colonies.

A study by Vinayak *et al*. (2004) using the Veterinary Medical Database, indicated 14.20 per cent brucellosis prevalence with ocular lesions among dogs. Chinyoka *et al*. (2014) conducted sero-prevalence study in dogs of urban and rural areas of Zimbabwe and reported 20.70 and 12.7 per cent, respectively.

Keid *et al*. (2017) cultured blood samples of 753 dogs and found 22.0 per cent of them had at least one of the clinical signs of brucellosis which included abortion/stillbirth, failure to conceive and enlargement of lymph nodes in female and recorded 20.90 per cent of positive cases. Further, they found 42.10 per cent of kennels screened in their study had positive dogs with the prevalence varied from 3.80 to 62.60 per cent. Abortion/stillbirth, failure to conceive and enlargement of lymph nodes were significantly associated with brucellosis in female in their study.

Seroprevalence rates in the south-eastern USA were estimated to be 7 to 8 per cent as reported by Bramlage *et al.* (2015). Cosford (2018) reported that *B. canis* infection has devastating effect in dogs of Canada though the disease is not common.

Buhmann *et al.* (2019) analysed the reports of central laboratory which tested dogs' samples that were received from 20 European countries for *B. canis.* Among the submitted samples, 3.70 per cent (61/1657) were PCR-positive for *Brucella* spp., and *B. canis* antibodies were identified in 5.40 per cent (150/2764) of submitted samples. An 11.10 per cent of submitted samples originated from Spain were *Brucella* spp. PCR-positive, 6.70 per cent from Poland and very less numbers from Italy and France. Samples positive for *B. canis* antibodies originated from 13 European countries - Sweden, Belgium, Austria, Switzerland, Italy, Finland, Germany, Denmark, Hungary, Norway, Poland, France and Netherlands.

Whitten *et al.* (2019) screened 943 dogs in Minnesota, of which 22 (3.10%) stray and 8 (3.50%) owner-surrendered dogs were positive by RSAT.

Mol *et al*. (2020) studied the frequency of brucellosis in dogs of 5 breeding kennels in the state of Minas Gerais, Brazil and detected frequency that varied from 6.30 per cent by AGID to 16.50 per cent by dot-ELISA. There was no positive serology for smooth Brucella. PCR testing was positive in 13.90 per cent of samples. Suarez-Esquivel *et al*. (2021) found 6.29 per cent dog cases positive in serological study in Costa Rica.

While evaluating the zoonotic importance of the *B. canis*, Krueger *et al*. (2014) conducted a seroprevalence study among canine-exposed individuals of Argentina and

recorded its prevalence ranging from 3.60 per cent (ME-RSAT) to 10.80 per cent (RSAT) and also observed higher RSAT seropositivity in employees working in kennel compared to other canine exposures.

Baek *et al*. (2003) subjected the dogs having only observed clinical sign (mild fever) that were maintained in a dairy farm with high prevalence of *Brucella abortus* infection for brucella testing and confirmed by PCR.

In India, Pillai *et al*. (1991) first reported *Brucella canis* infection in dogs from Small Animal Clinic of the Madras Veterinary College, Chennai. A seroprevalence study conducted in Karnataka state by Isloor *et al*. (1998) reported 3 per cent brucellosis in bovine. Renukaradhya *et al*. (2002) stated that bovine brucellosis is widespread in India and *Brucella melitensis* and *Brucella abortus* are the species of concern.

Aulakh *et al*. (1997) conducted seroprevalence of *Brucella canis* by subjecting 112 pet dogs for canine brucellosis antibody test across state of Punjab, India and found 9.80 per cent positivity. About 32.6 per cent of dogs that were with clinical symptoms such as persistant temperature, anorexia, abortions, orchitis, itching etc., were positive for the antibody test.

Mitra *et al*. (2013) screened 10 Labrador female dogs in a kennel at Kolkata, West Bengal (India) with complaints of conception failure, abortion and discospondylitis using 2-Mercapto-ethanol rapid slide agglutination test and lateral flow immunochromatographic assay and recorded *Brucella canis* infection by both tests in 50 per cent dogs.

Yoak *et al*. (2014) reported 10, 5.06 and 3.45 per cent seroprevalence of canine brucellosis in street dogs of Jaipur, Jodhpur and Sawai Madhopur cities of state of Rajasthan (India), respectively.

2.1.6 Diagnosis

2.1.6.1 Blood culture

Culture of the blood, urine, vaginal discharge, semen or aborted fluids/tissues samples has been the traditional and gold standard diagnostic test for *B. canis* (Wanke, 2004; Carmichael and Greene, 2006; Hollett, 2006; Bramlage *et al.*,2015; CFSPH, 2017). Samples should be collected following standard sterile precautions and procedures in an aerobic culture vial or heparinized tube (Carmichael and Greene, 2006; CFSPH, 2017). Due to low concentration of organism, intermittent shedding, poor sample choice for submission, inappropriate handling of sample, slow growing, fastidious forms and incorrect culture media make it very difficult to detect this organism (Carmichael and Greene, 2006).

Al Dahouk *et al*. (2003) suggested that a negative culture should not rule out infection as the low sensitivity corresponds to an unacceptable number of false negatives. A single negative blood culture may not be sufficient to rule out the disease in suspect animals but three blood samples collected at 24 hours intervals must be cultured negative to declare that a dog is not infected by *B. canis*. Serological tests are used for routine screening but culture of the organisms is the ideal and necessary for a definitive diagnosis (Serikawa *et al*., 1981; Carmichael and Shin, 1996; Baldi *et al*., 1997).

2.1.6.2 Serologic assays

Perusal of the literature revealed that the brucellosis in clinical cases of dogs is usually diagnosed by various serological tests, *viz*., (i) tube agglutination test (TAT), (ii) rapid plate agglutination test (RPAT), both with or without addition of 2-mercaptoethanol; and, (iii) agar gel immunodiffusion test (AGID) (iv) compliment fixation test (CFT) (v) Microagglutination test (MAT) (vi) Dot-ELISA, using antigens prepared either with *B. canis* or *B. ovis* (Serikawa *et al*., 1989; Baldi *et al*., 1994, 1997; Carmichael and Shin, 1996; Lucero *et al*., 2002).

Both nonspecific and specific cross reactions with shared surface antigens on *Pseudomonas aeruginosa*, *Bordetella bronchiseptica*, *Actinobacillus equuli*, *Streptococcus sp.*, *Staphylococcus sp.*, Moraxella-type organisms and gram-negative bacteria, lead to false positive results (Johnson and Walker, 1992; Mateu-De-Antonio *et al*., 1993; Baldi *et al*., 1994; Carmichael and Shin, 1996; Carmichael and Greene, 2006; Hollett, 2006; Yu and Nielsen, 2010; CFSPH, 2017).

Immunofluorescent antibody tests (IFA), Rapid slide agglutination tests (RSAT) and Tube agglutination tests (TAT) are commonly used as initial screening tools to rule out brucella infection (Carmichael and Greene, 2006; Hollett, 2006; Keid *et al*., 2009). Testing the dogs prior to seroconversion may yield false negative results and in some chronic cases of the infection due to low circulating antibody titres (Carmichael and Greene, 2006). Hence, in order to minimise the higher false positive or negative results inherited with the screening tests, a confirmatory test such as 2-mercaptoethanol RSAT (2ME-RSAT) or agar gel immunodiffusion assay using an internal cytoplasmic antigen

(AGIDcpa) should be carried out (Carmichael and Greene, 2006; Hollett, 2006; Keid *et al*., 2009).

Of the 13 dogs studied, 2 were found negative for *B. canis* infection by results of 2-ME RSAT and 3 by ICA. In a serodiagnosis report by Keid *et al*. (2007b), among the blood-culture positive dogs, only 82.81 per cent by RBT (rapid slide agglutination) and 39.06 per cent by 2-ME RSAT had positive reaction. In addition, as reported by Abernethy *et al*. (2012), the sensitivity of the RSAT was 76.60 per cent. Kim *et al*. (2007) reported that the kappa value between 2-ME RSAT and ICA was 0.89. Any single serological method cannot definitively diagnose canine brucellosis owing to low specificity and sensitivity, so substitutive or complementary diagnostic methods are required.

2.1.6.3 Immunochromatographic assay (ICA)

The assay consists of a nitrocellulose detection strip flanked at one end by a reagent pad and at the other end by an absorption pad. A simple application of pad flanks the reagent pad in turn. The composite strip is contained in a plastic assay device. The detection strip contains *B. canis*-specific antigen as a capture probe as well as a reagent control applied in distinct lines. The reagent pad contains dried and stabilized detection reagent consisting of a colloidal gold immune conjugate. Recently, an ICA using colloidal gold particles serving as detectors were described for detecting antibodies and antigen gold labels act as markers for molecules that are otherwise invisible by naked eye or through other detecting systems. A colloidal gold conjugate consists of suspension of gold particle coated with a selected protein or macromolecule such as an antibody. Sensitivity and

specificity are two important characteristics of a diagnostic test (Jung *et al*., 2005; Xiulan *et al*., 2005).

Kim *et al*. (2007) experimentally infected dogs with *B. canis* and found that the samples yielded positive results in haemoculture, 2-ME RSAT and ICA after 5 weeks, 7 weeks and 3 weeks, respectively. They also noticed a prevalence of 24.80, 39.50 and 39.10 per cent by Hemoculture, 2-ME RSAT and ICA, respectively in dogs tested from 10 different breed kennels for brucellosis, which are equivalent of 0.89 between 2- ME RSAT and ICA kappa values obtained. Landis and Koch (1977) reported that the kappa value of 0.01–0.2 indicated slight, 0.21–0.40 fair, 0.41–0.60 moderate, 0.61–0.80 substantial and 0.81– 0.99 almost perfect agreement. Therefore, this result indicated 2-ME RSAT and ICA were highly agreement. Based on the results of their evaluation Kim *et al*. (2007) concluded that sensitivity and specificity of the ICA are in par with conventional serological and bacteriological test used to detect *B. canis* infection in dogs.

Wanke *et al*. (2012) evaluated a commercial immunochromatographic assay called FASTest and the preliminary results indicated 100 per cent specificity of the FASTest. However, in acute and subacute cases, the sensitivity of the test was 89 per cent and 17 per cent in cases of chronic brucellosis which is lower than that of ELISAs (100%) and AGID (67%) tests. Suja (2014) conducted a study on incidence of canine brucellosis using ICA and RBPT which showed 0 and 26 per cent incidence, respectively.

Keid *et al*. (2015) evaluated the accuracy of immunochromatographic test (ICT) with that of the rapid slide agglutination test with and without the use of 2- mercaptoethanol and the agar gel immunodiffusion test (AGID) for the detection of *Brucella canis* infection.

References used for the test were microbiological culture (blood, semen and vaginal swab), PCR (blood, semen and vaginal swab) and clinical examination. A total of 102 dogs were divided into three groups based on results obtained in reference studies *viz*., *B. canis* infected dogs (Group 1), *B. canis* non-infected dogs (Group 2) and dogs with suspected brucellosis (Group 3). Evaluation of dogs of Group 1 proved that the diagnostic sensitivity to be 75.00, 37.50, 27.80 and 89.58 per cent by RSAT, 2ME-RSAT, AGID and ICT, respectively. Evaluation of Group 2 dogs proved the diagnostic specificity to be 91, 100, 100 and 100 per cent, respectively by RSAT, 2ME-RSAT, AGID and ICT. In the Group III, 9.67, 0.00, 3.22 and 6.45 per cent were positive by RSAT, 2ME-RSAT, AGID and ICT, respectively.

Brucellosis diagnosis in canines is difficult due to lack of a screening test with high sensitivity that can be used for rapid detection of infection. Keid *et al*. (2015) proved high diagnostic specificity and sensitivity of ICT compared to other serological tests but it failed to detect infection in 10.41 per cent in infected dogs proved positive by microbiological culture and/or PCR indicating active infection. Such failures can give way to potential spread of the disease by infected dogs that were tested false negative. Keid *et al*. (2015) concluded that though ICT being an easy to perform test but it does not complete the requirements of screening test.

2.1.6.4 Polymerase chain reaction (PCR)

Since 1987 numerous PCR-based assays for Brucella have been developed and published. The earliest assays were designed to exploit single unique genetic locus that was highly conserved in Brucella (eg: the *bcsp* 31 or the 16s rRNA genes). The advantage of

these types of assays is that they tend to have simple design and are very robust. Such tests are useful for screening or for identification when species or biovar designations are not critical (Bricker, 2002).

PCR assays have been developed to differentiate among Brucella species and/or biovars. These assays are directed toward genetic loci that are variable among species/biovars. Such targets are uncommon in Brucella since the genus is remarkably homogenous and has been proposed to be single species (Verger *et al*., 1985, 1987). While some deletions or rearrangements have been reported within species or biovar (Cloeckaert *et al*., 2000; Bricker *et al*., 2000), most genetic differences consist of single nucleotide polymorphisms. Particularly rare are the regions of hypervariability among species and biovars.

The first published PCR based diagnostic assay was reported by Fekete *et al*. (1990). This assay was based on the amplification of 635bp sequence from a gene encoding a 43-kDa outer membrane protein of Brucella abortus Strain-19. They were able to demonstrate the assay was specific for Brucella, applicable to all species and biovars, and very sensitive (less than 100 bacteria)

The next brucella gene target to be explored was the 16S rRNA gene (Herman and De Ridder, 1992). They were able to successfully amplify 800bp amplicon in all species of Brucella thus demonstrating it can be used for entire genus. To assess specificity, the assay was applied to a panel of 17 closely related bacteria to Brucella. No products were amplified from any non-Brucella species except *Orchobactrum anthropi* the closest relative of Brucella. Romero *et al.* (1995) got similar results using nine different primer

combinations. Rijpens *et al.* (1996) arrived at similar inference using a 16S-23S intergenic spacer region.

Baily *et al.* (1992) published a new PCR assay based on the gene encoding *bcsp* 31. The *bcsp* 31 gene is the first published brucella loci to be cloned and sequenced (Mayfield *et al*., 1998). This encodes an antigenic, periplasmic protein of unknown function. It is conserved in all species and biovars of Brucella (Bricker *et al*., 1988). The PCR assay developed by Baily *et al*. (1992) consisted of single pair of oligonucleotide primers designed to amplify a 223-bp product. Da Costa *et al.* (1996) confirmed the specificity of this assay.

