Characterization of isolates of *Bordetella bronchiseptica* from horses

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*Bordetella bronchiseptica* is a well-known Gram-negative bacterial pathogen causing a plethora of diseases in different animals. Although its infection has been reported from pigs and dogs in India, no report of *B. bronchiseptica* from horses is described. We report for the first time, isolation, identification and characterization of strains of *B. bronchiseptica* from respiratory infection in horses from different states in India. The antimicrobial susceptibility testing showed resistance to penicillins, ceftazidime, and chloramphenicol. The virulence capability of the strains was confirmed by sequencing genes such as adenylate cyclase toxin (*cyaA*), bordetella virulence gene (*bvgA*) and by PCR detection of flagellin gene (*fla*). We demonstrate the involvement of *B. bronchiseptica* strains in respiratory tract infection in horses in India.

**Key words:** *Bordetella bronchiseptica*, horse, India, phylogeny

*Bordetella bronchiseptica* chronically infects the upper respiratory tract, primarily as a respiratory pathogen of veterinary importance causing kennel cough in dogs, atrophic rhinitis in swine, and snuffles in rabbits [9]. However, the worldwide reporting of *B. bronchiseptica* infection in horses has been rare and sporadic [1, 11, 13]. In spite of reports of *B. bronchiseptica* from animals in India [22, 23], its isolation has so far not been reported from horses. Furthermore as the organism is often resistant to routinely used antimicrobial agents [8, 21], it is important to isolate and identify the pathogen to choose appropriate antimicrobial therapy.

A Thoroughbred mare from an organised farm, and a Marwari foal, were reported suffering from respiratory illness. After clinical examination, bilateral samples for culture were obtained using Transport Amies medium charcoal swabs and streaked onto 5% Sheep Blood Agar (SBA), MacConkey No. 2 (MLA) and Sabouraud’s Dextrose Agar (SDA) (HiMedia, Mumbai, India) media, and incubated aerobically for 1–5 days at 37°C. Bacterial yields were preliminarily identified by phenotypic characteristics and Biochemical identification strips (HiMedia) following standard procedures [15]. Isolates were subjected to carbohydrate utilization tests using 16 sugars at 1% concentration in Andrade’s Peptone water [15] and Biolog semi-automatic Microbiology Analysis System (GenIII, Biolog Inc., Hayward, CA, U.S.A.), as per the manufacturers protocol. Swabs were also tested for equine respiratory viruses *viz.*, Equid herpesvirus 1 (EHV-1), and Equid herpesvirus 4 (EHV-4) employing molecular methods [3].

Antimicrobial susceptibility of *B. bronchiseptica* isolates was determined by disc diffusion method on Muller Hinton Agar No. 4 (HiMedia, Mumbai, India). Isolates were classified as susceptible, intermediate or resistant according to Clinical and Laboratory Standards Institute (CLSI) standards [6]. The 16S rRNA gene was amplified using published primer as per the protocol [26]. The two isolates were identified by sequencing of 16S rRNA PCR product and BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/) [16]. For detection of genotypic virulence markers of the strains, primers were designed to amplify three virulent genes such as adenylate cyclase toxin (*cyaA*), bordetella virulence gene (*bvgA*) and flagellin (*fla*) (Table 1). *cyaA* and *bvgA* genes were sequenced commercially employing
The consensus sequence was submitted to (National Centre for Biotechnology Information) NCBI GenBank and used for further analysis. Amino acid (aa) sequence was predicted using ExPASy Translate tool. Changes in aa sequences, and secondary structure of partial CyaA proteins of the two isolates was predicted employing PSIPRED, NetSurfP and ScanProsite programs [4, 7, 20]. Sequences were BLAST analyzed on Uniprot BLAST program. The phylogeny of the partial sequence of cyaA gene was constructed by UPGMA method using 1,000 replicates after retrieving 62 BLASTn aligned sequences from the NCBI genome database [24] in MEGA5 [25]. The B. bronchiseptica strains, Eq24E and Eq128 were accessioned in National Centre for Veterinary Type Cultures (NCVT) repository.

