International Journal of Veterinary Sciences and Animal Husbandry

ISSN: 2456-2912
VET 2024; SP-9(2): 420-424

www.veterinarypaper.com
Received: 13-01-2024
Accepted: 16-03-2024

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Comparative evaluation of micro-agglutination test (MAT), whole cell antigen ELISA (wcELISA) and precipitated protein antigen ELISA (ppELISA) for detection of *Bordetella bronchiseptica* antibodies in dog population of India

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Abstract
Canine infectious tracheobronchitis (kennel cough) has worldwide distribution and is one of the most prevalent respiratory infections of dogs causing mild to severe pulmonary diseases in combination with other infectious agents. In the present study, sero-prevalence of *Bordetella bronchiseptica* was calculated on the basis of micro-agglutination test (MAT), whole cell antigen ELISA (wcELISA) and precipitated protein antigen ELISA (ppELISA). Total 136 serum samples were collected from dogs of different breed, age groups and both sexes from different geographical areas of India (Ahmedabad, Amroha, Chennai, Delhi, Lucknow, Meerut, Mumbai, Palampur, Bareilly). Out of 136 serum samples, 38 (27.9%), 69 (50.7%), 132 (97.1%) were detected positive with MAT, wcELISA and ppELISA, respectively, for the presence of *B. bronchiseptica* antibodies. Although health status, age and breed had no association with detection of *Bordetella* antibodies, yet females appeared to be significantly (p, 0.033) more prone for *Bordetella bronchiseptica* infection. It was found that MAT was the better test than wcELISA and ppELISA in terms of the specificity required for diagnosis. Detection of *Bordetella* antibodies with wcELISA and ppELISA had no significant correlation with clinical sickness. It is concluded that MAT is better test for serodiagnosis of *Bordetella bronchiseptica* infection than ELISA.

Keywords: *Bordetella bronchiseptica*, Serology, Micro agglutination test, ELISA

1. Introduction
Respiratory tract infections (RTI) are quite common in dogs especially in dogs housed together in re-homing centres, boarding or training kennels, pet shops, shelters and veterinary clinics. The contagious RTI are commonly diagnosed as canine infectious tracheobronchitis or kennel cough that affects dogs of all ages [1]. Kennel cough is a multi-etiological disease but *Bordetella bronchiseptica* is one of the major pathogen associated with disease [2]. The disease is mild, self-limiting characterized by paroxysmal, "honking" or hacking cough in dogs and spread through close contact with infected dogs [3]. The clinical signs develop after 5-10 days of infection having morbidity as high as 80% [4] but mortality rate is low. Kennel cough has been described in two clinical forms *viz.* uncomplicated form characterized by dry hacking cough, gagging and retching behavior and complicated form which usually is described in puppies or immuno-compromised dogs with wet cough, mucoid discharges and signs of systemic disease i.e. pyrexia, anorexia, chorioretinitis, vomiting and diarrhea [1, 5]. Experimental infection of *B. bronchiseptica* in dogs produces clinical diseases with respiratory signs and cough as major clinical symptoms [6, 7].
B. bronchiseptica is a small, Gram-negative coco-bacillary, non–motile or motile by lateral peritrichous flagella, non-spore forming, pleomorphic, aerobic bacteria belongs to family *Alcaligenaceae* and colonizes the upper respiratory tract of mammals [8]. Most common route of transmission is via aerosol droplets [9] and may infect immune-compromised humans [10].

Many researchers have tried to isolate the organism and evolve diagnostic methods based on molecular tools for the disease [11]. Although the organism is considered to be distributed worldwide, there have been very few sero-prevalence studies on *B. bronchiseptica* in canine population. Under several circumstances when it is not possible to identify the causal organism, specifically those organisms which are difficult to culture, presence of specific antibodies is used as the diagnostic criteria.

Kennel cough is traditionally diagnosed clinically, without the laboratory identification of the agent or agents involved. As a result, the prevalence of this infection in Indian dog population has been unknown. The objectives of this study were to assess the sero-prevalence of antibodies against *B. bronchiseptica* in apparently healthy and diseased dogs by using MAT, wcELISA and ppELISA.