Bricker and Halling (1994) developed a multipex PCR using multi-copy element IS711 also known as IS6501 (Ouahrani *et al*., 1993). Although the number and placement of IS element is typically conserved most species contain atleast one copy of IS element at a unique chromosomal location. The assay was named the AMOS-PCR assay for the Brucella species it can identify and differentiate (*B.abortus biovars 1,2 and 4; B.melitensis; B.ovis; and B.suis biovar 1*). The multiplex design consists of one common primer anchored in the IS element and a species-specific primer that binds to the unique sequence flanking that insertion site. The assay primers were chosen so that the species discrimination was determined by the size of the amplicon. Bricker *et al.* (2003) improvised this assay with certain changes in primer length and renamed it as *Brucella abortus* species–specific polymerase chain reaction (BaSS PCR) assay for the identification and discrimination of *B. abortus* field strains (wild-type biovars 1, 2, and 4) from 1) *B. abortus* vaccine strains, 2) other Brucella species, and 3) non-Brucella bacteria. Identical samples

were tested in two laboratories. Half the samples were fully viable, and half were bacteria that had been killed by methanol fixation. The results in one laboratory correctly identified 100 per cent of the samples, resulting in a predictive value of 100 per cent for all categories and 100 per cent sensitivity and specificity under the prescribed conditions. The second laboratory misidentified 31 samples, resulting in a range of 66.7–100 per cent sensitivity, 93.2–99.7 per cent specificity, and 77.3–98.2 per cent predictive values depending on the category. There was no significant difference in viable versus fixed bacteria for either laboratory. Subsequent review of the protocol indicated that contamination was the likely cause of 26 of the 31 erroneous identifications.

Fourteen dogs were obtained from 10 farms with Brucella-infected cattle and were studied for periods ranging from 2 to 81 days. At necropsy, Brucella abortus biovar 4 was isolated from all 14 dogs. (Forbes, 1990). Three dogs reared on a dairy farm with a high incidence for *Brucella abortus* were serologically positive for *B. abortus* and no other Brucella spp. The identity of the organism was confirmed to be *B. abortus* by AMOS (*abortus melitensis ovis suis*)-polymerase chain reaction with specific primers for *B. canis*. One hundred percent homology of the canine isolate and the bovine pathogen isolated from the farm was demonstrated. The only possible source of infection was infected cattle on the same farm. It is suggested that dogs be routinely included in brucellosis surveillance and eradication programs (Baek *et al*., 2003).

Keid *et al*. (2007b) used a pair of primers directed to 16S-23S rDNA interspacer (ITS) was designed directed to Brucella genetic sequences in order to develop a polymerase chain reaction (PCR) putatively capable of amplifying DNA from any Brucella species.

Nucleic acid extracts from whole-blood from naive dogs were spiked with decreasing amounts of *Brucella canis* RM6/66 DNA and the resulting solutions were tested by PCR. In addition, the ability of PCR to amplify Brucella spp. genetic sequences from naturally infected dogs was evaluated using 210 whole-blood samples of dogs from 19 kennels. The whole-blood samples collected were subjected to blood culture and PCR. Serodiagnosis was performed using the rapid slide agglutination test with and without 2-mercaptoethanol. The DNA from whole blood was extracted using proteinase-K, sodium dodecyl sulphate and cetyl trimethyl ammonium bromide followed by phenol–chloroform purification. The PCR was capable of detecting as little as 3.8 fg of Brucella DNA mixed with 450 ng of host DNA. Theoretically, 3.8 fg of Brucella DNA represents the total genomic mass of fewer than two bacterial cells. The PCR diagnostic sensitivity and specificity were 100 per cent. From the results observed in the present study, we conclude that PCR could be used as confirmatory test for diagnosis of *B. canis* infection.

A PCR assay for the detection of *Brucella canis* in canine vaginal swab samples was evaluated, comparing its performance with that of bacterial isolation, serological tests, and a blood PCR assay. One hundred and forty-four female dogs were clinically examined to detect reproductive problems and they were tested by the rapid slide agglutination test, with and without 2-mercaptoethanol (2ME-RSAT and RSAT, respectively). In addition, microbiological culture and PCR were performed on blood and vaginal swab samples. The results of the vaginal swab PCR were compared to those of the other tests using the Kappa coefficient and McNemar test. Of the 144 females that were examined, 66 (45.80%) were RSAT positive, 23 (15.90%) were 2ME-RSAT positive, 49 (34.02%) were blood culture positive, 6 (4.10%) were vaginal swab culture positive, 54 (37.50%) were blood PCR

positive, 52 (36.20%) were vaginal swab PCR positive, and 50.69 per cent (73/144) were positive by the combined PCR.

The PCR was able to detect as few as 3.8 fg of *B. canis* DNA experimentally diluted in 54 ng of canine DNA, extracted from vaginal swab samples of non-infected bitches. In addition, the PCR assay amplified *B. canis* genetic sequences from vaginal swab samples containing 1.0 x 10^0 cfu/mL. In conclusion, vaginal swab PCR was a good candidate as a confirmatory test for brucellosis diagnosis in bitches suspected to be infected, especially those negative on blood culture or blood PCR; these animals may be important reservoirs of infection and could complicate attempts to eradicate the disease in confined populations (Keid *et al*., 2007a).

Aras and Ucan, (2010) evaluated the performance of the PCR by comparing with the results of bacteriological culture as reference method. During the years 2007–2008, forty-eight inguinal lymph node samples were collected from 48 dogs (18 males and 30 females) that died in the city's pound for the study. *B. canis* was isolated from 4 (8.3%) of 48 lymph node samples. Forty-four (91.7%) of the samples were bacteriological culture negative. *B. canis* DNA was directly detected from all culture positive lymph node samples (n = 4) by PCR. All of the culture negative samples were confirmed as negative by PCR. When the culture method was used as a gold standard, sensitivity and specificity of the PCR assay were found to be 100%. The limit of PCR detection of *B. canis* DNA was 1.4 × 10^1 CFU/g at least. In conclusion, the PCR assay has been shown to have a diagnostic performance equal to bacteriological culture for detection of *B. canis.*

Baek *et al*. (2012) collected twenty mammary lymph node samples from cattle on a farm in the Republic of Korea. These cattle were serologically negative for *Brucella* by tube agglutination test (≤1:50) and serum agglutination test (≤1:50). Out of 20 lymph node samples, two samples were positive for *Brucella* growth on *Brucella* agar as well as blood agar. Tests for urease, hydrogen sulphide and reactions against monospecific sera A and M indicated that these two isolates (No. 15 and 16) belong to the genus *Brucella*. Genus specific, AMOS (abortus, melitensis, ovis, suis) and Bruce-ladder multiplex polymerase chain reaction (PCR) assays confirmed the *Brucella* isolates as either a *B. abortus* or a *B. canis* strain.

Kauffman *et al*. (2014) collected 105 whole-blood samples, 65 vaginal swab, 6 urine and 7 genitourinary tract tissue at necropsy from 107 dogs from two kennels having clinical signs. Comparative analysis by serology and qPCR was performed. Based on qPCR findings, 45 (42.06%) dogs were found infected with canine brucellosis and obtained sensitivity of 92.31 per cent and specificity of 51.92 per cent using DNA extracted from vaginal swabs whereas, sensitivity of 16.67 per cent and specificity of 100 per cent was obtained by using DNA of whole blood. Kauffman *et al*. (2014) opined that the *B. canis* outer membrane protein 25 DNA qPCR collected from non-invasive vaginal swab and urine samples helped early detection of *B. canis* infection in dogs even before the detection of antibodies.

Sánchez-Jiménez *et al*., (2014) collected blood samples from 499 dogs from kennels in two Colombian regions and 91 co-inhabiting humans. The 2-mercaptoethanol rapid slide agglutination test (2ME-RSAT), blood culture and PCR tests were performed

on all samples. They reported a prevalence of 9.9 per cent in co-inhabiting humans by serological survey and 0 per cent by PCR or blood culture tests. In dogs it was 10.8 per cent, 19 per cent and 13 per cent by blood culture, PCR and 2ME-RSAT respectively. Compared with blood culture, PCR had a sensitivity of 92.6 per cent and a specificity of 90 per cent for canine samples. Compared with 2ME-RSAT, it had a sensitivity of 77.4 per cent and a specificity of 89.2 per cent.

Kang *et al*. (2014) developed a new *Brucella canis* species-specific (BcSS) PCR assay and evaluated its specificity and sensitivity. A specific PCR primer set was designed based on the BCAN B0548-0549 region in chromosome II of *B. canis*. The PCR detection for *B. canis* included amplification of a 300-bp product that is, not found on other Brucella species or, genetically or serologically related bacteria. The detection limit of BcSSPCR assay was 6 pg/L by DNA dilution or 3 × 103 colony-forming units (CFU) in the buffy coats separated from whole blood experimentally inoculated with *B. canis*. Using the buffy coat in this PCR assay resulted in approximately 100-times higher sensitivity for *B. canis* as compared to detect directly from whole blood. This is the first report of a species-specific PCR assay to detect *B. canis*, and the new assay will provide a valuable tool for the diagnosis of *B. canis* infection.

Although *Brucella canis* is more common, the infection by *Brucella abortus* is more frequent in dogs sharing habitats with livestock and wild animals. De Oliveria *et al*. (2019) collected Serum and whole blood samples from 167 dogs. Antibodies against *B. abortus* and *B. canis* were detected using, buffered acidified plate antigen (BAPA) and agar gel immunodiffusion (AGID)., PCR was performed using the *bcsp 31*, *BruAb2_0168*,

and *BR00953* genes to detect *Brucella* spp., *B. abortus* and *B. canis* DNA, respectively. Sequencing was done to confirm the PCR results in three *bcsp 31* PCR products and compared with sequences deposited in GenBank. The seropositivity rates of 7.8 per cent and 9 per cent were observed for the AGID and BAPA tests, respectively. Positivity rates of 45.5 per cent and 10.8 per cent were observed when testing *bcsp 31* and *BruAb2_0168*, respectively, while there was no positivity for *BR00953*. The sequenced products had 110 base pairs that aligned with 100% identity to *B. abortus*, *B. canis*, and *B. suis*.

Jamil *et al*. (2019) collected a total of 181 serum samples from stray and working dogs in two different prefectures *viz*. Faisalabad (n = 87) and Bahawalpur (n = 94). Presence of antibodies against *B. canis* and *B. abortus/B. melitensis* was determined using the slow agglutination test (SAT) and ELISA, respectively. Real-time PCR was performed to detect and differentiate Brucella DNA at the species level. In Faisalabad, the serological prevalence was found to be 9.2 per cent (8/87) and 10.3 per cent (9/87) by SAT and ELISA, respectively. Only one of the ELISA positive samples (1.15%) yielded amplification for *B. abortus* DNA. In Bahawalpur, 63.8 per cent (60/94) samples were found positive by SAT; however, none of the samples was positive by ELISA or by real-time PCR.

Mol *et al*. (2020) evaluated 254 samples by PCR and found 31 (12.20 %) of the samples were -positive for Brucella sp. using, vaginal swabs, placental tissue and whole blood, which was more accurate than other tests employed *viz*., AGID, CFT, RBPT and 2ME MAT.

Alamian and Dadar (2020) sampled 173 dog blood specimens from herding dogs in three different provinces including Tehran (n = 127), Qom (n = 40) and Alborz (n = 6)

provinces. The presence of Brucella antibodies was determined using Rose Bengal plate test (RBPT), slow agglutination test (SAT) and 2-mercaptoethanol (2-ME), respectively. The seropositive samples were further screened using blood culture and PCR tests to identify and differentiate the implicated Brucella species. They found that, 24.3 per cent (42/173), 13.8 per cent (24/173) and 6.3 per cent (11/173) of blood samples tested positive using RBPT, SAT and 2-ME, respectively. However, among 42 seropositive samples, only 38.1 per cent (16/42) and 14.2 per cent (6/42) were positive by PCR and culture, respectively. *Brucella melitensis* biovar 1 and biovar 2 was isolated from the bacterial cultures of 6 blood samples and confirmed by bio typing, AMOS PCR and Bruce-ladder PCR assays.

2.1.6.5 Haematological parameters

The results of routine laboratory tests included complete blood count (CBC), serum biochemical tests and urinalysis are often within physiological reference ranges in dogs with brucellosis. However, the analysis of haematobiochemical parameters may show leukocytosis due to a neutrophilia, hyperglobulinemia, and hypoalbuminemia (Davidson and Sykes, 2014; Ledbetter *et al*., 2009). Degenerative left shifts, monocytosis, and/or lymphopenia have been uncommonly reported (Henderson *et al*., 1974; Dawkins *et al*., 1982).

Villalba *et al*. (1990) noticed pronounced leukocytosis in the acute phase of infection (first 3-4 weeks) and the counts then fell to leukopenic values at 2.5-3 months. When they studied weekly haemogram of 3 dogs for 3 months after innoculationg them with *B. canis* organisms.

Kustritz (2003) and Leadbetter *et al*. (2009) reported similar non-significant complete blood count and serum biochemistry; however, an elevated TLC of $24.50x10^3$/cmm with neutrophil count of 82.86 per cent has been reported (Leadbetter *et al*., 2009).

Non-significant variations or the haematobiochemical parameters in normal physiological limit in brucella positive dogs but with clinical symptoms of Pyogranulomatous dermatitis (Dawkins *et al*., 1982), Uveitis (Blouin, 1984) has been reported.

Total leukocyte count of 9.3×10^3/cmm in brucella positive male dogs with high titres suffering from disco-spondylitis and orchitis has been reported (Anderson and Binnington, 1983) and the TLC was within the physiological range. They have also recorded the non-significant variation in TEC (7.18×10^6/cmm), Hb (17.8 g/dL), HCT (47%), basophils (0%), eosinophils (6%), neutrophils (54%), lymphocytes (30%) and monocytes (10%) values in brucella positive male dogs.

A non-significant variation in haematobiochemical parameters of dogs with RBPT positive and negative has been reported by Suja (2014).

