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Detection and Phylogenetic Characterization of Virulence Genes of *Escherichia coli* Associated with Canine Urinary Tract Infections in India

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Abstract

BACKGROUND: *Escherichia coli* (*E.coli*) is one of the most common and important causative bacterium of urinary tract infections (UTIs) in both dogs and humans.

OBJECTIVES: This study aimed to identify virulence genes and a phylogenetic group of *E. coli* isolated from the urine of dogs suffering from UTIs.

METHODS: *E. coli* were isolated from urine of dogs suffering from UTIs and tested for the presence of the virulence genes using conventional polymerase chain reaction (PCR) and sequencing method.

RESULTS: Out of a total of 103 samples, 25 were found to be positive for *E. coli*, of these 20 (80.0%) were identified as *aer*, 14(56.0%) as *pap*, 12(48.0%) as *sfa*, 8(32.0%) as *afa*, 5(20.0%) as *hly* and 5(20.0%) as *cnf1* genes. None of the isolates carried *cnf2* genes.

CONCLUSIONS: The study demonstrated a high occurrence of virulence genes. The phylogenetic comparisons of these gene sequences detected in uropathogenic *E. coli* isolated from dogs showed high similarity to those present in the urine of humans with urinary tract infection. Phylogenetic comparisons of the virulence genes revealed that *hly*, *sfa* , *cnf1* and *pap* matched to group B2, *afa* to group A and *aer* to group B2 and D.

KEYWORDS: Dog, *Escherichia coli*, Polymerase Chain Reaction, Urinary Tract Infection, Virulence genes

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Introduction

Dogs are perhaps the most favoured domestic animal among all the pet animals. In developed countries, companion animals have become an integral part of the household (Chomel and Sun, 2011). Companion animals, especially dogs, suffer from various bacterial diseases affecting urinary tract leading to malfunctioning of this system, followed by critical medical emergency or death of the animal (Davison et al., 1999). Bacterial urinary tract infections (UTIs) are the most common cause of urinary tract disease in dogs. Approximately 14% of all dogs will acquire bacterial UTIs during their lifetimes with variable age of onset (Osborne, 1999).

Escherichia coli (*E. coli*) is the most prevalent and important causative agent of UTIs in dogs and humans, strains of this species are often abundant in the gastrointestinal tract at the time of infection (Johnson et al., 2003; Momoh et al., 2011; Tramuta et al., 2011 Saraylu et al., 2012).

However, *E. coli* that are associated with UTIs are commonly named uropathogenic isolates (UPEC), although there is evidence that different pathotypes may be related to UTIs (Marrs et al., 2005). Urinary tract infections are mostly common in dogs, but clinical signs are often minimal, and the infection may go undetected (Ling, 1995). Uropathogenic *Escherichia coli* is one of the most common bacteria isolated from canine and feline UTIs (Osugui et al., 2014).

Uropathogenic *Escherichia coli* contain several virulence factors that facilitate its colonization and invasion of host cells (Ejrnæs, 2011; Kudinha etal, 2012; Agarwal et al., 2013). Surface virulence factors (adhesins) of UPEC are among the most important virulence factors (Nicole, 2008; Bien et al., 2012).The main attachment factor, P fimbriae are particularly associated with pyelonephritis and are encoded by pap genes (Jadhav et al., 2011). Another adhesion that acts as a virulence factor is S fimbrial adhesion, which is coded by *sfa* genes (Bien et al., 2012; Pobiega et al., 2013). Also, afimbrial adhesions (*afa*) of *E. coli*, coded by *afa* genes, have been reported in cases of pyelonephritis (Servin, 2005). Other important virulence factors of UPEC strains are the toxins that act as secretory virulence factors (Bien et al., 2012). The most important secretory virulence factor is A-hemolysin (*hlyA*), which is encoded by the *hly* gene. Also, cytotoxic necrotizing factor 1 (*cnf1*) is reported in a third of pyelonephrogenic strains (Johnson, 1991). Other virulence factors also have important roles in the development of UTIs, including serum resistance ability due to the outer membrane protein *traT* encoded by *traT* genes (Mellata et al., 2003; Kawamura-Sato et al., 2010). Aerobactin is a bacterial siderophore encoded by aer genes and has recently been documented as a virulence factor in UPEC strains (Slaychev et al., 2009). The virulence factors are carried by pathogenicity islands (PAIs), which are mobile genetic elements that contribute to the horizontal transfer of virulence determinants (Hacker et al., 1997; Sabate et al., 2006). Phylogenetic analyses showed that *E. coli* strains and virulence genes were divided into four major phylogenetic groups (A, BI, B2, and D) based on their genetic polymorphisms (Clermont et al., 2000). It is known that the expression of virulence genes and phylotypes varies with geographical location (Agarwal et al., 2013). However, phylogenetic char-

acterization is an important tool to improve the understanding of *E. coli* populations and their relationship between strains and disease (Coura et al., 2015). Molecular characterization of *E. coli* virulence genes that are associated with UTIs in dogs has been studied in America and most parts of the world. However, in India, there is relatively less documented information on *E. coli* virulence genes associated with UTIs in dogs. The current study aimed to determine the virulence genes and their phylogenetic grouping in *E. coli* isolates from dogs with UTIs.

