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Poor Association of *Bordetella Bronchiseptica* Infection with Kennel Cough in Dogs in Northern India

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Abstract In the study nasal and throat swabs were collected from 78 apparently healthy and 69 cases of kennel cough from dogs of different age, sex, breed and places and analysed for presence of *Bordetella bronchiseptica* through bacterial isolation and multiplex PCR (mPCR). The pathogen could be isolated from an apparently healthy Labrador bitch while with mPCR, 7 (4.76%, 4 from healthy dogs and 3 from sick dogs) samples were positive for genus specific amplicon and three (2.04%, one from healthy and two from sick Labrador bitches) were positive for *B. bronchiseptica* species specific amplicons. The kennel cough had no significant association (p, 0.49) with the detection of *B. bronchiseptica* but sex (p, 0.03) appeared to be the significant predisposing factors associated with the infection.

Keywords Kennel Cough, *Bordetella Bronchiseptica*, Dog, Bitch

1. Introduction

Contagious respiratory tract infections in dogs are commonly diagnosed as canine infectious tracheobronchitis or kennel cough. Kennel cough affects dogs of all ages and breeds [1, 2]. Of the several pathogens associated with kennel cough *Bordetella bronchiseptica* is considered to be the most important one [3, 4]. *Bordetella bronchiseptica*, a zoonotic respiratory pathogen, causes several other disease syndromes in human and other animals [5, 6]. It is world-wide in occurrence but its status is little understood in India [7, 8]. Thus to understand the association of *B. bronchiseptica* with kennel cough in dogs this pilot study was undertaken.

2. Materials and Methods

In the study, nasal and throat swabs were collected from 78 apparently healthy and 69 cases of kennel cough from dogs of different age, sex and breed (Table. 1). The swabs were processed for bacterial isolation and also for detection of DNA of *B. bronchiseptica* with multiplex PCR standardized in the study. For isolation of *B. bronchiseptica*, swabs were resuscitated in 5 ml buffered peptone water (Hi-Media, Mumbai) for 6-8 h at 37°C and then the growth was streaked on to blood agar (Hi-Media) and MacConkey lactose agar (Hi-Media) plates and incubated for 24-48 h at 37°C. One ml of the growth from peptone water tube was transferred into eppendorf tube and kept in water bath at 100°C for 10 min. Thereafter, the eppendorf was transferred to -20°C for 10 min, and centrifuged at 5000 rpm for 5 min to collect supernatant. The supernatant was stored at -20°C till used in polymerase chain reaction (PCR) as template to detect *B. bronchiseptica*. Typical, flat, non-haemolytic, oxidase and catalase positive colonies were characterised [9] on the basis of phenotypic biochemical and morphological characteristics. The isolates positive for urease and nitratase production and citrate utilization and negative for fermentation of sugars, production of gelatinase, DNase, indole and H_2S was identified as *B. bronchiseptica* [10, 11].

For detection of *B. bronchiseptica* DNA, multiplex-PCR was carried out in thermal cycler (Eppendorf, Germany) by using 5 µl of template and one set of in house designed genus specific primers (Table. 2) and two sets of species specific primers [12, 13]. The PCR amplification was carried out with an initial denaturation at 95 °C for 10 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s and extension at 72 °C for 45 s followed by a final extension step at 72 °C for 7 min. Amplicons, 324 bp (from *alc* gene) for genus specificity, 237 bp (from *fla* gene) and 425 bp (from *fim* gene) for species specificity, were visualized under UV light after electrophoresis on 1% agarose gel (IBI scientific, Peosta Lowa) with 10 mg/ml ethidium bromide at 100 volts using 1× TBE electrophoresis buffer (Bio Basic Inc.).

Bordetella bronchiseptica strain (MTCC 6838) available in the laboratory was used in the study as control in all procedures for detection of the pathogen in clinical samples. The multiplex-PCR (mPCR) standardized in the study was sensitive to detect up to 5cfu of *B. bronchiseptica* (Fig. 1) and was specific as it did not yielded any amplicon using template from cultures of other common respiratory pathogens including *E. coli*, *Pseudomonas alcaligenes*, *Pasteurella multocida*, *Streptococcus pyogenes* and *Staphylococcus aureus* (Fig. 2).

Table 1. Detection of *Bordetella bronchiseptic* from deep nasal and throat samples of apparently healthy and dogs suffering from kennel cough.