Materials and Methods

III. MATERIALS AND METHODS

Female dogs presented to the Department of Veterinary Gynaecology and Obstetrics clinics, Veterinary College, Hebbal, Bengaluru from January 2021 to December 2021 were categorized based on the complaint and / or history of infertility in female dogs. **Group I** consisted of the dogs with complaints of abortion, **Group II** dogs had history of abortion and mummification, whereas dogs with complaint of premature/still birth and conception failure were included in **Group III.**

Permission was obtained from institutional animal ethics committee (No, VCH/IAEC/2021/38).

3.1 Materials

3.1.1 Collection of samples

About 5 mL of blood was collected from cephalic vein using fresh sterilized syringes and needles and was stored in EDTA and serum vacutainers (Levram Lifesciences, Silvassa India) at room temperature until the serum was separated. Further, blood in serum vacutainers was centrifuged at 2000 rpm for 10-15 minutes for complete separation of serum. Vaginal washings/discharges/tissues (placenta/liver/spleen) were collected in cryovials (M/s. Tarsons Ltd). These samples were stored in deep freezer (-20 ^{o}C) until further examination (Plate-1).

3.1.2 quickVET Canine Brucella Antibody Rapid Test Kit

The quickVET Canine Brucella Antibody Rapid Test Kit procured from ubio Biotechnology Systems Pvt. Ltd, Cochin, Kerala was used for the detection of *Brucella canis* antibodies in serum for initial screening.

3.1.3 Haematobiochemical analysis

Hematological parameters were determined using semiautomatic hematology analyser (Mindray BC- 2800Vet, India) using diluting, rinsing and lysing reagents procured from Hospimed Diagnostics, Bengaluru (Plate 2).

Biochemical parameters were determined with the help of biochemical analyser (Micro Lab, Ahmedabad) using commercially available reagent kits procured from Erba Mannheim®, Transasia Biomedicals Ltd., Sikkim (Plate 3).

3.1.4 PCR Assay

Plastic wares used in the present study were procured from M/s. Tarsons Ltd. Oligonucleotide primers were custom synthesized from Eurofins Genomics India Pvt. Ltd., Bengaluru and Bioserve Biotechnologies (India) Pvt. Ltd., Bengaluru. Biological deep freezer (Haier, bioline technologies, India) Biosafety cabinet (Thermofischer), digital electronic balance (Shimadzu BL2204), Eppendorf centrifuge 5430R and Eppendorf minispin were used for processing samples. DNA Extraction kit from Favorgen and EmaraldAMP® GT PCR master mix, Japan for PCR were used. Eppendorf flexlid nexus GX2 thermal cycler was used to perform PCR (Plate 5).

Plate 1: Deep freezer (Haier)

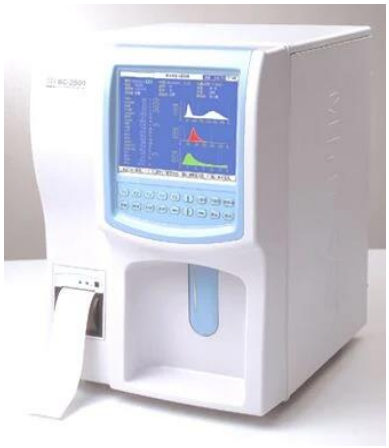

Plate 2: Mindray BC- 2800Vet, India

Plate 3: Biochemical analyser Micro Lab, Ahmedabad

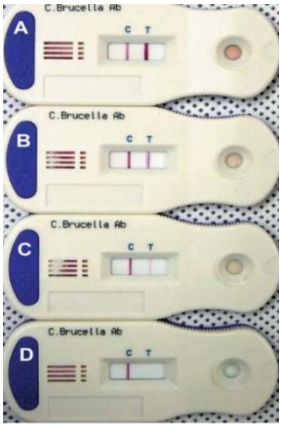

Plate 4: Canine Brucella Cassette T-Test Line (Canine Brucella Ag), C- Control Line (Goat Anti Mouse) Photo adapted from Kim *et al*, (2007)

Plate 5: Eppendorf flexlid nexus GX2 thermal cycler

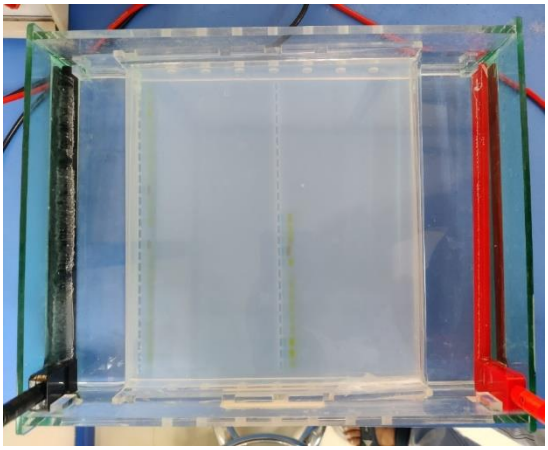

Plate 6: Horizontal electrophoresis unit

Gel electrophoresis on 2 per cent agarose gel (GeNei^{TM} GeNei laboratories Pvt Ltd. Bengaluru), was carried out by loading 10 µL of PCR product with a 100-bp ladder (Bioneer Co., Taejon, South Korea) as a molecular size marker and ethidium bromide (10 mg/mL) (HiMedia laboratories Pvt Ltd, India) for staining in a horizontal electrophoresis unit (GeNei laboratories Pvt Ltd. Bengaluru) (Plate-6). Stained gels were visualized and photographed under UV light with a UV transilluminator (Bio-Rad Laboratories, Milan, Italy) with image lab software.

Plastic ware like micropipette tip, micro centrifuge tube, and PCR tubes were sterilized by autoclaving at 15 psi at 121°C for 15 minutes.

3.2 Immunochromatographic assay

quickVET Canine Brucella Antibody Rapid Test Kit, a semi-quantitative immunochromatographic assay was used for the detection of *Brucella canis* antibodies in serum for initial screening.

3.2.1 Test principle

The test works on chromatographic immunoassay. Basic components of test strip include: a) Conjugate pad, which contains detection antibody (colloidal gold conjugated), b) A nitrocellulose membrane strip containing two lines

Test sample that is added to the sample well, with adequate amount of buffer migrates from the sample pad along the conjugate pad, where any antibody present in the sample will bind to the colloidal gold conjugate. The sample then continues to migrate across the membrane until it reaches the capture zone, where the antibody-antibody

conjugate complex will bind to the immobilized Canine Brucella Antigen (on test line) producing a visible line on the membrane. If the respective antibody is not present in the sample, no reaction occurs in the capture zone and no test line is formed in the zone corresponding to Antigen. The sample then migrates further along the strip until it reaches the control zone, where it produces a second visible line on the membrane. This control line indicates that the sample has migrated across the membrane as intended.

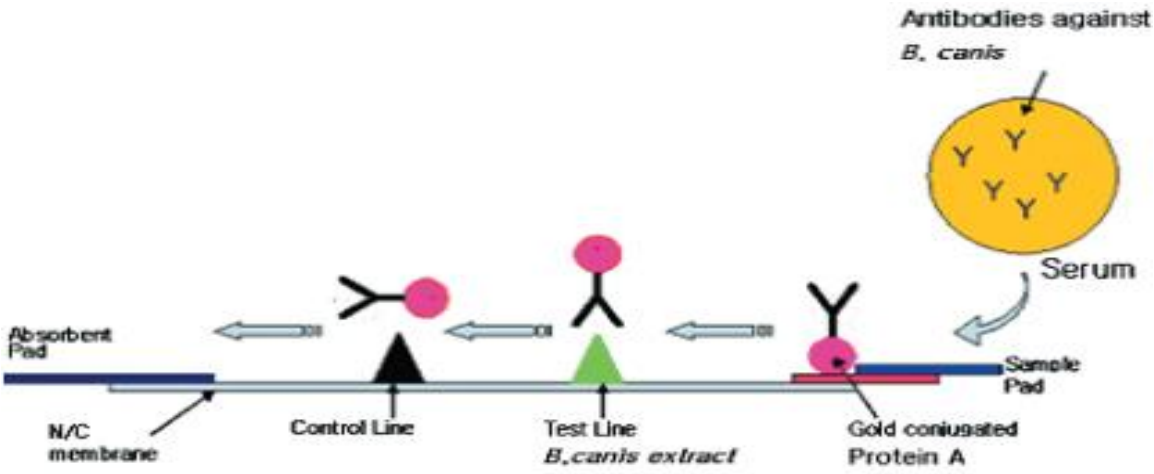

Figure 1: Principle of ICA (Photo adapted from Kim *et al*, (2007)

3.2.2 Test procedure

1. Test card was placed on a horizontal surface.
2. 10µL of the specimen was aspirated using the dropper provided and was added into the sample hole 'S'.
3. Two drops of diluent was added after the sample was completely absorbed.
4. Test was interpreted after 10 minutes.

3.2.3 Interpretation of test result+

Positive: If color bands at position C and T, Canine Brucella antibody is present in the sample.

Negative: If color band only at position C. Canine Brucella antibody is not present in the sample. Invalid if color band at C does not appear.

3.3 Determination of haematological parameters

The peripheral blood collected in EDTA vacutainer was subjected to immediate haematological examination using semi-automatic haematology analyser and the values were expressed as follow

a. Total Erythrocyte Count (10^6 /cmm)

b. Total Leucocyte Count (10^3 /cmm)

c. Differential Leukocyte Count (%)

d. Haemoglobin (g/dL)

e. Packed Cell Volume (%)

f. Thrombocyte count (10^3 /cmm)

3.4 Determination of biochemical profile

Following blood biochemical parameters were determined with the help of biochemical analyser using commercially available reagent kits.

a. Blood Urea Nitrogen (mg/dL)

b. Serum creatinine (mg/dL)

3.5 Polymerase chain reaction (PCR)

Plastic wares like micropipette tip, micro centrifuge tube and PCR tubes were sterilized by autoclaving at 15 psi at 121°C for 15 minutes.

3.5.1 Isolation of Genomic DNA from blood

The procedure for the genomic DNA extraction from blood is as follows:

1) 200 µL sample was transferred to a microcentrifuge tube.
2) 20 µL Proteinase K was added to the sample, and then 200 µL FATG2 buffer was added to the sample.
3) It was incubated at 60 °C for 30 min after mixing it thoroughly by pulse-vortexing. Occasional vortexing during incubation was done.
4) Later the mixture was incubated at 70 °C for 10 min.
5) 200 µL ethanol (96-100%) was added to the sample mixture and mixed thoroughly by pulse-vortexing.
6) The tube was briefly spinned to remove drops from the inside of the lid.
7) FATG Mini Column was placed in a collection tube and mixture was transferred to it. It was centrifuged at full speed (~18,000 x g) for 1 min Column and placed in to a new collection tube.

8) 400 µL W1 Buffer was added to the FATG Mini Column and centrifuged at full speed for 1 min then flow-through was discarded.

9) 750 µL Wash Buffer was added to the FATG Mini Column and centrifuged at full speed for 1 min, then the flow-through was discarded.

10) Centrifugation was done at full speed for an additional 3 min to dry the column.

11) 100 µL of preheated Elution buffer or ddH_2O (pH 7.5-9.0) was added to the membrane of the FATG Mini Column. The FATG Mini Column was kept static for 3 min.

12) Centrifuged at full speed for 2 min to elute DNA.

3.5.2 Isolation of genomic DNA from Animal Tissue

The steps for the genomic DNA extraction from the animal tissue is as follows:

1. 25 mg of tissue sample was cut and transferred to a microcentrifuge tube. Tissue was ground using a Micro pestle.

   Vaginal discharge was centrifuged at 7500 rpm for 10 min and the supernatant was discarded completely.

2. 200 µL of FATG1 Buffer was added and mixed well using Micro pestle or pipette tip.

3. 20 µL Proteinase K (10mg/ml) was added to the sample mixture and was mixed thoroughly by vortexing.

4. Incubation was done at 60ºC until the tissue was lysed completely (1-3 h). Vortexing was done occasionally during incubation. Sample was also incubated overnight for complete lysis.

5. 200 µL of FATG2 Buffer was added to the sample mixture, mixed thoroughly by pulse-vortexing and was incubated at 70 °C for 10 min.
6. 200 µL of ethanol (96-100%) was added to the sample mixture and mixed thoroughly by pulse-vortexing.
7. The tube was briefly spinned to remove drops from the inside of the lid.
8. FATG Mini Column was placed in a collection tube and the mixture (including any precipitate) was transferred to it. Centrifugation was done at full speed (~18,000 x g) for 1 min then the FATG Mini Column was placed to a new Collection Tube.
9. 400 µL of W1 Buffer was added to the FATG Mini Column and centrifugation was done at full speed for 1 min then the flow-through was discarded.
10. 750 µL of Wash Buffer was added to the FATG Mini Column and centrifugation was done at full speed for 1 min then the flow-through was discarded.
11. Centrifugation was done at full speed for an additional 3 min to dry the column. Important Step! This step removes the residual liquid.
12. 100 µL of preheated Elution Butter or ddH_2O (pH 7.5-9.0) was added to the membrane of the FATG Mini Column and kept static for 3 min.
13. Centrifugation was done at full speed for 2 min to elute the DNA.

3.5.3 Confirmation of Brucella genus using PCR

Brucella genus was confirmed by using B4 and B5 primers (Baily *et al*., 1992), which encodes a protein of Brucella abortus 31 kDa, *bcsp* 31. *bcsp* 31-PCR reaction

consisted of 10 µL 2x PCR master mix, 3 µg DNA template, 1 µL (10 pmol) of each primer, and nuclease free water up to 20 µL (Table 1).

3.5.4 Confirmation of Brucella species using AMOS PCR

Multiplex PCR was performed for the confirmation of *Brucella abortus*, *Brucella militensis, Brucella ovis* and *Brucella suis* using AMOS PCR. Each DNA amplification was performed in the 25 µL reaction mixture consisting 3 µL DNA template, 12.5 µL PCR master mix, 1 µL of each forward nucleotide primers (10 pmol), 2 µL of reverse nucleotide primer and water to make up to 25 µL (Table 2).

3.5.5 Confirmation of *Brucella canis* using species specific PCR

Species specific PCR reaction was performed for the confirmation of *Brucella canis* using a 20-µL reaction mixture containing 3 µL of template DNA and 1 µL of each of the primers (10 pmol) (Table 3).