Materials and Methods

Sample Collection

From February 2017 to January 2018, a total of 103 urine samples were collected aseptically through cystocentesis from dogs that were diagnosed with urinary tract infection in small animal section of Veterinary Clinical Complex (VCC), Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS) Hisar Haryana, India.

Bacteriological Examination

The fresh urine samples collected aseptically were inoculated and streaked onto a 5% sheep blood agar (BA) (HiMedia, Mumbai, India) and MacConkey's lactose agar (MLA) (HiMedia, Mumbai, India) plates separately with the help of a 4 mm diameter platinum loop. The plates were incubated aerobically at 37°c for 24-48h till adequate growth was observed. Suspected colonies were streaked onto Eosine Methylene Blue agar (EMB), (HiMedia, Mumbai, India) which is a selective medium for *E. coli* and the plates were incubated aerobically at 37°c for 24h. The appearance of blue, green colonies with a metallic luster on EMB was presumptively considered as indicating the presence of *Escherichia coli*.

Biochemical Examination

Escherichia coli was identified using Gram staining technique. Samples with rod-shaped arrangements were subjected to biochemical tests. (Indole, Methyl Red, Voges Proskauer, Citrates tests, Glucuronidase, Nitrate reduction, ONPG, Lysine utilization, Lactose, Glucose, Sucrose and Sorbitol) using commercially KB010 Hi E.coli™ Identification Kit (HiMedia Mumbai, India) following the manufacturer's instructions.

Isolation of Bacterial DNA

DNA of *Escherichia coli* from all the positive isolates was extracted using commercial available PureLink™ Genomic DNA mini kit (Invitrogen, USA) following the manufacturer's instructions as shown below.

DNA of *Escherichia coli* from all the positive isolates was extracted using commercial available PureLink™ Genomic DNA mini-kit (Invitrogen, USA). Briefly, overnight grown culture in BHI broth (10 ml) was centrifuged at 10000 rpm for 10 min at 4°C. Supernatant was discarded and pellet was re-suspended in 200µl of digestion buffer. Then 20µl of proteinase K was added and vortexed vigorously. The solution was then transferred into 2ml micro centrifuge tube and incubated in water bath (Bench Top Lab System, USA) for 2 h at 55°c. A total 200µl of both lysis/binding buffer and absolute ethanol was then added and each tube was carefully vortexed in order to mix the contents of the tube evenly. The solution was then carefully transferred into a PureLink™ column and then centrifuged. Five hundred microliters (500µl) of buffer I was added to the samples, mixed and centrifuged for 2 min at 12000 rpm,

the wash trough was discarded. Then 500µl of buffer II was added and the column was centrifuged for 2 min at 12000 rpm, the wash trough was discarded and the empty column was centrifuged for 2 min. The column was then transferred into a fresh 1.5ml micro centrifuge tube. Finally, for eluting the DNA, 30µl of elution buffer was added at the centre of the column, the column was incubated for 10 min at room temperature and then centrifuged at 12000 rpm for 2 min. The flow through was collected in a 1.5ml micro centrifuge tube as the DNA. The obtained DNA was stored at -20 °c for further use.

Detection of Virulence Genes

The presence of virulence genes in *E. coli* DNA extracts was determined by conventional PCR (Manage et al., 2019). Primers sequences, target genes, products size, and references are given in Table 1. The conventional PCR was performed in veriti thermo cycler (ABI, USA) in 25 volume reaction containing 6 µl of template DNA, 1µl of each of the primers (10 pmoles), 12.5 µl Phusion PCR Master-mix (2X) (High Fidelity, USA), 1µl DMSO and 3.5 µl of nuclease-free water. Amplification procedure consisted of initial denaturation at 98 °c for 30 s, followed by 35 cycles of denaturation at 98 °c for 10 s, annealing at 60 °c for 30 s, extension at 72°C for 30 s and a final extension at 72°c for 5 min. The PCR products were analyzed on 1.5% agarose gel electrophoresis and visualized under UV trans-illuminator GEL-DOC™ (BIO-RAD, India) and documented by photography for further analyses.