In both the clinical cases, B. bronchiseptica was isolated from nasal swab of horses along with other pathogens, which were suffering from respiratory distress, nasal discharge and febrile illness (Table 2). The predominant isolates obtained from adult mare (Eq24E) and foal (Eq128) grew as small 1–2 mm, grey, shiny, circular, isolated colonies (Fig. 1a and 1b). Isolates were Gram-negative cocobacillary rods (Fig. 1c), catalse positive and strong oxidase positive. Eq24E isolate showed poor haemolysis on SBA, whereas Eq128 isolate was haemolytic. On MLA, the cultures were observed as pin-point non-lactose fermenting yellowish colonies in 24 hr, which grew to 1–2 mm size after 72 hr incubation. Both isolates were Simmon’s citrate positive, lysine and ornithine decarboxylase positive, deamination negative and nitrate positive which failed to ferment any sugars. Urea was broken very rapidly within 3–4 hr. No fungi, and equine herpesvirus were detected in nasal swabs. The organisms were identified as B. bronchiseptica by routine phenotypic, biochemical and Biolog system. On antimicrobial susceptibility testing, the B. bronchiseptica Eq24E isolate was resistant to ceftazidime, penicillin and chloramphenicol; the Eq128 isolate was resistant to ceftazidime, penicillin and ampicillin (Table 3).

Amplification of 16S rRNA gene yielded 1.5 kbp products. The 16S rRNA sequences of both isolates showed the highest similarity (>99.7%) with that of B. bronchiseptica strain RB50 (99.72%), ATCC 19395 T (99.71%) followed by Bordetella parapertussis strain NCTC 5952 T (99.72%).

Table 1. Details of genes targeted and accession numbers

| Gene   | Primer sequences              | Amplicon size (bp) | Cycling conditions                  | Accession Nos.                  |
|--------|-------------------------------|--------------------|-------------------------------------|---------------------------------|
| 16S rRNA | F: 5′- AGAGTTGTGATCCTGCTGCCAGCAGCAG-3′ | 1,522              | [29]                                | Eq24E- KT336825; Eq128- KT368942 |
| bvgA   | F:5′-AATTTGCGAGCATTTCCGTCAG-3′ | 768                | 95°C-5 min, 95°C-50 sec, 57°C-1 min, 72°C-1:30 min, 35 cycles, 72°C-10 min, for final extension | Eq24E-bvgA KY215870; Eq128-bvgA KY215871 |
| cyaA   | F:5′-GGTGCAGTCAATCGACTA-3′   | 1,185              |                                     | Eq24E-cyaA KY215872; Eq128-cyaA KY215873 |
| flaA   | F:5′-GGTGCCGAACCAGTCCGCGGACGTTCTCAG-3′ | 736                |                                     | Not sequenced                   |

Table 2. Clinical and microbiological characteristic due to B. bronchiseptica

| Case, Age, Sex | Geographical area | Clinical presentation | Additional microflora | Antimicrobial treatment and prognosis |
|---------------|-------------------|-----------------------|-----------------------|--------------------------------------|
| Case 1, 5 years, Male | Tohana, Hisar, Haryana | Respiratory distress, nasal and ocular discharge, dull, poor feed intake | Streptococcus equi, Alcaligenes spp., Micrococcus spp., Staphylococcus spp. | Trimethoprim/Sulfonamide; Gentamicin; Recovered |
| Case 2, 6 months, Female | Pushkar, Rajasthan | Febrile condition, dull, depressed, anaerobic, coat ruffled, nasal discharge, head hanging low | Staphylococcus spp., Escherichia coli, Pseudomonas spp. | Gentamicin; Unknown |
CHARACTERIZATION OF ISOLATES OF *B. BRONCHISEPTICA* FROM HORSES

(Q9L469) with 98.4% identity at residue level, however, the sequence of Eq128 isolate has much variation at aa level with 97.9% identity with closest strain (Q9L469). The *bvga* partial sequence matched 99.5% with closest *B. bronchiseptica* strain 99-R-0433 (A0A058YBJ0) Uniprot entry for both the isolates.

