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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 chloramphanicol. 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*

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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 flaggelin (*fla*) (Table 1). *cyaA* and *bvgA* genes were sequenced commercially employing

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Table 1. Details of genes targeted and accession numbers

Gene	Primer sequences	Amplicon size (bp)	Cycling conditions	Accession Nos.
16S rRNA	F: 5′- AGAGTTTGATCCTGGCTCAG-3′ R: 5′- GACGGGCGGTGTGTRCA-3′	1,522	[29]	Eq24E- KT336825; Eq128- KT368942
bvgA	F:5′-AATTTCGCAGCCATTCCTTTGAC-3′ R: 5′-GATCAGACTGCGGGGGTACAG-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′-GGTGCGAATCCGTTCAATCGACTA-3′ F:5′-TTCCAGTACATCCGGCGAGGACTTC-3′	1,185		Eq24E-*cyaA* KY215872; Eq128-*cyaA* KY215873
flaA	F: 5′- CGCCGCCAACCAGTC-3′ R: 5′- GTACGTACTGCCATGGCCCCG-3′	736		Not sequenced

Table 2. Clinical and microbiological characteristic due to *B. bronchispetica*

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, anoerexic, coat ruffled, nasal discharge, head hanging low	*Staphylococcus* spp., *Escherichia coli, Pseudomonas* spp.	Gentamicin; Unknown

automated DNA sequencer (ABI 3130 Genetic Analyzer). 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 (NCVTC) 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 coccobacillary rods (Fig. 1c), catalase 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 decaroboxylase 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 chloramphanicol; 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%).

Amplification of genes *cyaA*, *bvgA* and *flaA* using DNA isolated from purified culture yielded 1,185, 768 and 736 bp products, respectively for both Eq24E and Eq128 strains of *B. bordetella*. The *bvgA* and *cyaA* genes showed identity of 98.87 to 99.37% at nucleotide (nt) level among compared isolates of *Bordetella* spp. The N-terminal partial sequence length of *cyaA* and *bvgA* genes sequenced was of 381 and 209 aa residues length, respectively. On Uniprot BLAST program analysis of *cyaA* partial sequences, the sequence of Eq24E isolate matched with *cyaA* of *B. parapertussis*

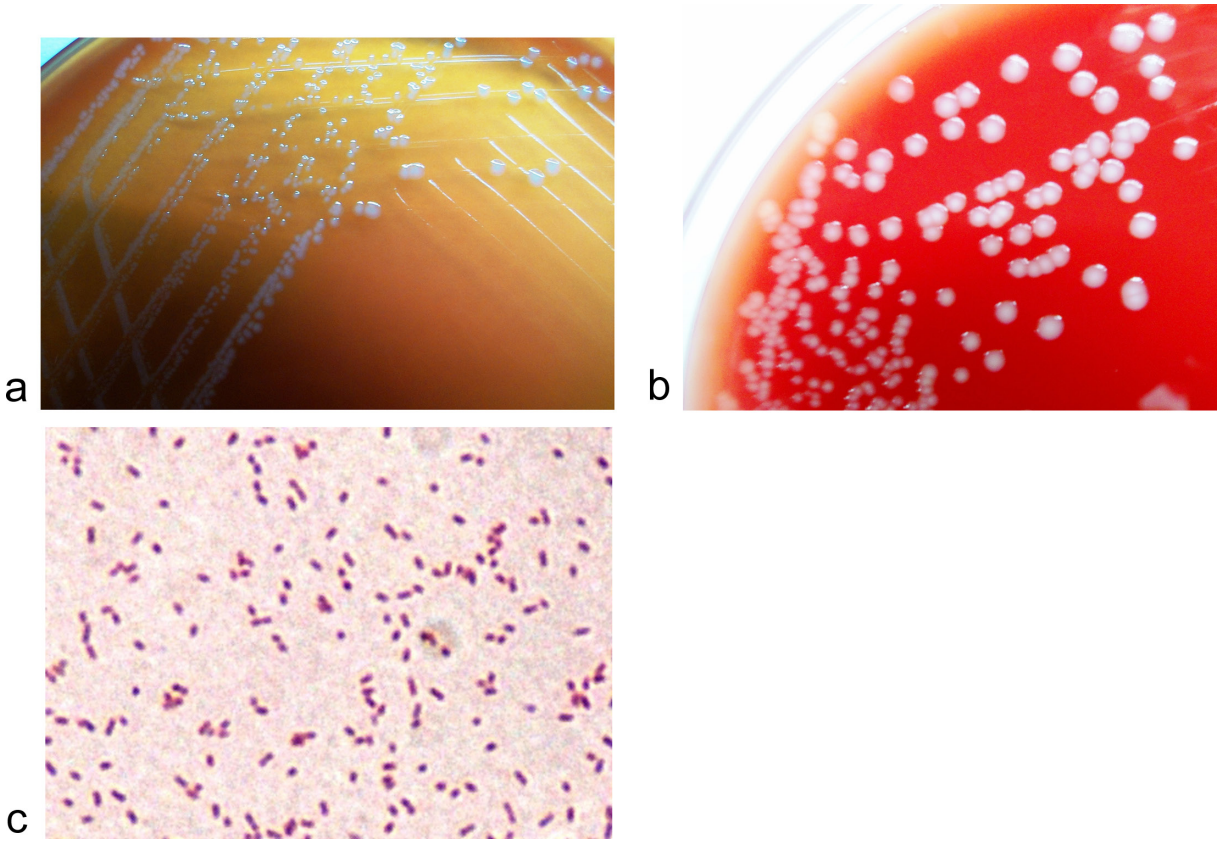

Fig. 1. a) Colony morphology of *B. bronchiseptica* Eq128 strain on SBA. b) *B. bronchiseptica* strain Eq24E shows poor hemolysis c) Microscopic morphology of *B. bronchiseptica.* Gram stain. 100 ×.