2. Materials and Methods

2.1 Sample collection: Samples were collected from apparently healthy and sick (having cough) dogs from different regions of India including Ahmedabad, Amroha, Chennai, Delhi, Lucknow, Meerut, Mumbai, Palampur, and Bareilly (Table 1). The veterinary clinics were selected on the basis of large number of cases which are examined regularly for respiratory syndromes. The dogs' owners consent has been taken before sample collection. The samples were collected from different breeds and age groups as well as both sexes (Table 1). Samples were collected up to one week from on-set of characteristic clinical signs of kennel cough. Information about the complete history from each dog including vaccination status, duration of clinical signs and dog activities (reared in kennels, natural mating, hunting or field sports or participation in dog shows) was collected. Each dog was examined physically before being sampled for presence of any kind of respiratory disease such as bronchopneumonia, chronic bronchitis, laryngeal paralysis, airway collapse etc. Whole blood was collected from a superficial vein into a glass tube, centrifuged at 4500 rpm for 30 minutes. The separated serum in the tubes was collected in the vials and stored at -20 °C until further use.

Table 1: Detection of *B. bronchiseptica* antibodies in dogs considering various parameters

Parameter	Sections	No. of dogs	Positive (%) for		
		Serum	MAT	ELISA (wc)	ELISA (pp)
Health	Apparently Healthy	73	16 (21.9)	42 (57.5)	69 (94.5)
	Sick	63	22 (34.9)	27 (42.8)	63 (100)
Sex	Male	76	27 (35.5)	39 (51.3)	73 (96.0)
	Female	48	11 (23.9)	17 (36.9)	45 (97.8)
Age group	Up to 3 months	3	1 (33.3)	1 (33.3)	1 (33.3)
	Up to 6 months	8	2 (25.0)	1 (12.5)	8 (100.0)
	Up to 1 year	21	6 (28.5)	8 (38.1)	19 (90.5)
	1 to 2 year	27	10 (37.0)	13 (48.1)	27 (100.0)
	2-3 year	10	2 (20.0)	3 (30.0)	10 (100.0)
	>3 year	53	17 (32.0)	30 (56.6)	53 (100.0)
Place	Ahmedabad	10	3 (30.0)	6 (60.0)	10 (100.0)
	Bareilly	58	19 (32.7)	21 (36.2)	58 (100.0)
	Chennai	14	0 (0.0)	13 (92.8)	14 (100.0)
	Delhi	7	4 (57.1)	7 (100.0)	7 (100.0)
	Amroha	9	4 (44.4)	7 (77.7)	6 (66.6)
	Lucknow	3	1 (33.3)	1 (33.3)	3 (100.0)
	Meerut	22	1 (4.5)	7 (31.8)	21 (95.4)
	Mumbai	7	4 (57.1)	5 (71.4)	7 (100.0)
	Palampur	6	2 (33.3)	2 (33.3)	6 (100.0)
Breed	Beagle	7	1 (14.3)	2 (28.5)	7 (100.0)
	Boxer	1	0 (0.0)	0 (0.0)	1 (100.0)
	Bull Mastiff	1	0 (0.0)	0 (0.0)	1 (100.0)
	Doberman	6	1 (16.6)	4 (66.6)	6 (100.0)
	German Shepherd	9	4 (44.4)	6 (66.6)	9 (100.0)
	Great Dane	2	2 (100.0)	1 (50.0%)	2 (100.0)
	Labrador	41	11 (26.8)	16 (39.0)	40 (97.5)
	Mongrel	12	3 (30.0)	5 (41.7)	11 (91.7)
	Napoleon Mastiff	1	0 (0.0)	0 (0.0)	0 (0.0)
	Pomeranian	20	6 (30.0)	12 (60.0)	19 (95.0)
	Pug	6	2 (33.3)	3 (50.0)	6 (100.0)
	Rottweiler	5	4 (80.0)	2 (40.0)	5 (100.0)
	Spaniel	1	0 (0.0)	1 (100.0)	1 (100.0)
	Spitz	9	3 (33.3)	3 (33.3)	9 (100.0)
	Terrier	1	1 (100.0)	1 (100.0)	1 (100.0)

2.2 Determination of *B. bronchiseptica* antibodies in dog serum

2.2.1 Antigen preparation and micro agglutination test (MAT)

The *B. bronchiseptica* MAT phase I antigen preparation as well as MAT was performed from *B. bronchiseptica* (MTCC-6838) as described by Kumar *et al.* [12]. The highest dilution up to which MAT formation was there, considered as positive Titre. Titre above and equal to 1:64 was considered as positive.