3.5.6 Confirmation by agarose gel electrophoresis

Gel electrophoresis on 2 per cent agarose gel was carried out by loading 10 µL of PCR product. A 100-bp ladder (Bioneer Co., Taejon, South Korea) as a molecular size marker and ethidium bromide (10 mg/mL) used for staining. Stained gels were visualized and photographed under UV light with a UV transilluminator (Bio-Rad Laboratories, Milan, Italy).

Table 1: Primers and PCR conditions for Brucella genus specific PCR

Target gene	Sequence (5' to 3')	Size (bp)	Reference
bcsp 31	B4: 5' -TGGCTCGGTTGCCAATATCAA-3' B5: 5 -CGCGCTTGCCTTTCAGGTCTG-3	223	Garshasbi *et al.* (2014)

Cycling conditions				
Initial Denaturation	**Denaturation**	**Annealing**	**Extension**	**Final extension**
94 ˚C for 5 min	94 ˚C for 1min	64 ˚C for 30 s	72 ˚C for 1min	72 ˚C for 7 min
	Repeat for 35 cycles			

Table 2: Primers and PCR conditions for Species specific (AMOS) PCR

Target gene	Sequence (5' to 3')	Size (bp)	Reference
BA	5' -GAC GAA CGG AAT TTT TCC AAT CCC- 3'	498	Bricker *et al.* (2003)
BM	5' -AAA TCG CGT CCT TGC TGG TCT GA- 3'	731	
BO	5' -CGG GTT CTG GCA CCA TCG TCG- 3'	976	
BS	5' -GCG CGG TTT TCT GAA GGT GGT TCA GG- 3'	285	
IS711	R-5' -TGC CGA TCA CTT AAG GGC CTT CAT- 3'		

Cycling conditions				
Initial Denaturation	**Denaturation**	**Annealing**	**Extension**	**Final extension**
94 ˚C for 5 min	95 ˚C for 1min	55.5 ˚C for 2 min	72 ˚C for 2 min	72 ˚C for 10 min
	Repeat for 35 cycles			

Table 3: Primers and PCR conditions for *Brucella canis* specific PCR

Target gene	Sequence (5' to 3')	Size (bp)	Reference
B. canis specific gene	F- 5' -CCAGATAGACCTCTCTGGA-3' R- 5' -TGGCCTTTTCTGATCTGTTCTT-3'	300	Kang *et al.* (2014)

Cycling conditions				
Initial Denaturation	**Denaturation**	**Annealing**	**Extension**	**Final extension**
94 ˚C for 7 min	94 ˚C for 35 s	59 ˚C for 40 s	72 ˚C for 35 s	72 ˚C for 10 min
	Repeat for 35 cycles			

The amplified PCR products were checked in 2% agarose gel along with the 100bp DNA ladder as molecular size marker. Electrophoresis was performed at 5 V/cm for one hour. The gels were visualized under UV light with an UV transilluminator and recorded in the gel documentation system.

3.5.6.1 Procedure

a. Required quantity of Agarose was weighed and dissolved in proportionate volume of 0.5X TBE buffer and melted in microwave oven for one minute or until agarose was completely dissolved into a uniform suspension.

b. The gel tray was sealed at both ends using a casting damp clip. The comb was placed into gel tray in appropriate position.

c. Ethidium bromide 10mg/mL was added to the molten agarose when it cooled approximately to 50 ˚C and poured into the gel tray carefully, avoiding air bubbles.

d. The comb and casting damp clip were removed after solidification.

e. The gel tray was held in electrophoresis tank and 0.5X TBE buffer was poured to submerge the gel in the tank.

f. The PCR products were carefully loaded into wells using micropipette and 2 µL of 100bp DNA ladder was added alongside the sample wells.

g. The electrophoresis was carried out at 5 V/cm at room temperature till the dye had reached the end of the gel and the bands were visualized with the help of gel documentation system and images were captured.

3.6 Statistical Analysis

Haematobiochemical parameters of positive and negative cases in different groups were evaluated using t-test and between positive cases of different groups using one-way ANOVA. Relative efficacy of ICA was derived as per the methods described by Pieterse *et al*. (1990).

Results

IV. RESULTS

A study was conducted in the dogs presented with complaint of abortion (Group I), complaints/history of abortion and mummification (Group II), premature/still birth and conception failure (Group III) presented to the Dept. of Veterinery Gynaecology and Obstetrics clinics of Veterinary College, Hebbal, Bengaluru. Initially, blood samples collected from the dogs with the above complaints/history were subjected for haematobiochemical analysis using semiautomatic hematology analyser and biochemical analyser and also were screened for *B.canis* using immunochromatographic assay using quickVET rapid kits. The tissues collected included vaginal discharge or washings, aborted tissues like placenta or fetus and blood for PCR assay. The results are portrayed in Table 4-8.

4.1 Haematobiochemical profile

The mean values of TEC, hemoglobin, HCT, platelet, TLC, DLC, ALT and creatinine are presented in Table 4-8.

4.1.1 Total erythrocyte count (TEC)

The mean total erythrocyte count (TEC) levels of dogs recorded in brucella positive and negative were 4.93±0.31 and 5.28 ± 0.35 $x10^6$/cmm, respectively and the difference was non-significant.

In the dogs found positive for brucella in Group I, Group II and Group III, the mean TEC level recorded was 4.71 ± 0.41, 5.48 ± 0.41 and 5.33 ± 0.74 x 10^6/cmm, respectively. Whereas, the mean level of TEC in brucella negative dogs of Group I, Group II and Group

III was respectively 4.65 ± 0.56, 5.84 ± 0.47 and 5.74 ± 0.53 x10^6 /cmm. Further, there was no significant variation between either the groups or within groups.

4.1.2 Haemoglobin (Hb)

The mean haemoglobin levels of dogs recorded in brucella positive and negative were 10.49 ± 0.70 and 11.93 ± 0.88 g/dL, respectively and the difference was non-significant.

In the dogs found positive for brucella in Group I, Group II and Group III, the mean haemoglobin levels recorded was 9.89 ± 0.84, 11.70 ± 1.31 and 11.82 ± 2.10 g/dL, respectively. Whereas, the mean level of haemoglobin in brucella negative dogs of Group I, Group II and Group III was respectively 9.84 ± 1.20, 13.59 ± 1.01and 13.85 ± 1.25 g/dL. Further, there was no significant variation between either the groups or within groups.

4.1.3 Haematocrit (HCT)

The mean percentage of haematocrit levels of dogs recorded in brucella positive and negative were 32.18 ± 2.16 and 34.30 ± 2.87 per cent, respectively and the difference was non-significant.

In the dogs found positive for brucella in Group I, Group II and Group III, the mean haematocrit level recorded was 30.75 ± 2.90, 35.73 ± 4.17 and 34.53 ± 4.47 per cent, respectively. Whereas, the mean level of HCT in brucella negative dogs of Group I, Group II and Group III was respectively 29.82 ± 5.50, 37.78 ± 2.94 and 38.55 ± 2.35 per cent. Further, there was no significant variation between either the groups or within groups.

4.1.4 Platelet count (PLT)

The mean platelet counts of dogs with brucella positive cases obtained was 291.10 ± 30.14 and 233.70 ± 49.76 $x10^3$/cmm in negative cases without any significant difference.

In Group I, Group II and Group III, mean platelet count of dogs found positive and negative for brucella was 305.70 ± 42.80 vs.199.60 ± 92.75, 284.00 ± 37.03 vs. 256.00 ± 82.08 and 241.80 ± 33.83 vs. 274.5 ± 64.50$x10^3$ /cmm, respectively. There was no significant difference between either the groups or within groups.

4.1.5 Total leukocyte count (TLC)

The mean total leukocyte count recorded in dogs that were found positive and negative for brucellosis was 25.95 ± 4.48 and 12.91 ± 1.54$x10^3$ /cmm, respectively and significant difference ($p<0.05$) between the positive and negative cases was found (Table 4).

The mean total leukocyte counts of dogs found positive and negative for brucella in Group I, Group II and Group III were recorded as 24.88 ± 5.29 vs. 16.82 ± 2.20, 39.15 ± 17.31 vs. 9.09 ± 0.83 $x10^3$ and 14.79 ± 3.52 vs.10.80 ± 2.40 $x10^3$ /cmm, respectively and the TLC did not vary significantly between either the groups or within groups.

4.1.6 Differential leukocyte count (DLC)

The differential leukocyte counts in dogs found brucella positive and negative did not vary significantly ($p>0.05$). Further, the percentages of DLC of dogs within or between the groups were non-significant (Table 4-8).

4.1.7 Alanine amino transferase (ALT)

The Alanine amino transferase (ALT) levels of dogs with brucella positive cases obtained was 44.04 ± 6.69 and 38.80 ± 9.15 U/L in negative cases without any significant difference between them.

In Group I, Group II and Group III, the mean ALT levels (U/L) of dogs found positive and negative for brucella was 39.17 ± 7.00 vs. 21.64 ± 2.55, 35.34 ± 11.79 vs. 38.30 ± 8.36 and 66.38 ± 22.34 vs. 81.50 ± 39.50, respectively. There was no significant difference between the brucella positive and negative cases of each group.

4.1.8 Creatinine

The mean creatinine levels (mg/dL) of dogs with brucella positive and negative cases obtained was 1.20 ± 0.11 and 1.31 ± 0.16, respectively and did not observe any significant difference.

In Group I, Group II and Group III, mean creatinine levels of dogs found positive and negative for brucella was 1.05 ± 0.08 vs. 1.14 ± 0.09, 1.24 ± 0.14 vs. 1.53 ± 0.36 and 1.67 ± 0.40 vs. 1.18 ± 0.24 (mg/dL), respectively and the difference between either the groups or within groups were non-significant.

Table 4: Mean Haematobiochemical parameters of dogs with or without brucellosis

Parameters	Positive (n=26)	Negative (n=18)
TEC (10^6 /cmm)	4.93 ± 0.31	5.28 ± 0.35
Hb (g/dL)	10.49 ± 0.70	11.93 ± 0.88
HCT (%)	32.18 ± 2.16	34.30 ± 2.87
PLT (10^3 /cmm)	291.1 ± 30.14	233.7 ± 49.76
TLC (10^3 /cmm)	25.95 ± 4.48[a]	12.91 ± 1.54[b]
N (%)	71.91 ± 2.08	70.04 ± 3.26
L (%)	21.25 ± 2.20	20.82 ± 1.30
M (%)	3.86 ± 0.24	4.48 ± 1.13
E (%)	3.18 ± 0.69	4.10 ± 4.10
B (%)	0.38 ± 0.17	0.40 ± 0.40
ALT (U/L)	44.04 ± 6.69	38.80 ± 9.15
CRT (mg/dL)	1.20 ± 0.11	1.31 ± 0.16

Note: The Mean values bearing different superscripts (a, b) within the row vary significantly ($p<0.05$)

Table 5: Mean Haematobiochemical parameters of dogs with complaint of abortion with or without brucellosis (Group I)

Parameters	Positive (n=18)	Negative (n=9)	Significance
TEC (10^6 /cmm)	4.71 ± 0.41	4.65 ± 0.56	NS
Hb (g/dL)	9.89 ± 0.84	9.840 ± 1.20	NS
HCT (%)	30.75 ± 2.90	29.82 ± 5.50	NS
PLT (10^3 /cmm)	305.70 ± 42.80	199.6 ± 92.75	NS
TLC (10^3 /cmm)	24.88 ± 5.29	16.82 ± 2.20	NS
N (%)	73.08 ± 2.69	75.48 ± 1.78	NS
L (%)	19.93 ± 2.71	19.28 ± 1.25	NS
M (%)	3.72 ± 0.33	4.950 ± 0.95	NS
E (%)	4.50 ± 0.20	5.10 ± 3.30	NS
B (%)	0.50 ± 0.20	0.30 ± 0.15	NS
ALT (U/L)	39.17 ± 7.00	21.64 ± 2.55	NS
CRT (mg/dL)	1.05 ± 0.08	1.14 ± 0.09	NS

Note: NS = Non-significant ($p>0.05$)

Table 6: Mean Haematobiochemical parameters of dogs presented with history of abortion and mummification with or without brucellosis (Group II)

Parameters	Positive (n=3)	Negative (n=4)	Significance
TEC (10^6 /cmm)	5.48 ± 0.41	5.84 ± 0.47	NS
Hb(g/dL)	11.70 ± 1.31	13.59 ± 1.01	NS
HCT (%)	35.73 ± 4.17	37.78 ± 2.94	NS
PLT (10^3 /cmm)	284.00 ± 37.03	256.00 ± 82.08	NS
TLC (10^3 /cmm)	39.15 ± 17.31	9.09 ± 0.83	NS
N (%)	73.87 ± 5.85	72.27 ± 4.57	NS
L (%)	19.23 ± 7.23	19.27 ± 1.29	NS
M (%)	4.53 ± 0.59	7.13 ± 3.20	NS
E (%)	4.30 ± 0.20	8.10 ± 4.10	NS
B (%)	0.70 ± 0.20	0.40 ± 0.40	NS
ALT (U/L)	35.34 ± 11.79	38.30 ± 8.36	NS
CRT (mg/dL)	1.24 ± 0.14	1.53 ± 0.36	NS

Note: NS = Non-significant ($p>0.05$)

Table 7: Mean Haematobiochemical parameters of dogs presented with complaint of still birth, premature birth and conception failure with or without brucellosis (Group III)

Parameters	Positive (n=5)	Negative (n=5)	Significance
TEC (10^6 /cmm)	5.33 ± 0.74	5.74 ± 0.53	NS
Hb(g/dL)	11.82 ± 2.10	13.85 ± 1.25	NS
HCT (%)	34.53 ± 4.47	38.55 ± 2.35	NS
PLT (10^3 /cmm)	241.80 ± 33.83	274.50 ± 64.50	NS
TLC (10^3 /cmm)	14.79 ± 3.52	10.80 ± 2.40	NS
N (%)	65.30 ± 0.44	53.10 ± 4.00	NS
L (%)	28.57 ± 1.32	27.00 ± 2.70	NS
M (%)	3.63 ± 0.27	4.15 ± 0.35	NS
E (%)	2.70 ± 0.20	3.10 ± 2.70	NS
B (%)	0.60 ± 0.30	0.20 ± 0.10	NS
ALT (U/L)	66.38 ± 22.34	81.50 ± 39.50	NS
CRT (mg/dL)	1.67 ± 0.40	1.18 ± 0.24	NS

Note: NS = Non-significant ($p>0.05$)

Table 8: Comparitive haematobiochemical parameters in positive cases of different groups (Mean ± SE)

Parameters	Group I (n=18)	Group II (n=3)	Group III (n=5)	Significance
TEC (10^6 /cmm)	4.71 ± 0.41	5.48 ± 0.41	5.33 ± 0.74	NS
Hb(g/dL)	9.89 ± 0.83	11.70 ± 1.31	11.82 ± 2.10	NS
HCT (%)	30.75 ± 2.90	35.73 ± 4.17	34.53 ± 4.47	NS
PLT (10^3 /cmm)	305.70 ± 42.80	284.00 ± 37.03	241.80 ± 33.83	NS
TLC (10^3 /cmm)	24.88 ± 5.29	39.15 ± 17.31	14.79 ± 3.52	NS
N (%)	73.08 ± 2.69	73.87 ± 5.85	65.30 ± 0.44	NS
L (%)	19.93 ± 2.71	19.23 ± 7.23	28.57 ± 1.32	NS
M (%)	3.72 ± 0.33	4.53 ± 0.59	3.63 ± 0.27	NS
E (%)	4.50 ± 0.20	4.30 ± 0.20	2.70 ± 0.20	NS
B (%)	0.50 ± 0.20	0.70 ± 0.20	0.60 ± 0.30	NS
ALT (U/L)	39.17 ± 7.00	35.34 ± 11.79	66.38 ± 22.34	NS
CRT (mg/dL)	1.05 ± 0.08	1.24 ± 0.14	1.67 ± 0.40	NS

Note: NS = Non-significant ($p>0.05$)

4.2 Immunochromatographic assay

Lateral flow immunochromatographic assay was performed for 44 suspected samples of canine brucellosis to detect the presence of antibodies to *B. canis*. All samples showed color band at control (C) line and no reaction in the test line was observed indicating that the samples were negative (Plate 12).

