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Short Communication

Microbiological and molecular detection of *Canicola* (*Haemophilus*) *haemoglobinophilus* from the urine of a dog

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Abstract

Urinary tract infections (UTIs) are one of the most common bacterial infections in humans and animals. They are mostly caused by pathogens like *Escherichia coli, Enterococcus faecalis, Proteus mirabilis, Klebsiella pneumoniae* and *Staphylococcus* spp. The major concerns with UTIs are the emergence of rare uropathogens like *Haemophilus* spp., high recurrence rates and the increasing antimicrobial resistance in the uropathogens. Here we report the isolation, microbiological and molecular detection of *Canicola* (*Haemophilus*) *haemoglobinophilus* from a female dog with urinary tract infection. The study highlighted the need for the inclusion of *Canicola* (*Haemophilus*) spp. in routine clinical bacteriological examinations.

Key words: Canine, Urinary tract infections, 16s rRNA confirmation

Highlights

- Report of *Canicola* (*Haemophilus*) *haemoglobinophilus* in animals is very rare.
- *C. haemoglobinophilus* isolate was resistant to penicillin G, erythromycin, and nitrofurantoin only.
- The isolate was present in the same clade with NCTC 1659 *C. haemoglobinophilus* strain.

Urinary tract infections (UTIs) in dogs due to bacterial infections is a common cause of morbidity (Weese *et al*., 2019) and occur in approximately 14% of dogs at least once in a lifetime (Ling, 1984). Comparatively, females (26.6%) have more incidence of UTIs than males (6.2%) (Kivistö *et al*., 1977). To confirm the presence of bacterial UTIs in dogs with indications of lower urinary tract disease, cystocentesis is advised, followed by urinalysis and quantitative aerobic bacterial culture (Weese *et al*., 2019). The UTIs in dogs are sometimes persistent or recurrent but mostly uncomplicated and resolve with 2-3 weeks of treatment with antimicrobials (Thompson *et al*., 2011). As in humans and cats, the most common pathogen isolated from UTIs in dogs is *Escherichia coli*. Other pathogens like *Enterococcus* spp. and *Pseudomonas aeruginosa* are highly prevalent in persistent or recurrent infections compared to uncomplicated infections (Seguin *et al*., 2003). Recently, there has been a growing area of concern in the treatment of UTIs due to the development of multiple drug resistance. *Haemophilus* spp. is one of the members of the so-called HACEK (*Haemophilus* spp., *Aggregatibacter* spp, *Cardiobacterium* spp., *Eikenella* spp. and *Kingella* spp.) group, which are slow-growing bacteria (Dingle *et al*., 2014). They are commensal to their hosts and sometimes have been documented rarely on the variety of local upper respiratory and systemic infections (Murphy, 2015). *Canicola haemoglobinophilus,* previously known as *Haemophilus haemoglobinophilus* or

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Haemophilus canis, was first isolated from the prepuce of a dog in 1903 by Friedberger (Frazer and Rogers, 1972). Recently, the new genus *Canicola* was proposed with one species, *C. haemoglobinophilus*, which is reclassified from [*Haemophilus*] *haemoglobinophilus*, based on phylogenetic similarities (Christensen *et al*., 2021).

A sample of urine from a female dog (Sample ID: 4387DUO) with pyuria (>50 pus cells/ field), having recurrent pyrexia and not taking its food for last one week, though with normal thirst drinking water and straining while urinating frequently and in small amounts, was submitted to the clinical epidemiology laboratory, ICAR- Indian Veterinary Research Institute, Izatnagar for the bacteriological examination. The sample (2 µL) was initially plated onto 5% (sheep blood) chocolate agar (to support the growth of fastidious bacteria if any) and onto a common differential media, MacConkey agar (BBL-Difco, USA) and incubated at 37°C for 24-48 hours. Colonies were counted, and 5 isolated colonies were picked up for further characterization based on growth, staining and biological characteristics. Biochemical tests like catalase, oxidase, indole, methyl red, Voges Proskauer, citrate utilization, nitrate reduction, growth on triple sugar iron agar, urease, malonate, phenylalanine deaminase, gelatine liquefaction and DNase tests were carried out as per standard procedure (Singh, 2009). The isolates were subjected to antimicrobial susceptibility testing (AST) using the disc diffusion method (Bauer, 1966) on bovine serum (5%) supplemented Mueller-Hinton agar (BBL-Difco, USA). Commercial AST discs like amoxycillin (30 µg), amoxycillin (30 µg) + clavulanic acid (10 µg), ampicillin (10 µg), azithromycin (15 µg), aztreonam (30 µg), cefotaxime (10 µg), cefoxitin (10 µg), ceftazidime (30 µg), ceftriaxone (10 µg), chloramphenicol (25 µg), colistin (10 µg), cotrimoxazole (25 µg), doxycycline (30 µg), enrofloxacin (10 µg), erythromycin (15 µg), gentamicin (30 µg), imipenem (10 µg), meropenem (10 µg), nitrofurantoin (300 µg), penicillin G (10 IU), piperacillin (100 µg), piperacillin (100 µg) + tazobactam (10 µg), tetracycline (30 µg) and tigecycline (15 µg) (Difco, USA) were used. The isolates were initially confirmed by biochemical screening, and one of the isolates was deposited in the repository of the Veterinary Type Culture Collection Centre (VTCC), ICAR-IVRI, Izatnagar for future reference. The isolate was maintained on a chocolate agar medium till the end of the study.

