Diagnosis of canine brucellosis using Rose Bengal plate test[#]

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Abstract

Canine brucellosis or beagle fever is a zoonotic bacterial reproductive disease of dogs, caused by Brucella canis and occasionally by Brucella abortus, B. melitensis and B. suis. The actual seroprevalence of canine brucellosis in India is unknown and not yet studied in Kerala. A total of 131 animals presented to the outpatient unit of medicine, gynaecology and obstetrics of the two University Veterinary Hospitals at Mannuthy and Kokkala with clinical signs of epididymitis, orchitis, abortion, still birth, foetal resorption, foetal mummification, foetal maceration, neonatal death and infertility were randomly selected for the study. Paired sera samples were collected on the day of presentation and after three weeks of presentation for Rose Bengal Plate Test (RBPT). In this study, sera samples from forty-seven (35.88 per cent) infected dogs showed agglutination on RBPT using B. abortus S99 antigen. Out of forty-seven RBPT positive samples, 80.85 per cent (38/47) were female and 19.15 per cent (9/47) were male dogs. The high seroprevalence of canine brucellosis in this study is attributed to the endemicity of bovine brucellosis in the study area.

Keywords: *Canine brucellosis, epididymitis, orchitis, abortion, neonatal death, infertility, Rose Bengal Plate Test (RBPT)*

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Brucellosis is a classical anthropozoonosis endemic in India causing an annual loss of 58.8 million US dollar (Praseeda *et al.,* 2005; Aulakh *et al.,* 2008; Kollannur *et al.,* 2007). Canine brucellosis occurs worldwide and is one of the leading causes of infertility and abortion in dogs (Khosa, 2022). It is endemic in Asia, America and Africa, with a reported seroprevalence of six to 35 per cent (CDPH, 2023). It was a zoonotic infectious venereal disease of concern in canine reproduction, caused by *Brucella canis*, a small, gram-negative, non–spore-forming aerobic coccobacillus and occasionally by *Brucella abortus, Brucella melitensis,* and *Brucella suis* (Anyaoha *et al.,* 2020). *Brucella canis* causes reproductive failure in both male and female dogs worldwide (Davidson and Sykes, 2014). In dogs, canine brucellosis was usually manifested as repeated consecutive abortions, alternated abortions with further normal whelping, birth of weak pups, high neonatal mortality rates, infertility, reproductive failure, prolonged vaginal discharge, endometritis, metritis, placentitis, epididymitis, orchitis, and prostatitis (Santos *et al.,* 2021; Nicoletti, 2022; Hamdy *et al.,* 2023). Thus, the disease could lead to huge economic burden for breeders and breeding kennels (Mol *et al.,* 2020). Brucella infection increases with age and that most diseased animal carries the infection throughout their life (Lingam *et al.,* 2020).

Serological diagnosis of canine brucellosis is often challenging and should be coupled with bacterial isolation and other promising tests (Keid *et al.,* 2009). According to Santos *et al.* (2021), dogs may be positive reactors in serological tests for months, in the absence of bacteremia and associated clinical signs of infection. But considering its zoonotic potential, this procedure requires biosafety level 3 conditions (Wallach *et al.,* 2004). Greene and Carmichael (2012) recommended that dogs should be tested at least twice in a month, to reduce the chance of getting false negative results in the initial weeks of infection. Khosa (2022) reviewed a battery of virulence factors (Lipopolysaccharide (LPS), T4SS secretion system and BvrR/BvrS system) that was responsible for the survival in the host and transmission of the bacterium. Bacteraemia

in canine brucellosis was intermittent and usually decreases in chronic infections, leading to varying results in PCR (Mol *et al.,* 2020). *Brucella abortus, B. melitensis*, and *B. suis* infection in dogs could be diagnosed using the procedures described for cattle, except for ELISA, which was not widely employed in dogs (Corbel, 2006). The main serological tests used for the diagnosis of brucella infection were Rose Bengal Plate Test (RBPT) as a screening test and complement fixation test (CFT) as a confirmatory test (Baek *et al.,* 2003). But these researchers reported that, RBPT was more sensitive than the CFT when testing culture-positive animals.