4.3 Polymerase Chain reaction (PCR)

4.3.1 Genus specific PCR

In the present study, genus specific PCR for detection of *Brucella* was carried out targeting *bscp31* gene. A total of 88 DNA samples (44 blood and 44 tissue samples) from 44 suspected animals were subjected to PCR. It was noticed that 9 blood samples and 9 tissue samples were found positive for Brucella. In addition, both blood and tissue samples from 8 animals were positive for Brucella. It was observed that samples from 26 animals either blood, tissue (vaginal discharge/washing/tissue) or both the samples amplified 223 bp size specific for Brucella (Plate 7 and 8). The PCR product was confirmed by sequencing and submitted to NCBI data base with accession number OL739266 (VCHVGO1). The per cent occurrence of Brucella in group I, II and III were 66.67 (18/27), 42.86 (3/7) and 50.00 (5/10), respectively. The overall occurrence of Brucella among the various dogs screened in the present study was 59.09 per cent (26/44), (Plate 7 and 8 and Table 9)

4.3.2 Species specific PCR (AMOS PCR)

In the present study all the samples which were positive for genus specific PCR was screened by AMOS PCR to detect the species. It was evident that 11 blood samples

and 4 tissue samples from the screened animals amplified 498 bp specific for *Brucella abortus* (Plate 9 and 10). In addition, both blood and tissues samples of 6 animals were positive for *Brucella abortus*. None of the samples screened in the present study were found to be positive for *Brucella melitensis, Brucella ovis* and *Brucella suis*. The overall occurrence of *Brucella abortus* in this study was 47.73 per cent (21/44), (Plate 9 and 10)

4.3.3 *Brucella canis* specific PCR

All the samples positive for presence of Brucella sp. were screened for *Brucella canis* by species specific PCR. It was observed that none of the DNA isolated from blood samples were positive, whereas 10 tissue samples amplified 300 bp specific for *B. canis* (Plate 11). The overall occurrence of *B. canis* in the present study was 22.73 per cent (10/44).

In the present study co-occurrence of *Brucella canis* and *Brucella abortus* was observed in 11.36 per cent (5 out of 44 animals screened). (Plate 11)

4.3.4 PCR: findings in different groups

In the present study 18 dogs were found positive for brucellosis in group I. Of these, 9 (50.00%) were found positive for *Brucella abortus*, 5 for *Brucella canis* and 4 for both the species. In group II, 3 dogs were positive for brucellosis. Where, 2 were positive for *Brucella abortus* and 1 dog was positive for both the species. Group III consisted of 5 dogs positive for *Brucella abortus*. (Table 10)

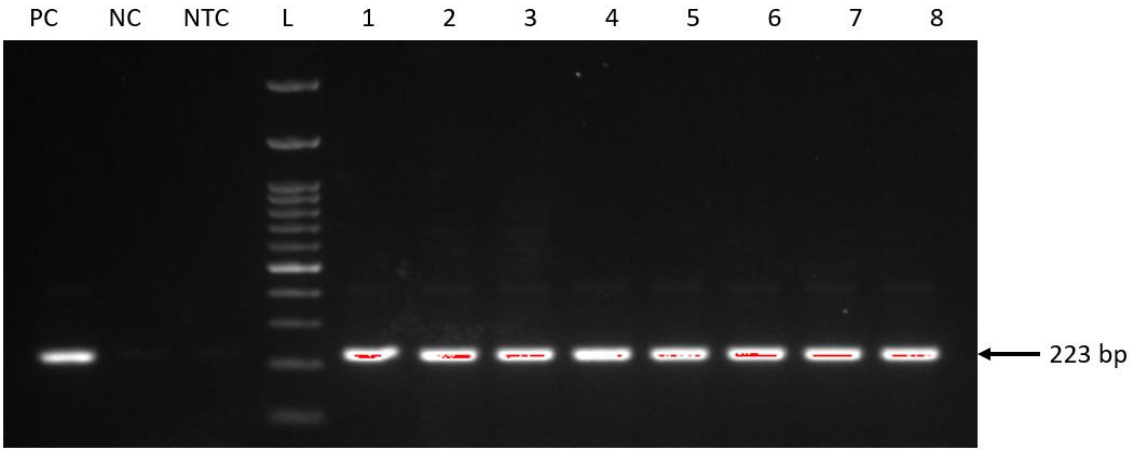

Plate 7: Amplification of *bcsp* 31 gene (223 bp) specific to Brucella (blood samples)

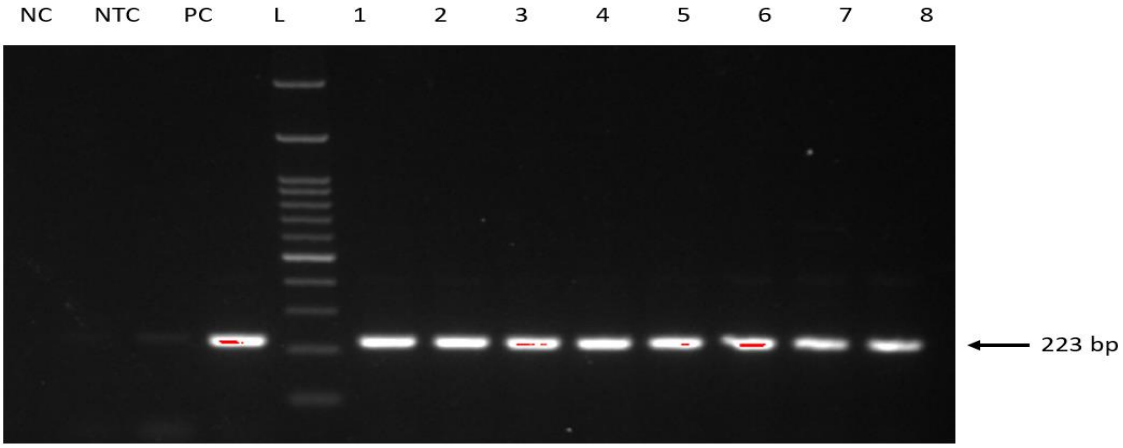

Plate 8: Amplification of *bcsp* 31 gene (223 bp) specific to Brucella (tissue samples)

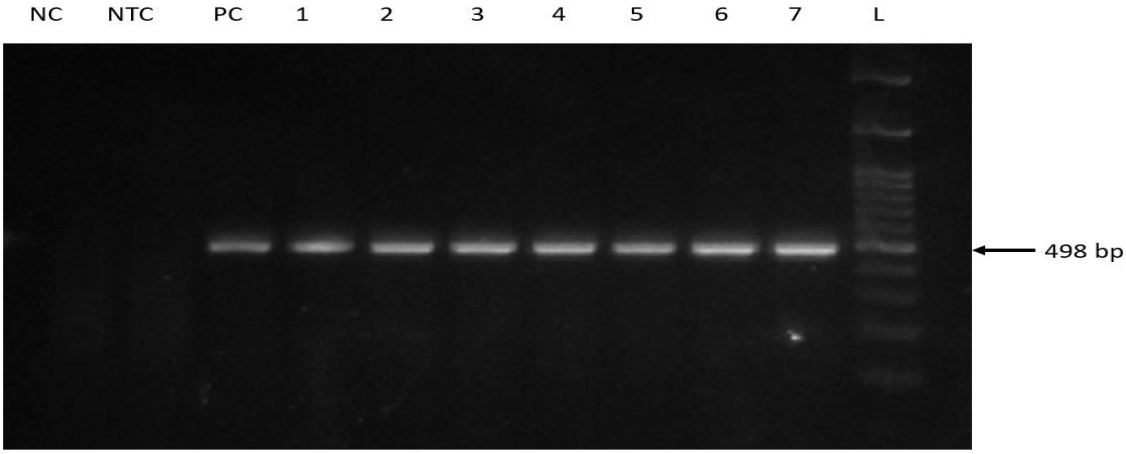

Plate 9: Amplification at 498 bp region specific to *Brucella abortus* (blood samples)

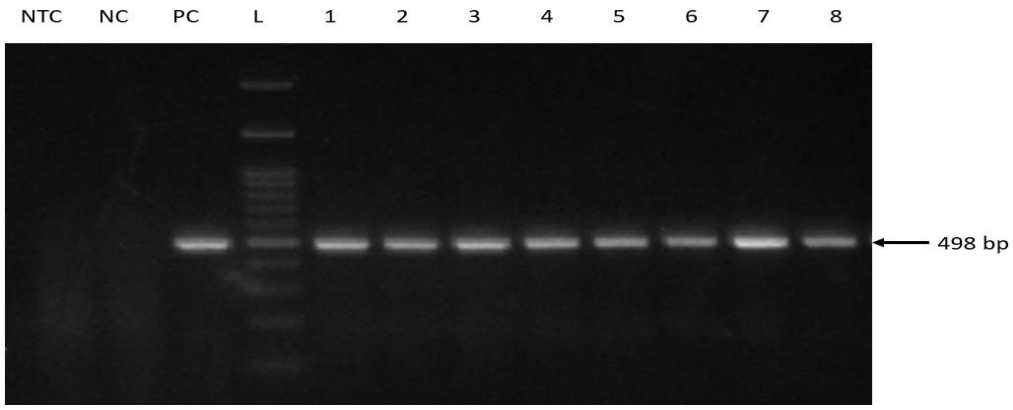

Plate 10: Amplification at 498 bp region specific to *Brucella abortus* (tissue samples).

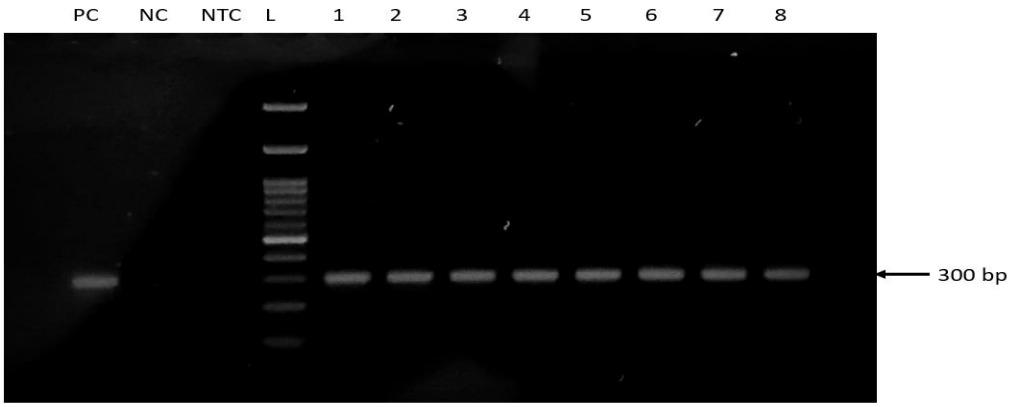

Plate 11: Amplification at 300 bp region specific to *Brucella canis* (tissue samples).