2.2.2 Preparation of antigen for ELISA

Whole cell antigen ELISA (wcELISA) and precipitated protein antigen ELISA (ppELISA) were prepared as explained by Boot *et al.* [13]. Briefly, *B. bronchiseptica* strains (4 pig isolates, 1 dog isolate & 1 MTCC-6838 isolate) were

grown aseptically and incubated for 48 hours at 37 °C. The growth was harvested in PBS (with 0.1% Merthiolate) and was centrifuged at 6000 rpm for 15 min after overnight incubation at room temperature. The supernatant was discarded and pellet was re-suspended in merthiolated PBS and stored at 4 °C as stock antigen. The final concentration of antigen used in ELISA was 10 µg/ml.

For ppELISA, the suspended bacterial cells were repeatedly sonicated for 30 min on ice using a Soniprep-150 sonicator (MSE UK Ltd.) at 5 µm amplitude. The cell lysate was precipitated at 60% concentration of ammonium per sulphate (SRL, India) at 4 ºC overnight, and then centrifuged at 15000 rpm for 15 min to collect the pellet. The pellet was re-suspended in sterile distilled water and stored at 4 ºC for further processing. The precipitate was dialyzed against sterile distilled water using dialysis tubing (Sigma Diagnostics, Mumbai) by standard protocol. Then the solutions in dialysis tubes were collected in two centrifuge tubes. The protein concentration determined at absorbance of 280nm (Thermo scientific Nanodrop) was 16.19 mg/ ml in solution and was stored as stock ppELISA antigens at -20 ºC till used.

2.2.3 ELISA

wcELISA as well as ppELISA antigen coated ELISA plates (each well with 100 µL of 10 µg/mL) were blocked for 2h with 2% bovine serum albumin (BSA). Single dilution ELISA (1: 200 diluted serums in PBS having 1% bovine serum albumin) was performed in triplicate. Goat-anti-dog-IgG alkaline phosphatase (Santacruz) conjugate was diluted 1: 1500 in PBS with 1% bovine serum albumin and 100 µL was used in each well. para-Nitrophenylphosphate, PNPP (Chem Cruz), dissolved (1 mg/mL) in carbonate buffer (0.1 M, pH 9.8) was used as substrate and plates were incubated in dark for 30 min after adding substrate. Then in each well 30 µL of 1 M NaOH was added to stop the reaction and plates were read with ELISA reader at 405 nm wavelength. For washing the plates at different steps PBS with 0.05% tween-20 was used. In each plate positive (confirmed infected dog through isolation/ PCR) and negative controls were kept in 8 wells each. Averages of OD reading of three test wells (for each serum) and negative were calculated.

Calculation of ELISA titres

ELISA titre= [(Average of test OD- average of negative control OD)*200]/ Average of negative control OD

ELISA titre values equal or more than the half of the positive control was considered as positive for all inferences.

2.3 Statistical analysis

Chi-squared test and bivariate correlation test were applied to analyze the data.

3. Results and Discussion

Kennel cough is a multi-etiological disease caused by *B. bronchiseptica* [7], canine parainfluenza virus [14] and canine adenovirus [15]. Due to close contact of dogs with humans and the disease being of zoonotic importance [10], bordetellosis is considered as one of economically, socially and psychologically important ailment. In the study, out of 136 serum samples, 38 (27.94%) serum samples were positive with MAT, when cut off titre was equal to 64. Number of MAT positives was significantly (p<0.092) higher in sick dogs (34.9%) than in apparently healthy dogs (29.1%) (Table 1). Further, *Bordetella* agglutinin titres ≥128 were exclusively detected in clinically sick dogs suffering from kennel cough. Although all the apparently healthy dogs were positive in MAT for *Bordetella* agglutinins, had titres ≤128 observations indicated that identification of *Bordetella* antigen may be from healthy dogs but for precipitation of disease enhanced immune response against the pathogen might be an important factor. Association of high *Bordetella* agglutinin titre with clinical disease indicated that *Bordetella* infection might be responsible as an important pathogen for kennel cough. All the sick dogs did not have high agglutinin titres indicating that *B. bronchiseptica* is not the only pathogen associated with kennel cough. Kennel cough has been shown to be a complex disease associated with multiple infections [1] and it is not clear at what level and how *B. bronchiseptica* play role in pathogenesis of kennel cough.