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

Antimicrobial	Eq24E	Eq128
Sulfafurazole	S	S
Co-trimoxazole (trimethoprim-sulphamethoxazole)	S	S
Cephalothin	S	S
Gentamicin	S	S
Ceftazidime	R	R
Tetracycline	S	S
Penicillin G	R	R
Ampicillin	S	R
Chloramphanicol	R	S
Amoxicillin	S	S
Amikacin	S	S
Ciprofloxacin	S	S

R, resistant; S, sensitive.

(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 *cyaA* 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 *cyaA* 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 *cyaA* gene fell into clade 2 only. Similarly all the pig isolates fell into clade 1b (Fig. 2). The *cyaA* 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

Table 4. Predictive changes in amino acid sequences and secondary structure of *cyaA* amino-terminal portion

Isolate name	Amino acid change	Secondary structure
Eq24E and Eq128	D234N (Aspartic acid to Asparagine)	Coil to coil
	E94A (Glutamic acid to Alanine)	Helix to helix
	R235P (Arginine to Proline)	Coil to helix
	E266D (Glutamic acid to Aspartic acid)	Coil to coil
	G278Q (Glycine to Glutamine)	Helix to helix
	E278Q (Glutamic acid to Glutamine)	Helix to helix
	A364T (Alanine to Threonine)	Coil to coil
	G370S (Glycine to Serine)	Coil to coil
	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

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 chloramphanicol. 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 [18] (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-*

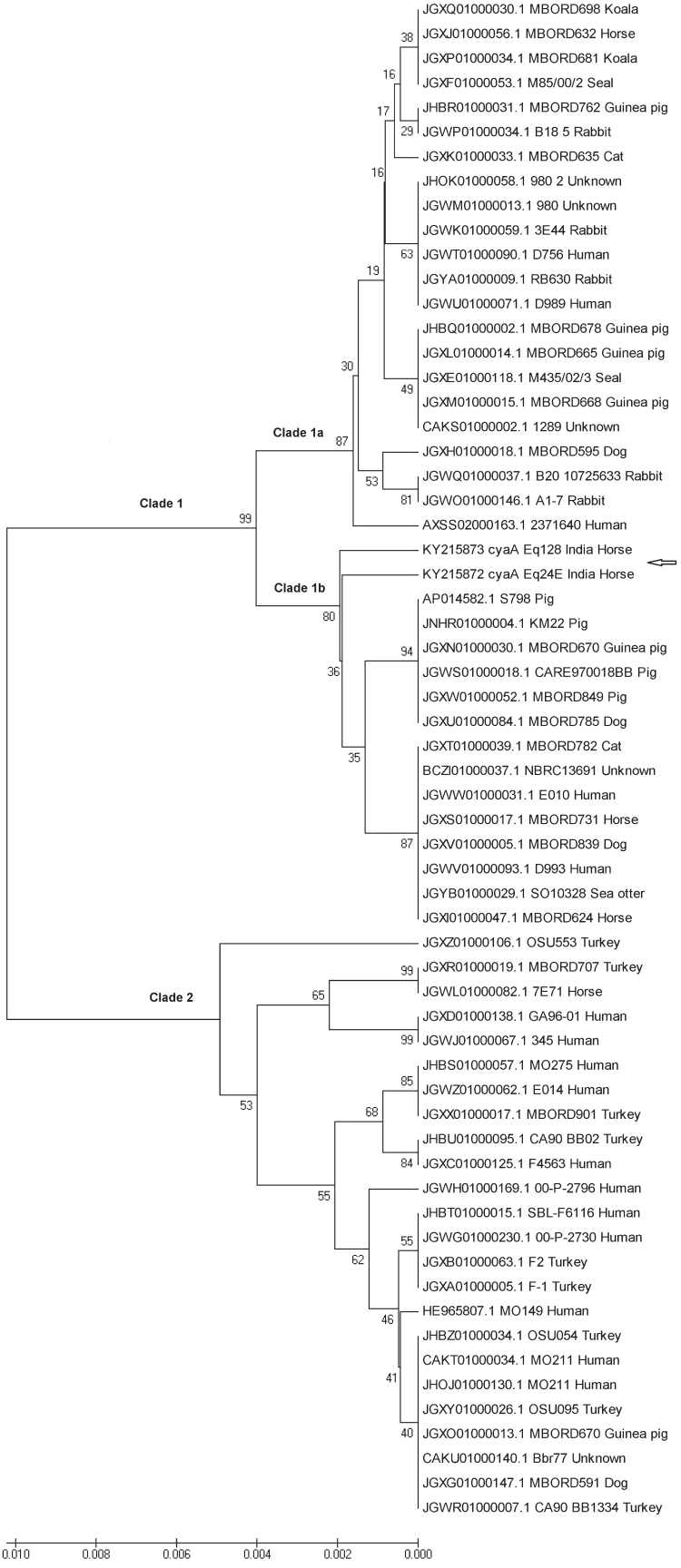

Fig. 2. Molecular phylogenetic analysis of partial *cyaA* nucleotide sequence by UPGMA method.

chiseptica. 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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