The DNA was extracted from freshly grown pure culture by snap chill method. Briefly, in 200 µL nuclease-free water a loopful of colonies from chocolate agar was heated at 95-98°C for 10 minutes, followed by immediate freezing at –20°C for 10 minutes and finally centrifuged at 10,000 rpm for 10 minutes to collect supernatant to be used as the template DNA for polymerase chain reaction (PCR). The PCR was carried out using universal primers (16s For - AGAGTTTGATCMTGGCTCAG and 16s Rev - GYTACCTTGTTACGACTT) targeting the 16s rRNA region (Lane, 1991). The amplification was performed for 35 cycles of denaturation, annealing, and extension at 94°C for one minute, 55°C for 30 seconds, and 72°C for two minutes, respectively, with a final extension of 72°C for 5 minutes. The amplified products were run on 1.2% agarose gel electrophoresis, and results were documented using Chemiluminescence gel documentation system (AlphaImager HP, Premas Life Sciences Pvt. Ltd New Delhi, India). The PCR product was custom sequenced from Eurofins Genomics India, New Delhi, India. The sequences were aligned, and NCBI BLAST search was carried out to find the matching sequences available in the data bank. For phylogenetic analysis, 16s rRNA gene sequences of different *C. haemoglobinophilus* strains and related species were retrieved from DNA Data Bank/GenBank, and a phylogenetic tree was built by the Maximum Likelihood method created on the Tamura-Nei model (Tamura and Nei, 1993) in MEGA 7.0.26 software.

Uncountable (>400) colonies were observed

Table 1. Biochemical characteristics of the *C. haemoglobinophilus* isolates

Test	Results	Test	Results
Catalase	+	Growth in triple sugar iron agar	+
Oxidase	+	Urease	-
Indole (I)	+	Malonate	-
Methyl red (MR)	-	Phenylalanine deaminase	-
Voges Proskauer (VP)	-	Gelatin liquefaction	-
Citrate utilization (C	-	DNAse tests	-
Nitrate reduction	+	H_2S production	+

Table 2. Antibiotic susceptibility assay results of the *C. haemoglobinophilus* isolates

Antimicrobials	Results	Antimicrobials	Results
Amoxycillin (30 µg),	S	Doxycycline (30 µg)	S
Amoxycillin (30 µg) + clavulanic acid (10 µg)	S	Enrofloxacin (10 µg)	S
Ampicillin (10 µg)	S	Erythromycin (15 µg)	R
Azithromycin (15 µg)	S	Gentamicin (30 µg)	S
Aztreonam (30 µg)	I	Imipenem (10 µg)	S
Cefotaxime (10 µg)	S	Meropenem (10 µg)	S
Cefoxitin (10 µg)	S	Nitrofurantoin (300 µg)	R
Ceftzidime (30 µg)	S	Penicillin G (10 IU)	R
Ceftiaxone (10 µg)	S	Piperacillin (100 µg)	S
Chloramphenicol (25 µg)	S	Piperacillin (100 µg) + tazobactam (10 µg)	S
Colistin (10µg)	S	Tetracycline (30 µg)	S
Cotrimoxazole (25µg)	S	Tigecycline (15 µg)	S

after 48 hours of incubation on a chocolate agar plate inoculated with 2 µL of urine. The *C. haemoglobinophilus* appeared as small, smooth, convex, pinpoint, transparent colonies on 5% chocolate blood agar (Fig. 1) but not on MacConkey agar after 48 hours of incubation at 37°C. On Gram's staining, the bacterium appeared as small gram-negative rods. The biochemical properties of all the five colonies were identified, evaluated, and compared, as described in Bergey's Manual of Determinative Bacteriology (Bergey, 1994) and were found to be in concordance with that of *C. haemoglobinophilus* (Nørskov Lauritsen, 2015) (Table 1).