Breed of the dog	Sex		Age (yrs)	Health status (numbers)		Positive for isolation (%)	Positive with PCR for genus specific amplicon (%)	Positive with PCR for species specific amplicon (%)
	Male	Female		Sick	Healthy			
Beagle	0	7	1 to 4	4	3	0.0	0.0	0.0
Boxer	2	0	1 to 3	1	1	0.0	0.0	0.0
Bull Mastiff	1	0	< 1	0	1	0.0	0.0	0.0
Doberman	5	0	1 to 7	3	2	0.0	0.0	0.0
German Shepherd	9	7	< 1 to 9	6	10	0.0	0.0	0.0
Great Dane	4	0	6 to 4	3	1	0.0	0.0	0.0
Labrador	24	22	< 1 to 7	20	26	1 (2.17)	5 (10.86)	3 (6.52)
Mongrel	12	5	< 1 to 14	6	11	0.0	1 (5.88)	0.0
Napoleon mastiff	1	0	< 1	0	1	0.0	0.0	0.0
Pomeranian	11	10	< 1 to 13	12	9	0.0	0.0	0.0
Pug	6	3	< 1 to 3	2	7	0.0	0.0	0.0
Rottweiler	6	0	1 to 6	3	3	0.0	1 (16.66)	0.0
Spaniel	1	0	5	0	1	0.0	0.0	0.0
pitz	5	5	2 to 10	8	2	0.0	0.0	0.0
Terrier	1	0	< 1	1	0	0.0	0.0	0.0
Total	88	59		69	78	1 (0.68)	7 (4.76)	3 (2.04)

Table 2. List of genus specific and species specific primers used in the study for *Bordetella bronchiseptica*

Name of primers	Sequence 5'─ 3'	Product length (bp)	References
Genus specific primers designed from *alc* gene			
A643BBalc-F	GCCGACCCACGCAGCGAATAT	213	This study
A856Bbalc-R	GGCCGGTGACGAGATAGCTGTG		
B688Bbalc-F	ACCAACCGCATTTATTCCTACTA	324	This study
B1012Bbalc-R	GGCCCTGGAGTTCGTATTTATG		
Species specific primers for B. bronchiseptica			
425BBfim-1 F	TGAACAATGGCGTGAAAGC	425	Xin *et al*., 2008
425BBfim-2 R	TCGATAGTAGGACG		
237BBFla 4 F	TGGCGCCTGCCCTATC	237	Hozbor *et al*., 1999
237BBFla 2 R	AGGCTCCCAAGAGAGAAAGGCTT		

Note: (F) = Forward primer; (R) = Reverse primer

Table 3. Detection of *Bordetella bronchiseptica* in dogs in relation to their sex and health status

Antigen detection in dogs			Positive for detection of B. *bronchiseptica* with		
Sex	Health Status	Total samples tested	Isolation	Genus specific PCR	Species specific PCR
Male	Sick	46	0	1	0
	Healthy	42	0	1	0
Female	Sick	23	0	2	2
	Healthy	36	1	3	1
Chi-square statistics with respect to			Isolation	Genus specific PCR	Species specific PCR
Male: Female			0.220	0.083	**0.033**
Sick: Healthy			0.345	0.825	0.489
Male:: Healthy: Sick			1.000	0.948	1.000
Female:: Healthy: Sick			0.420	0.961	0.313
Male: Female:: Healthy: sick			0.360	0.317	**0.050**

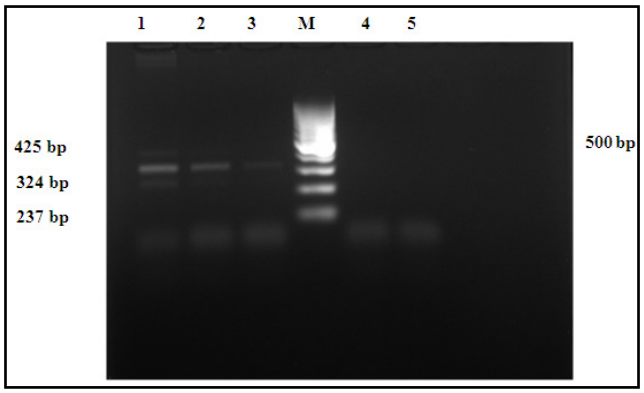

Figure 1. Sensitivity of PCR for Bordetella bronchiseptica using with 10 fold dilution.

Lane M, 100 bp ladder; Lane 1, Positive control (B. bronchiseptica snap chilled supernatant with 500cfu/5µl template); Lane 2, Positive control (B. bronchiseptica snap chilled supernatant DNA with 50cfu/5µl template); Lane 3, Positive control (B. bronchiseptica snap chilled supernatant DNA with 5cfu/5µl template); Lane 4, Positive control (B. bronchiseptica snap chilled supernatant DNA with 0.5cfu/5µl tem; Lane 5, Positive control (B. bronchiseptica snap chilled supernatant DNA with 0.05cfu/5µl template.

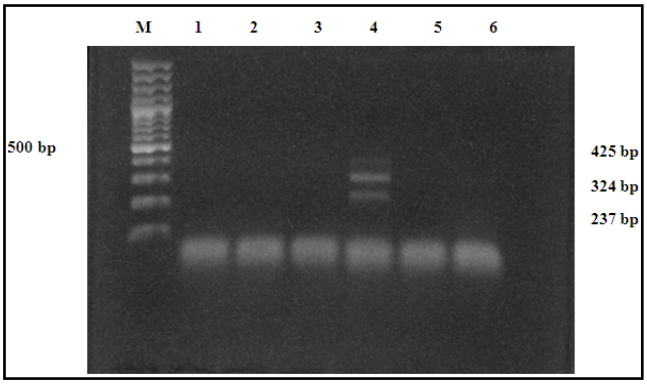

Figure 2. Specificity of PCR for Bordetella bronchiseptica tested with other commonly found respiratory bacteria.