The Rose Bengal Plate Test (RBPT) was a simple, rapid, inexpensive, spot agglutination test for diagnosing brucellosis in animals (Kustriz, 2003). Chothe and Saxena (2014) referred RBPT as the rapid screening test to diagnose brucellosis. In an investigation on the seroprevalence of brucellosis among infertile crossbred cows and slaughtered cows an overall incidence of four per cent and 20 per cent respectively was documented in and around Thrissur, Kerala using RBPT (Praseeda *et al.,* 2005). Reddy *et al.* (2014) reported a detection rate of bovine brucellosis among the slaughtered cattle of Kerala as 7.74 per cent in RBPT. *Brucella canis* shared antigenic components with the *B. ovis* and the vaccine strain *B. abortus* RB51, hence either of the strain can be used as antigen for diagnosis of canine brucellosis (Nielsen *et al.,* 2004; Escobar *et al.,* 2010). The sensitivity of RBPT was found to be very high (>99 per cent) but the specificity could be disappointingly low (Smits and Kadri, 2005). So, the history and clinical findings should be used along with the results of serological and bacteriological tests to get a confirmatory diagnosis. At two-week post-infection, antibodies developed against the wall and cytoplasmic proteins of brucella could be detected (Wanke, 2004). At neutral pH, RBPT could measure the presence of IgM, IgG1 and IgG2. At the buffered pH of 3.65, it prevented agglutination with IgM, and measured only IgG1. It was an internationally recommended test for the screening of brucellosis in small ruminants, but lacked standardisation of the antigen (Kaltungo *et al.,* 2014). Carmichael (2018)

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opined that, whenever there was a history of poor reproductive performance in either sex or abortion in bitches, canine brucellosis should be considered in the differential diagnosis. Even when multiple tests were employed, Mol *et al.* (2020) clearly demonstrated that the diagnosis of canine brucellosis remained as a big challenge now a days. Screening for brucellosis is an important part of the pre-breeding evaluation of any dog and it should be included in the initial diagnostics in cases of infertility, abortion, orchitis and epididymitis in dogs (Davidson and Sykes, 2014). Thus, the current study was conducted considering the endemic status of bovine brucellosis in India and the possibility of transmission of the disease from ruminants to pet dogs cannot be excluded. The actual seroprevalence of canine brucellosis in India is unknown and has not yet been studied in Kerala. The objectives of the study were to identify the positive reactors for canine brucellosis using the widely useful serological test for brucellosis, i.e., Rose Bengal Plate Test (RBPT).

Materials and methods

Study area

Kerala is the south-western coastal state of India and Thrissur (10.52ºN, 76.21ºE) is in the central part of Kerala in which the current study was conducted. A total of 131 animals presented to the outpatient unit of medicine, gynaecology and obstetrics of two University Veterinary Hospitals of Mannuthy and Kokkala were randomly selected for the study. It included both male and female dogs with clinical signs of epididymitis, orchitis, abortion, still birth, foetal resorption, foetal mummification, foetal maceration, neonatal death and infertility which failed to conceive even after three consecutive breeding's (natural/artificial insemination).

Collection of samples

On the day of presentation, blood (5ml) was aseptically collected from all the cases in non-vacuum blood collection tube (Ultimate Health Care, Kerala, India) without anticoagulant. Serum was separated by centrifugation within 24 hours of collection and was subjected for serological diagnosis. Paired sera samples for RBPT were collected on the 0th day and 21st day of presentation.

Rose Bengal Plate Test (RBPT)

Rose Bengal Antigen (killed suspension of smooth *B. abortus* S99 cells stained by Rose Bengal) and the control positive serum was procured from IAH & VB, Palode, Thiruvananthapuram, Kerala. The standard technique suggested by Alton *et al.* (1975) was adopted in the present study to perform RBPT in serum samples from dogs. Serum samples and antigen for the RBPT test were brought to room temperature. 30 µl of each serum sample and antigen were taken on a white glossy ceramic tile. Immediately after the last drop of antigen had been added to the plate, serum and antigen were mixed gently using a clean micro tip for each test to produce a circular or oval zone approximately two centimetres in diameter. The mixture was mixed gently for four minutes at ambient temperature by tilting the slide both in clockwise and anticlockwise direction. The plates were shaken for 8 minutes and any agglutination that appeared within this time was recorded as a positive reaction. Samples identified with no agglutination (0) were regarded as negative, while those with +, ++ and +++ were regarded as positive. The results were analysed statistically by the Chi-squared test and Wilcoxon signed-rank test, using the SPSS version 24.0.

Results and discussion

Canine brucellosis (beagle fever) initially emerged as an epidemic disease of venereal origin in beagle breeding kennels of New Jersey characterised by bitches with aborted pups and impotent male dogs, causing a huge economic burden to the beaglers (Faigel, 1969; Spink and Morisset, 1970). In 1966, the causative agent of beagle fever was first isolated by Dr. Leland Carmichael from aborted foetuses obtained from beagle breeding kennels of USA (Spink and Morisset, 1970; Hasso and Serian, 2012). Now the disease had been diagnosed in various breeds and it has got a world wide distribution. In India, the first report of canine brucellosis was made by Thanappa Pillai and his co-workers in 1991 (Athira *et al.,* 2021; Jasrotia *et al.,* 2021). Canine brucellosis outbreaks are

more likely to occur in kennels than households, but the same infection prevention and control strategies should be applied (CDPH, 2023). The Rose Bengal Plate Test (RBPT) is capable of detecting all the three main anti-brucella immunoglobulin isotypes (IgM, IgG1 and IgG2) in the earlier stage of brucella infection (Kustriz, 2003; Corbel, 2006).