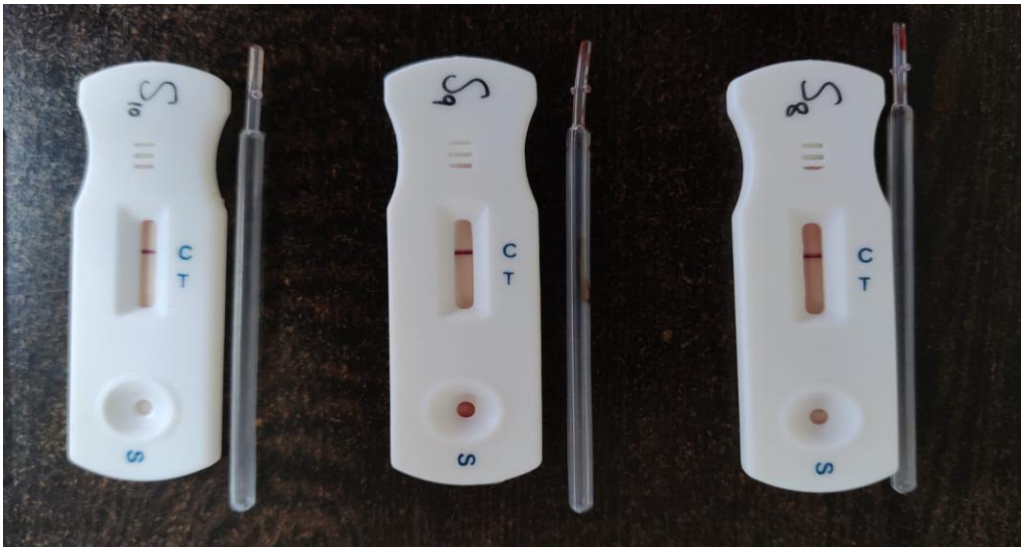

Plate 12: ICA cassette showing no reaction on test line (T) indicating negative results

Table 9: Classification of cases screened for Brucellosis (n=44)

Groups	Positive	Negative
Group I (27)	18 (66.67)	9 (33.33)
Group II (07)	3 (42.86)	4 (57.14)
Group III (10)	5 (50)	5 (50)
Total Cases (44)	26 (59.09)	18 (40.91)

Note: Values in the parentheses indicate percentage

Table 10: Number of positive cases detected by PCR assay

Groups	***B. abortus***	***B. canis***	Both
Group I (18)	9 (50.00)	5 (27.78)	4 (22.22)
Group II (03)	2 (66.67)	0	1 (33.33)
Group III (05)	5 (100)	0	0
Total positive cases (26)	16 (61.54)	5 (23.08)	5 (23.08)

Note: Values in the parentheses indicate percentage

Table 11: Number of *Brucella abortus* cases detected by PCR assay

Groups	Blood	Tissue	Both
Group I	8	2	3
Group II	1	1	1
Group III	2	1	2
Total positive cases (21)	11	4	6

Table 12: Number of *Brucella canis* cases detected by PCR assay

Groups	Blood	Tissue	Both
Group I	0	9	0
Group II	0	1	0
Group III	0	0	0
Total positive cases (10)	0	10	0