Lane M, 100 bp ladder; Lane 1, E. coli (snap chilled supernatant); Lane 2, Pseudomonas alcaligenes (snap chilled supernatant DNA); Lane 3, Pasteurella multocida (snap chilled supernatant DNA); Lane 4, Positive control (B. bronchiseptica (snap chilled supernatant DNA) Lane 5, Streptococcus pyogenes (snap chilled supernatant DNA); Lane 6, Staphylococcus aureus (snap chilled supernatant DNA).

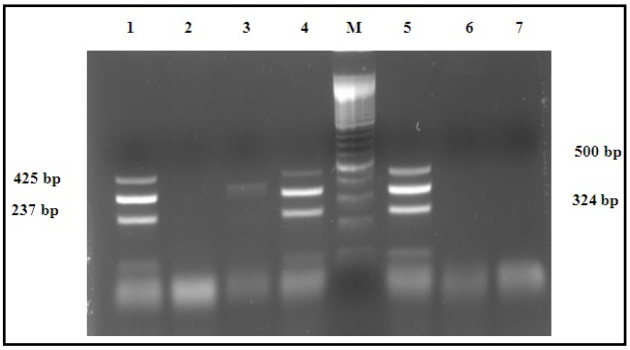

Figure 3. Multiplex PCR for Bordetella bronchiseptica (mPCR) targeting fim(425bp), alc(324bp) and fla(237bp) gene.

Lane M, 100 bp ladder; Lane 1, Positive control (B. bronchiseptica genomic DNA); Lane 2 and 3, Field sample (Snap chilled supernatant); Lane 4, Positive control (B. bronchiseptica snap chilled supernatant DNA); Lane 5, Field isolate of B. bronchiseptica; Lane 6, Field sample (snap chilled supernatant); Lane 7, Negative control.

3. Results and Discussion

On processing of nasal and throat swabs from 147 dogs, *B. bronchiseptica* could be isolated from an apparently healthy Labrador bitch aged 14 months from Mumbai but none of sample from sick dogs was positive for the pathogen (Table. 3). In contrast to standard strain, field isolate was sensitive to tetracycline, polymixin-B and nalidixic acid. However, with mPCR, 7 (4.76%, 4 from healthy dogs and 3 from sick dogs) samples were positive for genus specific amplicon and three (2.04%, one from healthy and two from sick dogs Labrador bitches) were positive for *B. bronchiseptica* specific amplicons (Fig. 3).

The study revealed (Table. 3) that neither the isolation of *B. bronchiseptica* nor detection of DNA of the pathogen with genus/ species specific PCR had significant association with presence or absence of cough symptoms in dogs (p, 0.49). However, all the dogs which were positive for *B. bronchiseptica* either through isolation or through mPCR were all Labrador bitches revealing a significantly more proneness (when results of MPCR were considered final) of females to *B. bronchiseptica* colonization (p, 0.03) however with respect to isolation or detection of pathogen with genus specific PCR no such difference was apparent (Table. 3). In mPCR, one sample each of dogs from Bareilly, Meerut and Mumbai were positive. In dogs below 6 months above two years of age *B. bronchiseptica* could not be detected. More detection of *B. bronchiseptica* in bitches might be instrumental in maintaining the infection in kennels due to more chances of transmission of infection from mother to pups. The positivity for *B. bronchiseptica* antigen was exclusively detected in Labrador bitches which might be due to some unexplored factors associated with more persistence of infection in Labrador bitches.

Low isolation and detection rate of *B. bronchiseptica* from dogs in India was in concurrence to earlier studies on dogs with kennel cough in India [7, 8] and Norway [14]. However in Japan, [15] out of 68 household dogs about 10.3% samples of dogs were reported positive for *B. bronchiseptica*. The lower isolation/ detection rate in the study might be due to low prevalence of *B. bronchiseptica* infection in India or due to problems associated with transport of samples over long distances [16]. The study indicated that *B. bronchiseptica* might not an important cause of kennel cough in India. However, for proving the point, more elaborate studies are needed. Detection of *B. bronchiseptica* by PCR in more number of dogs than isolation indicated that PCR was more sensitive than the conventional method as reported earlier in several studies [11, 12, 13, 17, 18]. Therefore, multiplex PCR may be recommended for rapid diagnosis of bordetellosis is suspected cases.

4. Conclusion

The study revealed that kennel cough had no significant association (p, 0.49) with the detection of *B. bronchiseptica* in nasal/ throat swabs of dogs. Further sex (p, 0.03) appeared to be the significant predisposing factors associated with the infection as detection of *B. bronchiseptica* could be isolated and detected with PCR only from the samples taken from bitches. More carriage of infection by bitches may be important factor in maintenance of infection in kennels.

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