A total of 131 serum samples were tested which includes 105 female and 26 male dogs. Thirteen different dog breeds in the age group of 18 to 36 months were represented in the study population with Labrador Retrievers being more commonly affected with reproductive problems. The predominance of Labrador Retrievers among pet dogs in the study area might have influenced this study. In this study, sera samples from forty seven (35.88 per cent) infected dogs showed agglutination on RBPT using *B. abortus* S99 antigen (Table 2). Hasso and Serian (2012) conducted a screening test for brucellosis among the stray dogs of Iraq and reported a seroprevalence of 30.7 per cent using the Rose Bengal Plate Test containing *B.abortus* antigen. Suja (2014) reported a twenty-six percent seropositivity for brucellosis in dogs of Bangalore, India using the *B. abortus* S99 antigen. Contrary to our findings, Lingam *et al.* (2020) reported sero prevalence of brucellosis in dogs from Telangana state, India as 2.75%, 3.00%, 2.25% and 3.25% by RBPT, Lateral flow assay (LFA), Standard Tube Agglutination Test (STAT) and Enzyme-Linked Immunosorbent Assay (ELISA), respectively.

The high seroprevalence of brucellosis in dogs of this study is attributed to the endemicity of bovine brucellosis, close contact with tissues/secretions from aborted foetuses and placenta of infected cattle and consumption of raw milk. The prevalence of canine brucellosis was slightly higher in female dogs compared to male dogs in this study (Table 1). This could be either due to the presence of higher concentration of erythritol (a carbohydrate that stimulates the multiplication of brucellae) in the placenta and foetal fluids of female than male reproductive organs or transmission of brucella organisms through semen of an infected champion stud dog that was used for mating different females of this study area (Anyaoha *et al.*, 2020; Lingam *et al.*, 2020). Using the Chi-square test, no significant difference between gender and occurrence of brucellosis in dogs of this study population were noticed.

The main clinical signs noticed in dogs with reproductive disorders were epididymitis and orchitis (19.15 per cent), abortion and still birth (51.06 per cent), foetal resorption, mummification and maceration (10.64 per cent), neonatal death (8.51 per cent) and infertility which failed to conceive even after three consecutive breeding's (10.64 per cent). The results of RBPT in these animals are depicted in the Fig. 1.

Samples identified with no agglutination on RBPT were regarded as negative while those with fine agglutination, clumping and definite clearing were regarded as positive reactors for brucellosis (Table 2). Chothe and Saxena (2014) reported that suitable modifications of RBPT are required to get accurate results to avoid a false positive result. Usually the reaction was observed up to four minutes. But in this study, the reaction was observed for a period of eight minutes as suggested by Diaz *et al.* (2011) in human *B. melitensis* cases. Visible agglutination with a typical rim was observed in positive cases within this period of time. Paired sera samples were collected on the 0th day and 21st day of presentation for RBPT. Score 0 was given to 64.12 per cent of samples at day 0 and day 21 of the presentation. Score 1, 2 and 3 were given to

Table 1. Results of RBPT in male and female dogs

RBPT	Male dogs	Female dogs	Chi square value	p value
	Number (%)	Number (%)		
Positive	9 (34.61)	38 (36.2)	0.022	0.881[ns]
Negative	17 (65.39)	67 (63.8)		

ns = There is no association between gender and occurrence of canine brucellosis

	RBPT Positive	RBPT Negative
Epididymitis & orchitis	9	17
Abortion & stillbirth	24	33
Foetal resorption, mummification & maceration	5	10
Neonatal death	4	9
Infertility	5	15