Similarly, age had no significant (p, 0.939) effect on MAT titres and had negative correlation (r, -0.039), between age and MAT results. MAT on samples of different regions (Table 1) revealed no significant difference in positivity among different places (p, 0.437). Breed-wise association indicated that excluding Great Dane and Rottweiler, no significant difference in different breeds for *B. bronchiseptica* positivity MAT (p, 0.944) observed (Fig. 1). A slightly negative correlation between (r, -0.039) advancement of age and titres for MAT might be associated with development of protective immunity preventing colonization of *B. bronchiseptica* to stimulate continuous production of agglutinins (IgA and IgM). The hypothesis corroborate with the observations as high agglutinin titre were not detectable in any of the dog positive for *B. bronchiseptica* antigen.

For detection of *B. bronchiseptica* infection, whole cell antigen ELISA [13] and LPS based ELISA [16] have been reported for different animals. In all earlier studies whole cell ELISA (wcELISA) has been found to be superior than LPS based ELISA [16]. In the present study, an attempt was made to compare whole cell antigen ELISA with an ELISA standardized using cell lysate protein (ppELISA) precipitated at 60% saturation of ammonium sulphate. At cut off titre 238 (≥ half of the titre of positive control serum from the dog positive for isolation of *B. bronchiseptica*), 69 (50.7%) out of 136 serum samples were positive in wcELISA, which is slightly less than the prevalence of *B. bronchiseptica* antibodies (68%) reported in earlier studies [17]. It might be due to number of factors such as geographical diversity for detection of *B. bronchiseptica* antibodies (31.8% to 100%), variable immune response, different casual factors of kennel cough and many unexplored factors. In contrast to *Bordetella* agglutinins (detected by MAT) there was no association of wcELISA results and health status of the dogs under study. Inability to differentiate / associate the results of wcELISA with clinical observations indicated that wcELISA may be of value to determine seroprevalence of bordetellosis in dogs but of no relevance for diagnosis. Comparatively less number of females (37%) were detected positive with wcELISA than males (51%), but in contrast to MAT significantly (p, 0.08%) more number of healthy dogs than sick dogs were detected positive with wcELISA. Further statistical analysis revealed the negative correlation between the results of the two tests (MAT, wcELISA).

In MAT, the age had slightly negative correlation with the agglutination titres while in wcELISA this relation was apparently positive indicating that as the age advances probability of exposure to infection with *B. bronchiseptica* increases. Thus the presence of anti *Bordetella* IgG antibodies. IgG class antibodies persist comparatively for longer period [18] than IgA and IgM (detected in MAT) and

have more probability of detection by ELISA. Although variation among results of wcELISA from dogs of different breed was apparent, statistically it was insignificant (p, 0.645). To effectively conclude about the effect of breed, sizable and comparable numbers of samples of dogs of different breeds are required.

Though ppELISA at the cut off limit used in the study was 100% sensitive in detecting all the sick cases as positive. Of 73 apparently healthy dogs 69 were detected positive with ppELISA and specificity could not be increased without decreasing the sensitivity. If we keep the sensitivity (keeping PCR as standard diagnostic test) 100% then ppELISA had specificity of about 2%. On reviewing the performance of all three serological tests and taking PCR detection of *B. bronchiseptica* as standard diagnostic test only MAT have the promise to evolve as a diagnostic test. Without compromising with sensitivity and specificity of about 90% (at the cut off limit of 128) can be achieved for MAT. Poor or no correlation between detection of antibodies in dog sera and clinical sickness (kennel cough) is not a novel finding it has been reported in earlier studies too either using whole cell ELISA or LPS ELISA [16]. Thus, serological methods, particularly ELISA may not be of diagnostic value with positive predictive strength. Therefore, further refinement in serological diagnosis is warranted through use of some more specific antigen(s) of *B. bronchiseptica* which should have expression in dogs with clinical disease only. The findings of the study corroborate with similar studies on pigs [12]. In pigs, serological tests including MAT, wcELISA and ppELISA have been reported to have low positive predictive value and poor sensitivity and specificity for detection of *B. bronchiseptica* infection (Fig. 1).