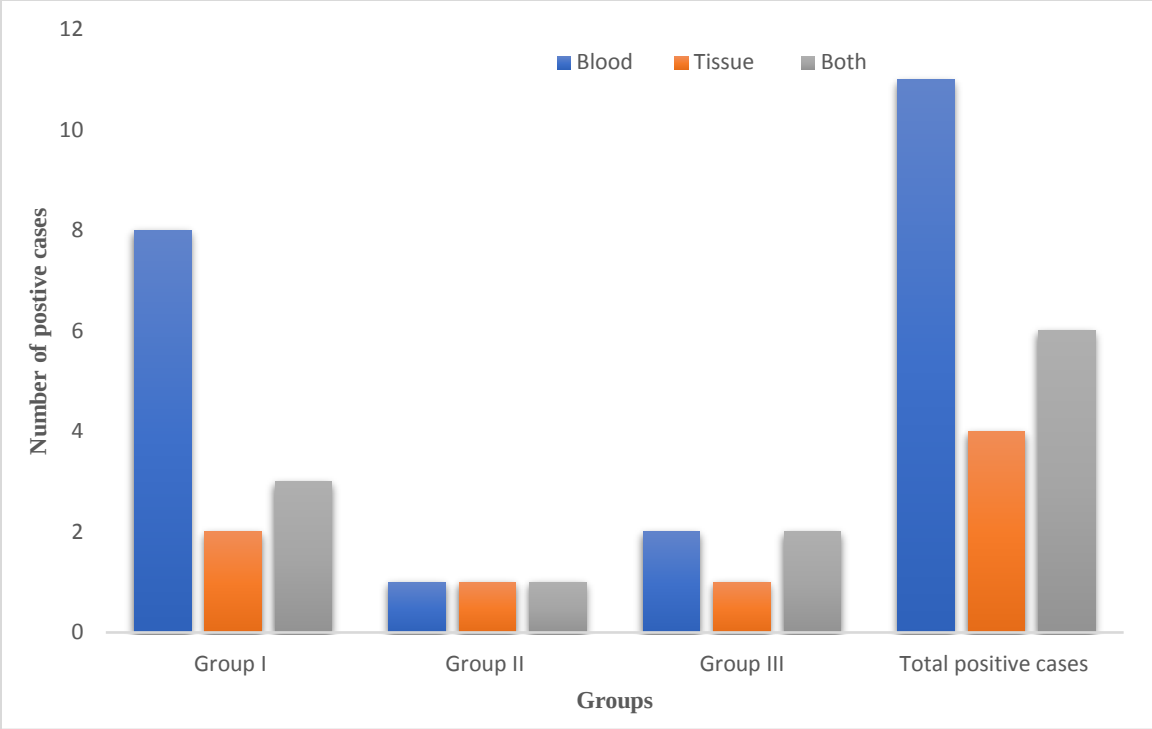

Figure 2: Number of *Brucella abortus* cases detected by PCR assay

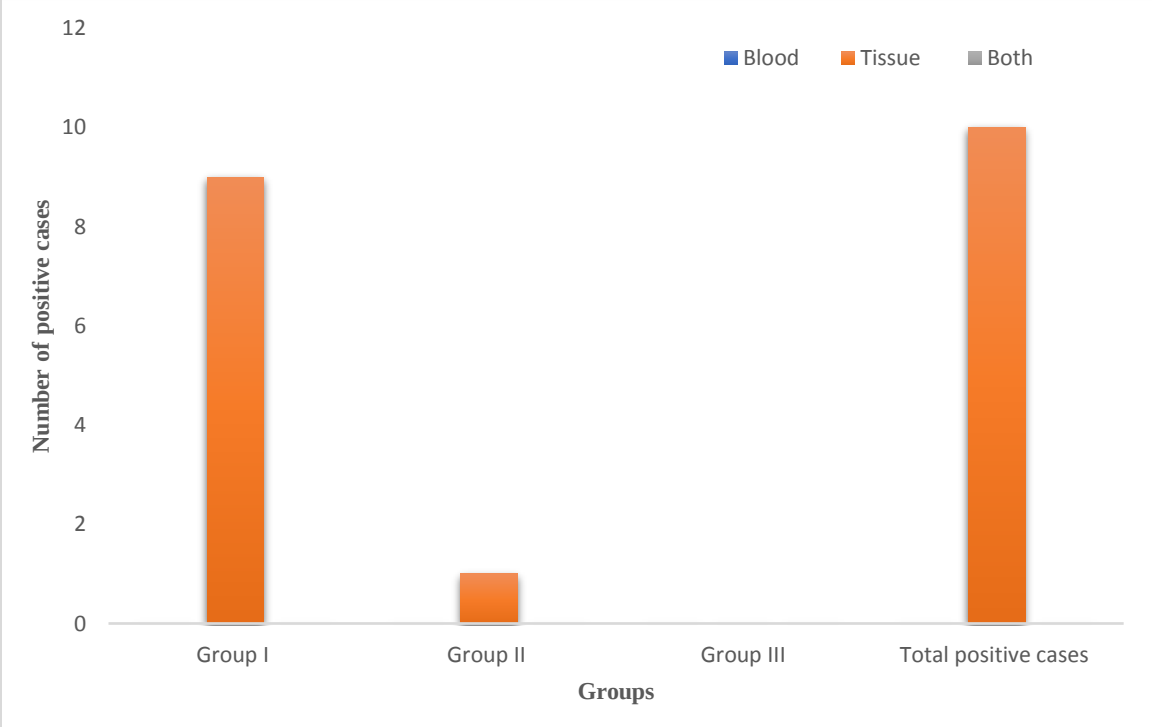

Figure 3: Number of *Brucella canis* cases detected by PCR assay

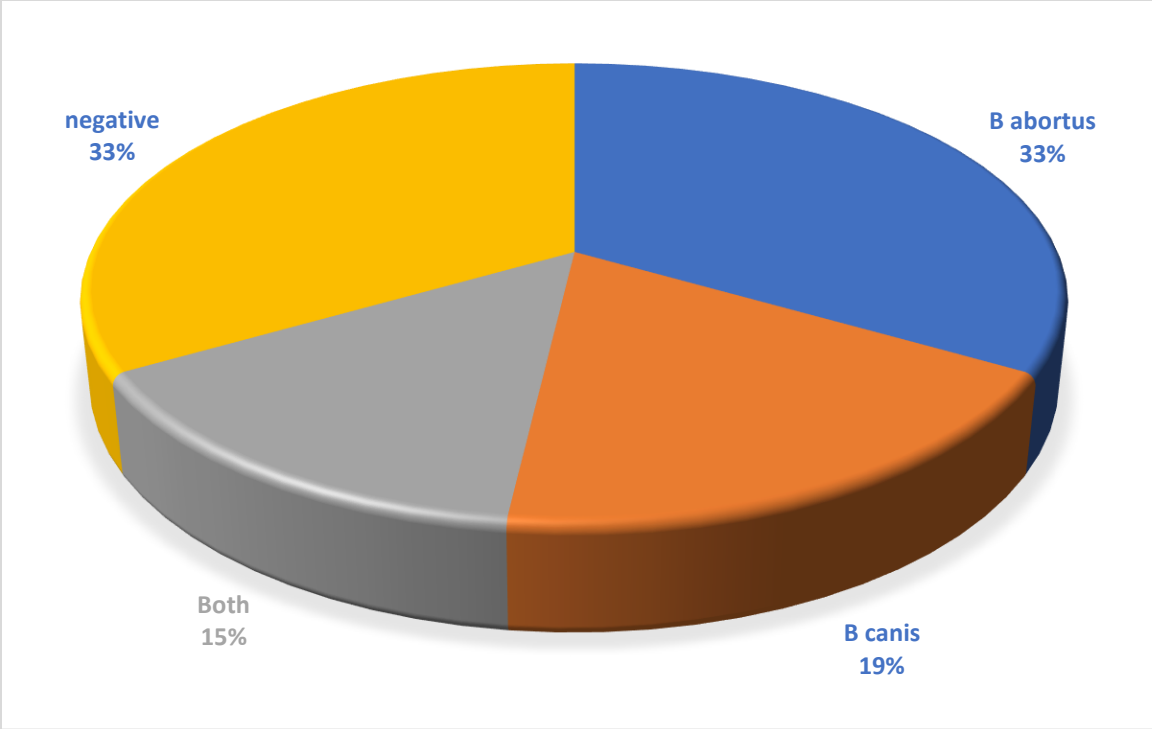

Figure 4: Positive cases detected by PCR assay in Group I

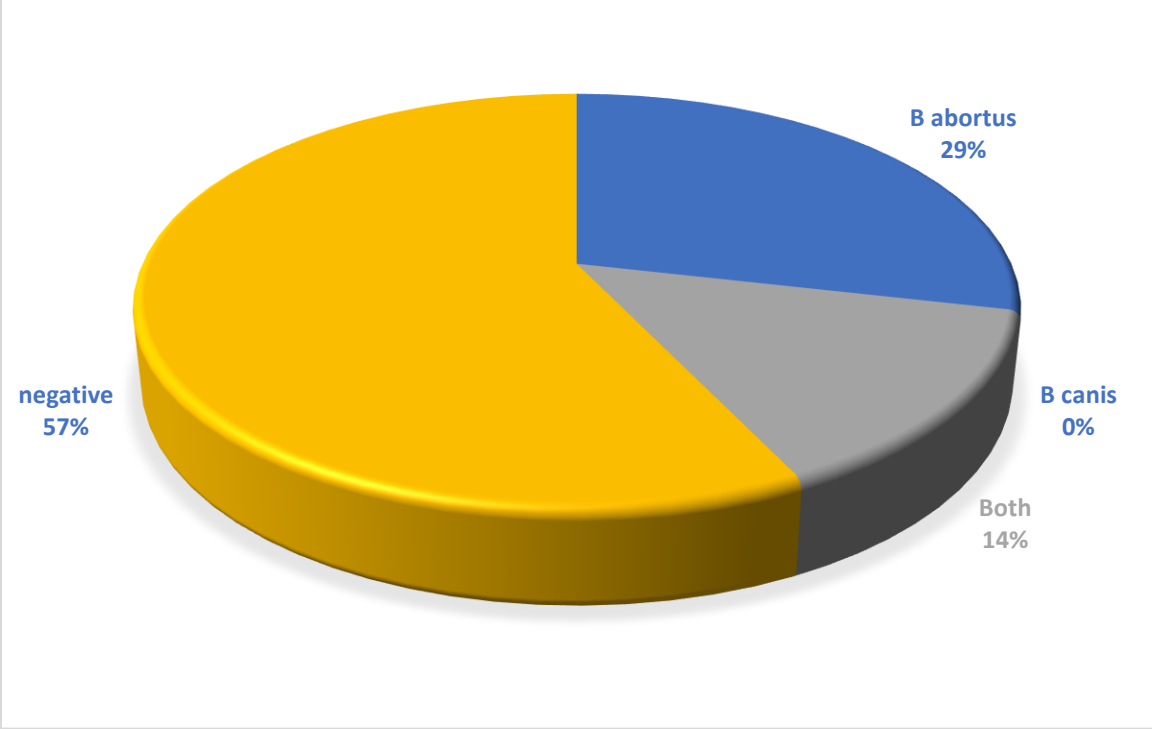

Figure 5: Positive cases detected by PCR assay in Group II

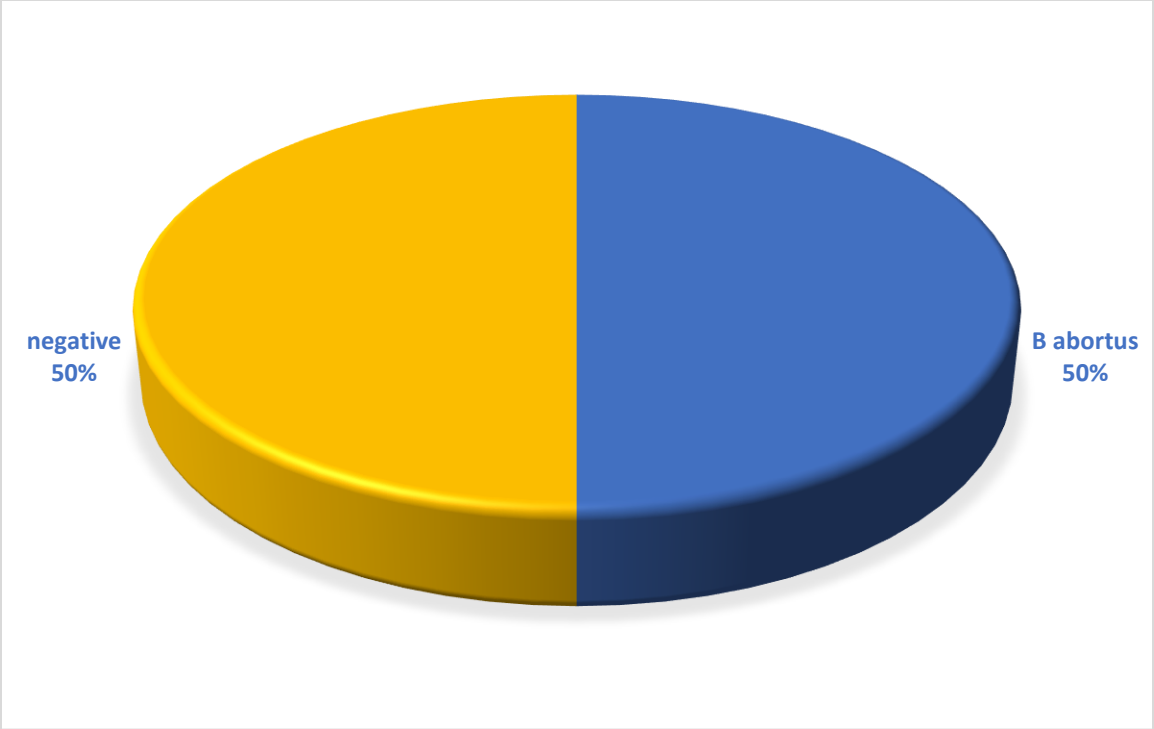

Figure 6: Positive cases detected by PCR assay in Group III

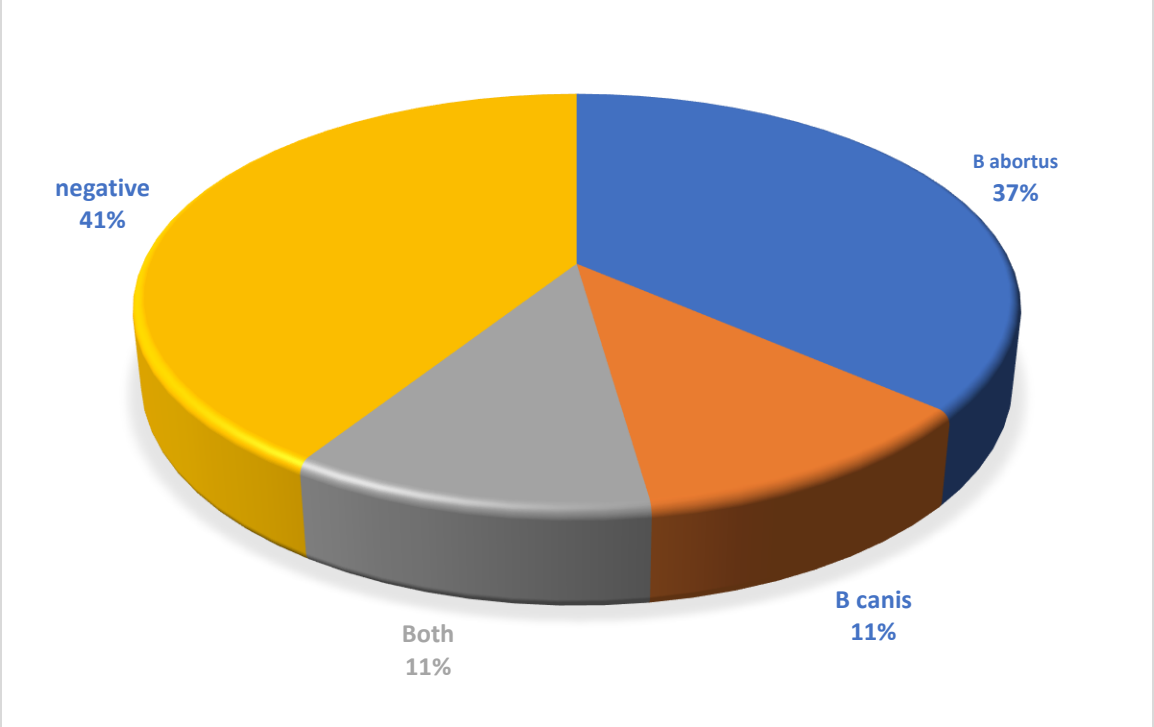

Figure 7: Positive cases detected by PCR assay in screened dogs

Discussion

V. DISCUSSION

The dogs presented with complaints of abortion, history of abortion and mummification and premature/still birth and conception failure were considered for the study. The blood samples collected from the dogs with above infertility problems were subjected to haematobiochemical analysis and for screening of *B.canis* using immunochromatographic assay using quickVET Rapid test kits. The tissues (vaginal discharge or washings, aborted tissues like placenta or fetus) and blood were used for the PCR assay.

5.1 Occurrence of canine brucellosis

In the present study, the suspected dogs were screened using ICA and PCR assay to study the occurrence of canine brucellosis. The occurrence of brucellosis recorded in the dogs subjected to the study was 59.09 per cent (26/44), which was obtained by *bcsp* 31 gene specific PCR assay for brucellosis. Similarly, Keid *et al*. (2007a) also found an overall occurrence of 50.70 per cent (73/144) of brucellosis in dogs by genus level PCR and are in accordance with the results obtained in present study. De Oliveria *et al*. (2019) reported similar occurrence of 45.50 per cent of canine brucellosis while screening 167 dogs in Pantanal region.

Kim *et al*. (2007) evaluated the immunochromatographic assay for screening of *B. canis* in naturally infected 463 kennel dogs and recorded 39.10 per cent occurrence using 2ME RSAT. Suja (2014) conducted a study on incidence of canine brucellosis using RBPT and reported 26 per cent incidence.

However, lower occurrence of brucellosis of 12.00 per cent (31/254) in dogs screened using PCR assay was reported by Mol *et al*. (2020).

Based on the species-specific PCR (AMOS) results the occurrence of *B. abortus* in the screened animals was found to be 47.73 per cent (21/44). Whereas, the occurrence of *Brucella canis* recorded in the present study was 22.73 per cent (10/44) as obtained by species specific PCR assay.

Forbes (1990) and Baek *et al*. (2003) recorded a higher occurrence of 100 percent *B. abortus* using necropsy tissue of 14 dogs and 3 dogs using blood cultures, respectively maintained in brucella affected cattle farms using AMOS PCR assay. De Oliveria *et al*. (2019) have reported a lower occurrence of 10.80 per cent after screening 167 herding dogs whereas, Jamil *et al*. (2019) obtained 1.15 per cent of occurrence of *Brucella abortus* by screening blood samples of 181 stray and herding dogs using species specific PCR assay.

Lower occurrence of 8.30 per cent of *Brucella canis* has been reported by Aras and Ucan (2010) after collecting lymph node samples from 48 dogs died in a city dog pound and performing species specific PCR using the lymph node samples. Kauffman *et al*. (2014) reported higher occurrence of 42.06 per cent (45/107) *Brucella canis* in screened dogs using blood and vaginal swab samples on species specific PCR.

It is to be noted that in all the studies tissue samples yielded good sensitivity compared to that of the blood samples for detection of *B. canis*. The epidemiologic results vary based on type of sample considered for testing, bacteremic stage/stage of infection at which the samples are collected, tests used for screening, protocols for DNA extraction and

amplification, different target gene and choice of reference test (Zoha and Carmichael, 1982; Keid et al., 2007b; Xavier *et al*., 2010 and Makloski, 2011). Infected bitches have the potential to shed large numbers of brucella organisms for variable periods following parturition or abortion (Makloski, 2011). In canine brucellosis bacteremia is intermittent and eventually decreases in chronic infections (Kawaguchi *et al*., 2011). The serologic methods for screening the brucellosis do not provide accurate results during first 12 weeks post infection because the seropositivity is highly dependent on bacteremia (Zoha and Carmichael, 1982).

5.2 Relative efficacy of diagnostic methods

Lateral flow immunochromatographic assay was performed for the samples collected from 44 brucellosis suspected dogs. All the samples showed color band at control (C) line and no reaction in the test line. Thus, it was indicated that all the samples were negative. The sensitivity and specificity of ICA were 0 and 100 per cent respectively whereas the relative efficacy of ICA was found to be 77.27 per cent when compared with results of PCR, as none of the animals were detected by this test (Pieterse *et al*., 1990). Though this is specific to *B. canis*, it failed to detect 10 *B. canis* dogs that were found positive by PCR.

Similar to the present findings, Suja (2014) conducted a study on canine brucellosis using ICA (quickVET Rapid test kits) and RBPT which showed 0 and 26 per cent incidence, respectively.

However, higher accuracy of the test is by Wanke *et al*. (2012) who have reported 100 per cent specificity of the FASTest, an immunochromatographic assay in 17 healthy

dogs, sensitivity of 89 per cent in 27 acute and subacute cases and 17 per cent in 6 chronic cases in confirmed healthy and with *Brucella canis* infection. Similarly, Keid *et al*. (2015) recorded 89.58 per cent sensitivity of Anigen Rapid Canine Brucella Ab Test® in 43 out 48 dogs (confirmed by bacteriological culture) and specificity of 100 per cent with ICA.

Keid *et al*. (2015) also evaluated an ICA (Anigen Rapid Canine Brucella Ab Test®) in a total of 102 dogs for *B. canis* in different kennels of a Brazilian state. They recorded ICA positive in 43 out 48 dogs (89.58 %) confirmed by bacteriological culture and specificity of 100 per cent with ICA.

Although the ICA is an acceptable and reliable screening test when the samples are tested in duplicate with higher sample size, but this is a poor test for confirmation compared to bacterial culture, RSAT, ELISA and PCR. Immunochromatographic test has low sensitivity in acute and sub-acute cases as it requires 2-4 weeks for seroconversion and low sensitivity is also noticed while diagnosing chronic cases due to low circulatory antibody titres in such cases (Wanke *et al*., 2012). Cases screened in present study consisted of the cases which were presented during the process of infection (exhibited with clinical symptoms) and the seroconversion probably might not have taken place. There were also cases presented with history of clinical symptoms during previous pregnancy suggesting chronic infections in which the antibody level might have reached non detectable levels to be identifed by the ICA (Carmichael and Greene, 2006; Wanke *et al*., 2012). In order to get proper accuracy of the test, Keid *et al*. (2015) opined that ICA test should be used to diagnose canine brucellosis after detectable levels of circulatory antibodies are produced.

However, diagnostic importance of ICA as a screening test needs to be proved with more number of studies.

5.3 Haematobiochemical parameters

In the present study, the mean total erythrocyte count (10^6/cmm), haemoglobin (g/dL), haematocrit value (%), platelet count (10^3/cmm), neutrophil (%), lymphocyte (%), basophil (%), alanine aminotransferase activity (U/L) and creatinine (mg/dL) recorded in the dogs that were brucella positive and negative did not show any significant difference. Further, there was no significant variation observed in the parameters recorded in dogs with brucella positive and negative within and between groups. The parameters studied in the dogs with clinical symptoms and positive cases were within physiological range.

However, the total leukocyte count in brucella positive cases was significantly ($p<0.05$) higher ($25.95 \pm 4.48 10^3$/cmm) compared to its count in brucella negative cases.

A non-significant variation in haematobiochemical parameters of dogs with RBPT positive and negative has been reported by Suja (2014).

Leadbetter *et al*. (2009) and Kustritz (2003) have reported similar non-significant complete blood count and serum biochemistry; however, an elevated TLC of 15.00-24.50x10^3/cmm with neutrophil count of 82.86% has been reported in two dogs (Leadbetter *et al*., 2009).

Similarly, Villalba *et al*. (1990) noticed pronounced leukocytosis in the acute phase of infection (first 3-4 weeks) and the counts then fell to leukopenic values at 2.5-3

months, when they studied weekly haemogram of 3 dogs for 3 months after innoculationg them with *B. canis* organisms.

Contrarily, total leukocyte count of 9.3 x 10^3/cmm in three brucella positive male dogs with high titres suffering from disco-spondylitis and orchitis has been reported (Anderson and Binnington, 1983) and the TLC was within the physiological range. They have also recorded the non-significant variation in TEC (7.18 x 10^6/cmm), Hb (17.8 g/dL), HCT (47%), basophils (0%), eosinophils (6%), neutrophils (54%), lymphocytes (30%) and monocytes (10%) values in brucella positive male dogs.

Similarly, non-significant variations or the haematobiochemical parameters in normal physiological limit in brucella positive dogs but with clinical symptoms of Pyogranulomatous dermatitis (Dawkins *et al*., 1982), Uveitis (Blouin, 1984) has been reported.

The leucocytosis recorded in the positive cases could be attributable to the inflammatory process due to brucella infection (Leadbetter *et al*., 2009).

5.4 PCR assay

Blood samples and tissue samples of 44 dogs with clinical symptoms subjected to *bcsp 31* gene specific PCR assay and found 66.67 (18/27), 42.86 (3/7) and 50.00 (5/10) per cent, found brucella positive respectively in Group I, Group II and Group III with an overall positivity of 59.09 per cent. Nine blood and tissue samples each were positive for brucella species and in addition 8 dogs were found positive both in blood and tissue samples.

Similarly, Keid *et al*., (2007a) recorded 50.70 per cent brucella positive by genus level PCR and 37.50 per cent of dogs were found positive through blood PCR and 36.20 per cent (52/144) dogs by vaginal swab PCR, and De Oliveria *et al*. (2019) reported similar results of 45.50 per cent of canine brucellosis while screening 167 dogs in Pantanal region. The reports are in agreement with the present findings where 59.09 per cent brucella positive cases and 38.63 (17/44) each by blood and tissue (vaginal discharge/washings, aborted tissue), respectively was recorded.

However, Mol *et al*. (2020) recorded lower incidence of brucellosis of 12.00 per cent (31/254) in dogs screened using PCR assay.

The species-specific PCR (AMOS) used to detect different species in the brucella positive samples revealed 11 blood and 4 tissue samples to be positive for *Brucella abortus*. In addition, 6 dogs were positive in both blood and tissue samples. The overall occurrence of *B. abortus* in the screened animals was found to be 47.73 per cent (21/44).