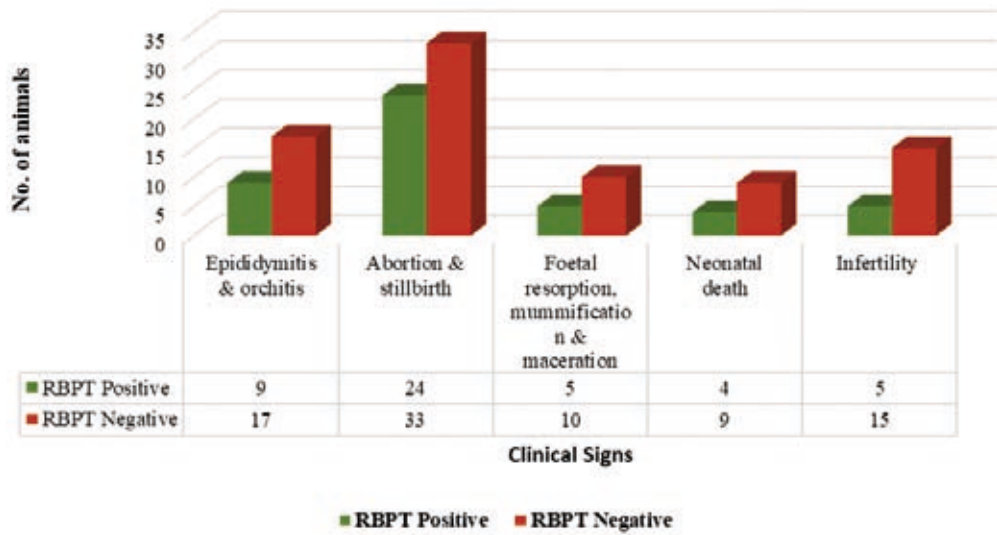**Clinical Signs**

■ RBPT Positive ■ RBPT Negative

Fig. 1. Results of RBPT in dogs with various clinical signs

Table 2. Scoring and interpretation of RBPT results (Yilma, 2016; MacMillan, 2018)

Reading	Observation	Score
No agglutination		Score 0
Barely perceptible agglutination (using magnifying lens), some rimming		Score +/1
Fine agglutination, definite rimming, some clearing		Score ++/2
Coarse clumping, definite clearing		Score +++/3

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Table 3. Results of RBPT in paired serum samples

Day of collection	RBPT Score							
	Score 0		Score 1		Score 2		Score 3	
	No.	%	No.	%	No.	%	No.	%
Day 0	84	64.12	37	28.25	10	7.63	0	0
Day 21	84	64.12	8	6.11	35	26.72	4	3.05

Table 4. Sensitivity of RBPT test results using paired serum samples

Day of collection	Sample size (N)	Mean ± SD	p value	Test value
Day 0	131	44 ± 0.634	0.001**	5.745
Day 21	131	69 ± 0.969		

** There exists a significant difference at 1% level between mean values of day 0 and day 21 RBPT results

28.25 per cent, 7.63 per cent and zero per cent respectively at day zero of presentation. On day 21 of presentation, RBPT score 1, 2 and 3 were given to 6.11 per cent, 26.72 per cent and 3.05 per cent of the samples tested respectively (Table 3). Using the Wilcoxon signed-rank test, there exists a significant difference at 1% level between mean values of day 0 and day 21 RBPT results.

Mol *et al.* (2020) pointed out the importance of screening dogs with reproductive disorders using serological tests that could detect antibodies against rough as well as smooth *Brucella* spp., because anti-smooth Brucella antibodies had been detected in dogs. For better diagnosis of Brucella infection, Saxena *et al.* (2015) suggested a combination of RBPT and ELISA to be used, especially in the case of those samples which were found negative by either RBPT or STAT used alone. From this study, it can be concluded that, when correlated with appropriate history and clinical findings, RBPT could be a very useful test to diagnose canine brucellosis. There is no treatment protocol for canine brucellosis and considering the potential animal and human health hazard, it is recommended to euthanize the tested positive animals (CDPH, 2023).

Conclusion

Brucellosis in India is a very common but often neglected re-emerging disease. It had gained a widespread attention among the dog breeders due to its significant economic losses due to infertility, birth of weak pups and high neonatal mortality rates. It is a zoonotic disease with high occupational risk to breeders and veterinarians. Dogs who are in close contact with cattle, pigs, sheep and goats may be infected by *B. abortus*, *B. suis and B. melitensis* respectively. In such animals, RBPT was performed to detect antibodies against these smooth Brucella organisms. In our study population, there were a high seroprevalence of canine brucellosis associated with smooth *Brucella* spp. The anti-smooth brucella antibodies in dogs detected in the current study emphasize the importance of performing tests capable of detecting antibodies to both rough and smooth *Brucella* spp. Pet handlers should be educated and trained to use personal protective gears while attending normal whelping as well as abortion cases. Efficiency of detecting canine brucellosis depends on proper sampling (foetal membranes, aborted and stillbirth foetuses, semen, urine and vaginal secretions) at defined time intervals (tested at least twice in 30 days interval), combination of more than one serological test and follow-up of dogs to detect clinical signs (bitches with repeated spontaneous abortions, prolonged vaginal discharge, infertility, episodes of abortions in breeding kennels, impotent male dogs) suggestive of brucellosis. To conclude, when complemented with appropriate anamnesis and clinical findings, RBPT is a very useful, rapid diagnostic tool for canine brucellosis. It needs no complicated infrastructure or sophisticated training and a comparatively cheap, highly sensitive and easily adaptable test.

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