On antimicrobial susceptibility assay, all the five colonies were found to be resistant to penicillin G, erythromycin, and nitrofurantoin (Table 2).

The PCR reaction performed using 16S rRNA gene primers showed the amplification of 16S rRNA genes at size approximately 1500 bp (base pairs) when compared with 100 bp markers. Amplified products were sequenced, which gave the size of a 1.15 kb gene sequence. Global alignment and comparative analysis were conducted at NCBI BLAST analyses using NCBI BLASTN for evolutionary analysis. The results showed that identified strain had 99% identity with *C. haemoglobinophilus* strain NCTC 1659 from the USA. The partial sequences were submitted to the NCBI, and the accession number "MW325980" was obtained

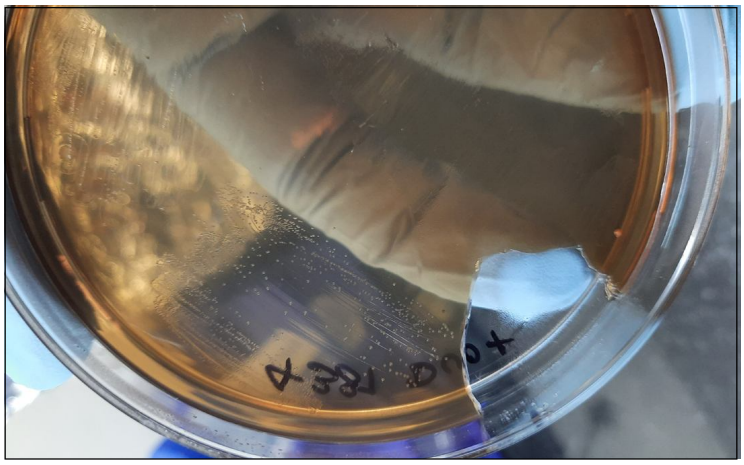

Fig. 1. ***Canicola (Haemophilus) haemoglobinophilus*** **in chocolate agar**

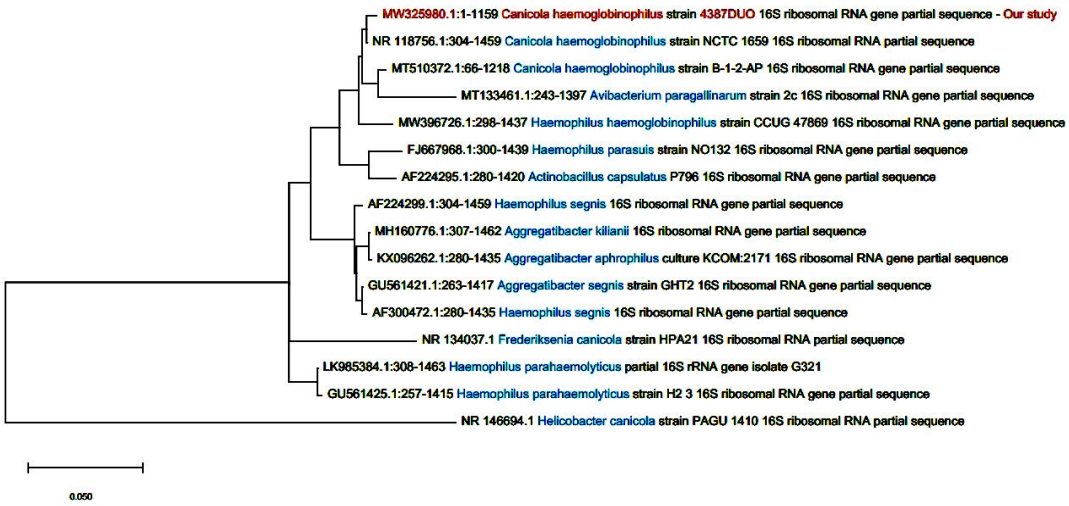

Fig. 2. The phylogenetic tree on the Maximum likelihood method based on partial sequence 16S Ribosomal RNA gene with different related species

based on the 16S rRNA gene of *C. haemoglobinophilus*. The phylogenetic analysis of the 16s ribosomal DNA partial sequence is shown in Fig. 2 using the maximum likelihood method.