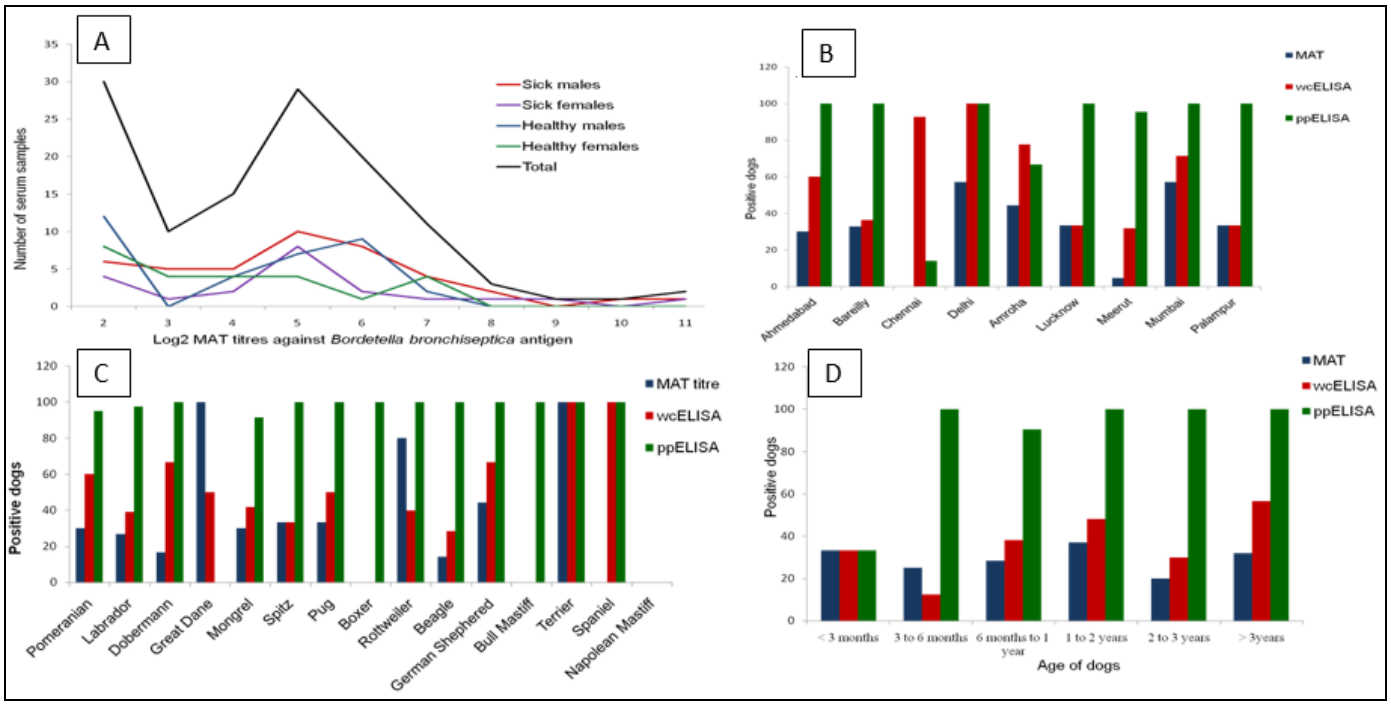

Fig 1: Health status and frequency of positivity for MAT titres in dog serum samples (Graph A); Comparison between MAT, wcELISA and ppELISA results of dogs samples in relation with their geographical location (Graph B), breeds (Graph C) and age (Graph D).

4. Conclusion

The present study revealed that there was poor correlation between *Bordetella* antibody titres and kennel cough in dogs. Of all the tests, MAT had the best diagnostic value as titres ≥128 were detected only in clinically sick dogs. Both of the ELISA though very sensitive had very low specificity and positive predictive value, thus were useless in diagnosis. Although health status, age and breed had no association with detection of *Bordetella* antibodies, females appeared to be significantly (p, 0.033) more prone for carriage of *Bordetella bronchiseptica* infection. More work on large number of serum samples from sick and healthy dogs is needed to standardize and evaluate diagnostic potential of MAT in dogs.

5. Acknowledgements

We are thankful to ICAR-IVRI for providing funds and facilities to carry out the research work.

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