The partial *cyA* sequence of Eq128 and Eq24E strains had 9 common aa changes whereas 3 additional aa changes were observed in Eq128 (Table 4). On analysis of partial 1,144 bp long N-terminal catalytic domain of *cyA* sequence of this study resulted in a clear-cut division of taxa into two clades; clade 1 and clade 2 (Fig. 2) with high bootstrap values of 99–100%. Clade 1 further divided neatly into 2 clades (Clade 1a and 1b). The Indian horse isolates fell into clade 1b along with 2 other horse sequences out of total 4 equine origin sequences. Significantly, clade 2 was predominated by mainly turkey and human isolates; moreover all of the turkey isolates sequence of *cyA* gene fell into clade 2 only. Similarly all the pig isolates fell into clade 1b (Fig. 2). The *cyA* sequences had 99% similarity to other *B. bronchiseptica* sequences.

In the present study, the *B. bronchiseptica* showed predominant growth and other bacteria were detected in lower numbers. *Bordetella bronchiseptica* has been reported to be isolated as the single or with other commensal or pathogenic bacteria [8]. The Thoroughbred mare which

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**Table 3.** Antimicrobial susceptibility of the *B. bronchiseptica* isolates by disc diffusion method

| Antimicrobial         | Eq24E | Eq128 |
|-----------------------|-------|-------|
| Sufasulfazole         | S     | S     |
| Co-trimoxazole        | S     | S     |
| (trimethoprim-sulphamethoxazole) |       |       |
| Cephalothin           | S     | S     |
| Gentamicin            | S     | S     |
| Ceftazidime           | R     | R     |
| Tetracycline          | S     | S     |
| Penicillin G          | R     | R     |
| Ampicillin            | S     | R     |
| Chloramphenicol       | R     | S     |
| Amoxicillin           | S     | S     |
| Amikacin              | S     | S     |
| Ciproflaxacin         | S     | S     |

R, resistant; S, sensitive.
yielded S. equi isolate with B. bronchiseptica was from a farm in which strangles was not present, although strangles is endemic in equines of India [17]. Streptococcus equi may also be yielded by inapparent carriers of the organisms from upper respiratory tract [19].

Infections of respiratory disease in horses due to B. bronchiseptica have been earlier reported as the primary cause of horse pneumonia [1, 13]. In detailed study in foals, B. bronchiseptica was second most common isolate after S. zooepidemicus from bronchial lavage specimens in which no viruses were isolated [11]. Although the bacterial respiratory infections are generally considered to be as result of secondary invasion following a viral disease (1), in present cases EHV-1, and EHV-4 were not detected. The isolation of different strains of B. bronchiseptica from animals indicates that both were primarily suffering from upper respiratory infection caused by B. bronchiseptica. Isolation from geographically distant locations shows prevalence of B. bronchiseptica infection in horses in India.

The antimicrobial susceptibility profiling revealed that both isolates were susceptible to sulpha drugs and gentamicin which were used in treatment. Bordetella sp., have either unpredictable susceptibility or are predictably resistant to particular antibiotics or classes of antimicrobials [10]. The two Bordetella isolates were resistant to ceftazidime, penicillins and chloramphenicol. Diminishing antimicrobial susceptibility of B. bronchiseptica isolates is being reported worldwide for β-lactams like penicillins and cephalosporins [12]. In a previous study in India, porcine isolates of B. bronchiseptica were resistant to varying classes of antimicrobials [14].