Forbes (1990) recorded a higher occurrence of 100 percent *B. abortus* using necropsy tissue of 14 dogs maintained in brucella affected cattle farms using AMOS PCR assay. Smilarly, Baek *et al*. (2003) reported a 100 per cent occurrence in 3 dogs screened from a cattle farm having high prevalence of *Brucella abortus* by AMOS PCR assay using blood cultures. The only possible source of infection was infected cattle on the same farm. It is suggested that dogs should be routinely included in brucellosis surveillance and eradication programs.

De Oliveria *et al*. (2019) have reported a lower occurrence of 10.80 per cent after screening 167 herding dogs whereas, Jamil *et al*. (2019) obtained 1.15 per cent of occurrence of *Brucella abortus* by screening blood samples of 181 stray and herding dogs using species specific PCR assay.

The overall occurrence of *Brucella canis* recorded in the present study was 22.73 per cent (10/44) by species specific PCR assay using tissue samples and co-occurrence of *B. abortus* and *B. canis* was also found in 11.36 per cent (5/44). None of the blood samples were positive for *B. canis*.

Lower occurrence of 8.30 per cent of *Brucella canis* has been reported by Aras and Ucan (2010) after collecting lymph node samples from 48 dogs died in a city dog pound and performing species specific PCR using the lymph node samples. When the culture method was used as a gold standard, sensitivity and specificity of the PCR assay were found to be 100%. The limit of PCR detection of *B. canis* DNA was 1.4×10^1 CFU/g at least. In conclusion, the PCR assay has been shown to have a diagnostic performance equal to bacteriological culture for detection of *B. canis.*

Kauffman *et al*. (2014) reported higher occurrence of 42.06 per cent (45/107) in screened dogs to be positive for *Brucella canis*. Of which 3.26 per cent of blood and 56.92 per cent of vaginal swab samples were positive on species specific PCR. Kauffman *et al*. (2014) obtained sensitivity of 92.31 per cent and specificity of 51.92 per cent using DNA extracted from vaginal swabs whereas, sensitivity of 16.67 per cent and specificity of 100 per cent was obtained by using DNA of whole blood.

Keid *et al*., (2007b) screened whole-blood samples of 210 dogs from 19 kennels by blood culture and PCR assay after initial serodiagnosis using the rapid slide agglutination test (RSAT) with and without 2-mercaptoethanol (2ME RSAT). The DNA (total genomic mass) extracted from whole blood using proteinase-K, sodium dodecyl sulphate and cetyl trimethyl ammonium bromide and purified by phenol–chloroform purification was even lesser than two bacterial and were able to detect about 3.8 fg of Brucella DNA in 450 ng of host DNA by PCR. They obtained the diagnostic sensitivity and specificity of PCR of 100 per cent and concluded that PCR could be used as confirmatory test for diagnosis of *B. canis* infection. Keid *et al*., (2007b) opined that PCR is a good alternative to blood culture for direct detection of brucellosis in dog, as it showed good concordance in detection of Brucella-infected dogs. Further, they suggested that PCR is a faster diagnostic tool in examination of confined populations thus, helping in segregation of potential sources of infection.

Sánchez-Jiménez *et al*., (2014) collected blood samples from 499 dogs from kennels in two Colombian regions and 91 co-inhabiting humans. The 2-mercaptoethanol rapid slide agglutination test (2ME-RSAT), blood culture and PCR tests were performed on all samples. They reported a prevalence of 9.9 per cent in co-inhabiting humans by serological survey and 0 per cent by PCR or blood culture tests. In dogs it was 10.8 per cent, 19 per cent and 13 per cent by blood culture, PCR and 2ME-RSAT respectively. Compared with blood culture, PCR had a sensitivity of 92.6 per cent and a specificity of 90 per cent for canine samples. Compared with 2ME-RSAT, it had a sensitivity of 77.4 per cent and a specificity of 89.2 per cent.

Alamian and Dadar, (2020) sampled 173 dog blood specimens from herding dogs in three different provinces. After confirming presence of Brucella antibodies using Rose Bengal plate test (RBPT), slow agglutination test (SAT) and 2-mercaptoethanol (2-ME). The seropositive samples were further screened using blood culture and PCR tests to identify and differentiate Brucella species. They reported, 24.30 (42/173), 13.80 (24/173) and 6.30 per cent (11/173) of blood samples to be positive using RBPT, SAT and 2-ME, respectively. However, among 42 seropositive samples, only 38.10 (16/42) and 14.20 per cent (6/42) were positive by PCR and culture, respectively. *Brucella melitensis* biovar 1 and biovar 2 was isolated from the bacterial cultures of 6 blood samples and confirmed by biotyping, AMOS PCR and Bruce-ladder PCR assays. These findings highlight the potential risk of Brucella transmission from dog to humans along with other livestock and reflect the critical role of infected dogs in the persistence of Brucella infections among ruminant farms.

Baek *et al*, (2012) collected twenty mammary lymph node samples from cattle on a farm in the Republic of Korea. These cattle were serologically negative for Brucella by TAT. Brucella agar as well as blood agar yielded positive results for two samples. Tests for urease, hydrogen sulphide and reactions against monospecific sera A and M indicated that these two isolates belong to the genus *Brucella*. Genus specific, AMOS (*abortus, melitensis, ovis, suis*) and Bruce-ladder multiplex polymerase chain reaction (PCR) assays confirmed the *Brucella* isolates as either a *B. abortus* or a *B. canis* strain. It was the first report of the occurrence of a *B. canis* infection in cattle in Korea.

Gyuranecz *et al*. (2011) selected three culture-positive cases and 3 culture-negative cases with histories of reproductive disorders and examined for the presence of *B. canis* infection using histopathology, IHC, and polymerase chain reaction (PCR) assays. Characteristic histologic lesions were noticed in all of the 6 animals, whereas IHC and PCR yielded positive results only in single cases from both groups. The results imply that all cases of canine abortion should be examined for brucellosis by bacterial culture of aborted fetuses and placentas. Immunohistochemical examination of placentas is also recommended because it is a quick and sensitive technique compared with bacterial culture. Multiple methods (i.e., serology, blood, and genital bacterial cultures) should be applied simultaneously and repeatedly for the reliable screening of *B. canis* infection in live individuals.

Stage of infection may influence the sensitivity of a particular test (Xavier *et al*., 2010). Infected bitches can shed large numbers of *Brucella canis* for variable periods after parturition or abortion (Makloski, 2011). Serologic methods do not provide accurate results during first 12 weeks post infection and seropositivity is highly dependent of bacteremia (Zoha and Carmichael, 1982). Diagnosis of *B. canis* limited by the time it takes to observe the *B. canis* specific antibodies in sera. Diagnosis is difficult because of unstable serum antibody titers that vary from individual to individual as well as between different methods used for their detection (Kim *et al*., 2007).

Varying level of diagnostic sensitivity and specificity of PCR have been obtained in different studies, which may be the consequences of different protocols used for DNA

extraction and amplification, different target gene and choice of reference test (Keid *et al.*, 2007b).

Bacteremia in canine brucellosis is intermittent and eventually decreases in chronic infections, PCR depends on whether the dog is in bacteremic phase or it is chronic (Kawaguchi *et al*., 2011). PCR results may also be influenced by inhibitors, use of antibiotics, haem group and anticoagulants such as heparin and EDTA which inhibit sensitivity of PCR (Keid et al., 2007b). Mol et al, (2020) stated that use of only laboratory methods may not be sufficient for confirmation, even though laborious bacteriological culture may be necessary.

Vaginal swab is considered as preferable sample during the post abortion period because large number of brucella organisms are shed during this period. Detection of *Brucella canis* by PCR in bitches post abortion, parturition or pregnancy may be expected because pregnant uterus is the site of brucella persistence (Johnson and Walker, 1992). *B. canis* was more frequently found in vaginal swabs as it has tropism for reproductive tissues (Kauffman *et al*., 2014).

PCR tests can be positive in acute cases with recent bacteremia and still no production of antibodies, causing the serological test to be negative. The opposite can also occur in chronic cases where bacteria get localized and antbodies may be present in circulation (Keid et al., 2009).

Brucella transmission from sheep, goat and cattle to dogs is possible by consumption of infected reproductive tissues, aborted fetus, placenta, contaminated dairy

products, meat from infected animals and contaminated meat. It is also possible that the dogs can act as reservoir and potential source of infection to farm animals as well as human beings (Wareth *et al*., 2017; Macpherson, 2012; Van *et al*., 2018; Zheludkov and Tsirelson, 2010; Lucero *et al.*, 2010).

Introduction of brucellosis in breeding kennels can be by different routes like imported bitch from a brucella prone area, crossing of female from a commercial male dog meant for breeding, use of infected or contaminated meat (Gyuranecz 2011).

In the present study, it was noted that haematobiochemical parameters were of no much use in diagnosis of canine brucellosis and there were no specific changes with regard to the disease. ICA should be used for diagnosis of *B. canis* during 2-4 weeks post infection. PCR was proved to be the better diagnostic tool as it could detect positive cases in both acute and chronic cases. It was also noted that *B. abortus* is better detected in blood samples where as *B. canis* detection was more optimum in tissue samples.

Summary

VI. SUMMARY

A study on occurrence and diagnosis of canine brucellosis was carried out on female dogs presented to Deparment of Veterinary Gynaecology and Obstetrics clinics, Veterinary College, Bengaluru. Dogs with complaints of abortion (Group I), history of abortion and mummification (Group II) and premature birth, still birth and conception failure (Group III) were screened using ICA (*Brucella canis* specific) and PCR (genus and species specific). The duration of the study was 12 months.

A total of 44 dogs with any of the above-mentioned infertility problem were screened. None of the dogs were found to be positive on Immunochromatographic assay. Heametobiochemical analysis of dogs screened revealed no significant difference in mean values between or within the groups or total cases except for mean TLC values of total positive and negative cases, where leukocytosis was noticed in positive cases.

DNA extraction was done from blood and tissues (vaginal discharge/washing/aborted tissue) samples of dogs being screened. A genus specific PCR assay (*bcsp* 31) was performed which reveals amplification of 223bp size. It was found that 9 animals were positive in blood, 9 in tissue (vaginal discharge/washing/aborted tissue) and 8 dogs were found positive on both blood and tissue samples. Out of which 18 animals belonged to Group I, 3 to Group II and 5 to Group III.

Samples were further subjected to AMOS PCR assay where blood samples of 11 dogs and tissue samples of four dogs were noticed to amplify product at 498 bp which were in par with positive control (DNA extracted from strain-19 live vaccine after heat killing)

which indicates presence of *Brucella abortus*. Also 6 animals were positive in both blood and tissue samples. In all, 21 animals were found positive for *Brucella abortus* in blood or tissue or both samples, which constituted 47.73% of the animals screened.

There was no amplification of with respect to *Brucella melitensis, Brucella ovis* and *Brucella suis.*

Also, 10 tissue samples were found positive for *Brucella canis* as they revealed amplification at 300 bp region which was further confirmed by sequencing of representative sample. This constituted 22.73% of animals screened. There was no amplification in any blood samples.

Overall, a total of 26 dogs were found to be positive for canine brucellosis, where 16 dogs were positive for *Brucella abortus*, 5 dogs for *Brucella canis* and 5 dogs for both the organisms. In Group I, 9 dogs were positive for *Brucella abortus*, 5 for *Brucella canis* and 4 animals for both the species. Group II had 2 *Brucella abortus* positive dogs and 1 dog positive for both the species. Group III revealed 5 *Brucella abortus* positive dogs.

It is to be noted that all the cases were presented to clinic during the course of infection (within 2 weeks) or with history of clinical signs in previous pregnancy, which indicates they were either acute, sub-acute or chronic cases. In such cases either the seroconversion might not have happened (acute and sub-acute cases) or might have undetectable levels of serum antibody titers (chronic cases). This could be the reason for none of the cases being detected on ICA.

PCR assay was found to have good sensitivity at both genus and species levels. Also, it was noticed that a greater number of the blood samples were positive for *Brucella abortus* compared to those of tissue samples. None of the blood samples were positive for *Brucella canis* but it was detected in tissue samples with good sensitivity. This proves that blood samples would be more appropriate to diagnose *Brucella abortus* whereas tissue sample should be the sample of choice to detect *Brucella canis* to achieve proper diagnosis.

Haemetobiochemical parameters did not reveal any specific change which would be helpful to suspect/diagnose canine brucellosis. However, they can be used as a routine procedure to monitor physiological status during the course of infection.

As occurrence of canine brucellosis was high in the present study, it is to be noted that there is a potential threat to public health as these animals will be in close contact with humans. The assays are also supposed to be evaluated with larger samples and at least in duplicates to find the reliability of assays.

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Abstract

VIII. ABSTRACT

A study on occurrence and diagnosis of canine brucellosis was carried out on female dogs presented to the Department of Veterinary Gynaecology Obstetrics clinics, Veterinary College, Bengaluru. A total of 44 female dogs were screened with complaints of abortion (Group-I), history of abortion and mummification (Group-II) and premature birth, still birth and conception failure (Group-III) using ICA (*Brucella canis* specific) and PCR (genus and species specific). Twenty-six dogs were found positive on *bcsp31* PCR (genus-specific) where 9 animals were positive in blood, 9 in tissue (vaginal discharge/washing/aborted tissue) and 8 dogs were found positive on both blood and tissue samples. Out of these, 18 animals belonged to Group-I, 3 to Group-II and 5 to Group-III. Further, the samples were subjected to AMOS and *B. canis* specific PCR and 16 dogs were found positive for *Brucella abortus*, 5 animals for *B. canis* and 5 dogs for both the organisms. In Group-I, 9 dogs were positive for *B. abortus*, 5 for *B. canis* and 4 animals for both the species. Group-II had 2 *B. abortus* positive dogs and 1 dog positive for both the species. Group-III revealed 5 brucella abortus positive dogs. None of the dogs were positive for *Brucella ovis and Brucella suis.* ICA didn't show any positive results. Haemetobiochemical studies revealed no significant variation within or between the groups except for mean TLC values between total brucella positive which was significantly higher compared to negative dogs with clinical signs. Current study revealed PCR is highly sensitive for diagnosis of canine brucellosis.

Key words: Immunochromatographic assay (ICA), Polymerase chain reaction (PCR), *bcsp* 31, AMOS, Canine brucellosis.