In canines, urinary tract infections are common and are multifactorial. Bacterial UTIs are caused mostly by gram negative organisms than gram positive (Rampacci *et al*., 2018). These organisms are usually commensals but invade the urinary system when the immune system of the host is compromised (Ettinger and Feldman, 2010). The infections are mostly uncomplicated in nature (Thompson *et al*., 2011). In our study, the observation indicated that >20,000 live bacteria might be present in each mL of urine. If the bacterial count in urine exceeds $>10^2$-10^3 CFU/mL, it is an indication of bacteriuria; however, in severe cases of UTIs, the count may exceed $>10^5$ CFU/mL of urine (Stamm *et al*., 1982). *Haemophilus* species have been isolated from a variety of unexpected sites, including gastrointestinal and gynaecological sites, as well as soft tissue, muscle tissue and bone (Christensen, 1990). In Hungary, *C. haemoglobinophilus* was isolated from the

pharyngeal swabs of seven canine cases presented for endoscopic examination (Ujvári *et al*., 2020). It may be present in the normal oral microflora (Kačírová *et al*., 2019; Maďar *et al*., 2021). *Haemophilus* spp. and *C. haemoglobinophilus* are usually fragile and cannot survive for a long time once it has been removed from the hosts (Christensen *et al*., 2021). The bacteria grow well on both chocolate agar and 5% sheep blood agar under aerobic and microaerophilic conditions (Mazurova and Kubankov, 2000). The incidence of *Haemophilus* spp. infection in UTIs is not well documented or underestimated, because it fails to grow in standard urine culture medium (Gabre-Kidan *et al*., 1984; Hansson *et al*., 2007). For example, if the samples won't be inoculated in the chocolate agar plate in the routine urine bacteriological analysis, you may miss the pathogen (Diedrich and Manby, 2017). *Haemophilus* spp. infections in humans are mostly associated with any urinary tract abnormalities (Morgan and Hamilton-Miller, 1990; Roth *et al*., 2009; Stærk *et al*., 2018) and children with urinary tract abnormalities or malfunction can be considered "compromised hosts" (Jantunen *et al*., 2001). The first report of *C. haemoglobinophilus* was of acute otitis media in a child (Frazer and Rogers, 1972). Though *C. haemoglobinophilus* organisms are reported as commensals in the lower urinary tract, especially in males, are associated with vaginitis (Osbaldiston, 1971) and cystitis (Diedrich and Manby, 2017) in females. In Spain, the incidence of *Haemophilus* spp. UTIs in human patients were 0.27% with 0.88% for paediatric ones (Galan *et al*., 1996). The observations of antimicrobial susceptibility test are like the previous studies on *Haemophilus* spp. (Diedrich and Manby, 2017). There are numerous antimicrobial agents that can be used in the clinical treatment of UTIs, including fluoroquinolones, aminoglycosides, β-lactams, tetracyclines, and potentiated sulfonamides, with the choice of drugs varying by region (Rampacci *et al*., 2018). In our study, antimicrobial susceptibility testing showed the susceptibility of the bacteria to various classes of antibiotics including the commonly used antibiotics for UTIs like potentiated amoxicillin, ceftriaxone, potentiated sulphonamides, etc. The phylogenetic analysis confirmed the isolate as *C. haemoglobinophilus*. The isolate was present in the same clade as NCTC 1659 *C. haemoglobinophilus* strain. Though *Haemophilus* spp. seems to be of zoonotic importance, it is not possible to estimate its real significance considering present or earlier studies. To the best of the authors' knowledge, this is the first report to show *C. haemoglobinophilus* in UTIs in bitch in India.

Conflict of interest: Authors have no conflict of interest in this study.

Author's contribution: RK: Carried out the experiment, draft, and revision of the manuscript; AY: Participated in the experiment; HA: Involved in the development of manuscript; VJ: Involved in the development of manuscript; AP: Sample collection; DKS: Involved in the development of manuscript; BRS: Conceptualized, isolated the bacteria, involved in the development of draft and revision of the manuscript.

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