Many virulence genes are associated with B. bronchiseptica, viz., adenylate cyclase-hemolysin (cyaA), filamentous hemagglutinin (fha), fimbriae (fim), and Bordetella virulence gene (bvgA) among others [5]. The virulence capability of strains (Eq24E & Eq128) was substantiated by detection of genes (cyaA, bvgA, flaA) involved in pathogenesis of B. bronchiseptica in respiratory infection. High amount of bacteria in mucous has been shown to be associated with increased inflammatory response in animals [29]. The predominant growth of B. bordetella culture on SBA in this study indicated inflammatory response in sick animals. Detection of fla confirms the adhesive capacity of our isolates, and cyaA detection and hemolysis on SBA shows the toxin, cyclolysin producing ability of isolates, thus producing the inflammation and mucopurulent discharge, which in addition to fever, and coughing found in present cases are consistent findings in equine bordetellosis [1].

The cyaA is a highly conserved virulence gene; and is expressed in all three bordetellae species [27]. Virulence factor expression level difference has been reported in different strains of B. bronchiseptica [18] which has been correlated with phylogeny [19] (Fig. 2). The phylogenetic analysis of partial cyaA gene sequence revealed a division showing an adaptive relation of strains with different hosts. Clade 2 predominantly had turkey (9) and human (11) cyaA sequences, showing that humans and turkeys may be sharing B. bronchiseptica isolates among themselves. Similar findings were reported earlier also [28].

The sequence analysis of N-terminal of cyaA has shown multi-clonal nature of B. bronchiseptica isolates. Variation has also been previously reported in this immunodominant region of B. bronchiseptica [2]. In our study, most of the substitutions did not bring about major changes in secondary structure of CyaA, except 1 (R235P) in Eq24E and Eq128 with changes from coil to helix, which may be functionally disruptive due to compromises on helical structure. Also conspicuous were 3 additional synonymous substitutions observed in Eq128 strain which separated the cyaA sequence of this strains from all other 39 cyaA sequences of B. bron-

| Isolate name | Amino acid change          | Secondary structure |
|--------------|----------------------------|---------------------|
| Eq24E and Eq128 | D234N (Aspartic acid to Asparagine) | Helix to helix       |
|              | E94A (Glutamic acid to Alanine)     | Helix to helix       |
|              | R235P (Arginine to Proline)        | Helix to helix       |
|              | E266D (Glutamic acid to Aspartic acid) | Helix to helix     |
|              | G278Q (Glycine to Glutamine)       | Helix to helix       |
|              | E278Q (Glutamic acid to Glutamine) | Helix to helix       |
|              | A364T (Alanine to Threonine)       | Helix to helix       |
|              | G370S (Glycine to Serine)          | Helix to helix       |
|              | D97N (Aspartic acid to Asparagine) | Helix to helix       |
| Eq128        | A160V (Alanine to Valine)          | Strand to strand     |
|              | N268S (Asparagine to Serine)       | Coil to coil         |
|              | K331R (Lysine to Arginine)         | Helix to helix       |
Fig. 2. Molecular phylogenetic analysis of partial cyaA nucleotide sequence by UPGMA method.
in *B. bronchiseptica*. The A160V, D268S and K331R synonymous substitutions in *B. bronchiseptica* Eq128 are also observed in the 27 sequence comparison with *Bordetella pertussis* and *B. parapertussis* CyaA protein sequences. Due to the constant immunological mediated changes observed, it can serve as marker gene for temporal and spatial tracking of *B. bronchiseptica* strains from different animal hosts.

This study confirms the presence of virulent *B. bronchiseptica* isolates, and their role in respiratory tract infection in horses in India. As *B. bronchiseptica* causes infection in a variety of companion and farm animals to which humans are frequently exposed, therefore zoonotic transmission to humans from horses can be possible, although such cases have been rare [30]. Studies are required to know the prevalence and epidemiology of *B. bronchiseptica* in Indian equines and their role in respiratory tract infections.

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