Nucleotide sequence analysis of the virulence genes and their phylogenetic comparisons

The PCR products obtained were purified using PureLink™ Quick Gel Extraction Kit (Invitrogen, Germany) and the cycle sequencing reactions were performed in a total volume of 10µl using Big Dye Terminator v 3.1 Cycle Sequencing Kit (ABI, USA) in automated DNA sequencer (Applied Biosystem 3130XL Genetic Analyzer). The DNA sequencing was performed in both directions with the PCR primers as sequencing primers (Table 1).

The resulting sequences obtained from the sequencer were used to make Contigs using SeqMan program in Lasergene suite (version 5). The contigs were then compared with those reported in GenBank (NCBI) by a BLAST search online. Clustal W (Bioedit software ver 9.0) was used for multiple sequence alignment, and Mega 6.0 bioinformatics sequence analysis tool was used for phylogenetic studies.

Statistical analysis

Vassarstat was used for the analysis of confidence interval (proportion).

Results

Examination of a total of 103 urine samples from dogs suffering from UTIs for detection of *Escherichia coli* revealed 25 (24.3%) positive isolates.

The cultural growth on MacConkey lactose agar was seen as typical purple/pink colonies. However, the positive isolates were further confirmed as *E. coli* by inoculation on Eosine-Methylene blue agar medium.

Microscopic examination of gram stained colonies shows a gram negative rod-shaped arrangement. The biochemical test showed that *E. coli* were positive for Indole, Methyl red, Glucuronidase, Nitrate, ONPG, Lysine utilization, Lactose, Glucose, Sucrose, and Sorbitol. On the other hand, all the strains were negative for Voges-Proskauer's and Ci-

Table 1. Primers for conventional PCR assays

Target Genes	Primers Sequences	Product Size (bp)	References
cnf1	F: AAGATGGAGTTTCCTATGCAGGAG R: CATTCAGAGTCCTGCCCTCATTATT	498	(Falbo et al.,1992)
cnf2	F:GTGAGGCTCAACGAGATTATGCACTG R:CCACGCTTCTTCTTCAGTTGTTCCTC	839	(Pass et al.,2000)
Pap	F: GCAACAGCAACGCTGGTTGCATCAT R:AGAGAGAGCCACTCTTATACGGACA	336	(Yamamoto et al., 1995)
Aer	F: TACCGGATTGTCATATGCAGACCGT R: AATATCTTCCTCCAGTCCGGAGAAG	602	(Herrero et al., 1988)
Afa	F: GCTGGGCAGCAAACTGATAACTCTC R: CATCAAGCTGTTTGTTCGTCCGCCG	750	(Le Bouguenec et al., 1992)
sfa	F: CTCCGGAGAACTGGGTGCATCTTAC R: CGGAGGAGTAATTACAAACCTGGCA	410	(Le Bouguenec et al., 1992)
hly	F: AACAAGGATAAGCACTGTTCTGGCT R: ACCATATAAGCGGTCATTCCCGTCA	1177	(Nam et al., 2013)

trate utilization test.

Of the 25 *E. coli* isolates assayed, 20(80.0%) showed the presence of *aer*, while 14 (56.0%), 12(48.0%), 8(32.0%) and 5(20.0%) were found to have *pap*, *sfa*, *afa*, *cnf1* and hly genes respectively. No *cnf2* gene was found in any of the *E. coli* isolates tested (Table 2).

The *aer, afa, cnf1, hly, sfa*, and *pap* gene sequences show the highest nucleotide similarity of 99% with several strains retrieved from the GenBank data base.

hly, sfa, cnf1, and *pap* gene sequence were highly similar to the sequences that were associated with the phylogenetic group B2 whereas *aer* gene sequences showed maximum similarity to the one in B2 and D phylogenetic group and *afa* sequence grouped within the phylogenetic group A.

Table 2. Distribution of virulence genes of *E. coli* isolated from 25 dogs diagnosed with UTIs in Hisar Haryana, India.

Virulent genes	(%) No. of isolates	Proportion	95% CI (Lower Limit)	95% CI (Upper Limit)
Cnf1	5	0.0485	0.0209	0.1086
cnf2	0	0.0	0.0	0.0
Pap	14	0.1359	0.0827	0.2153
Aer	20	0.1942	0.1294	0.281
Afa	8	0.0777	0.0399	0.1459
Sfa	12	0.1165	0.0679	0.1927
Hly	5	0.0485	0.0209	0.1086

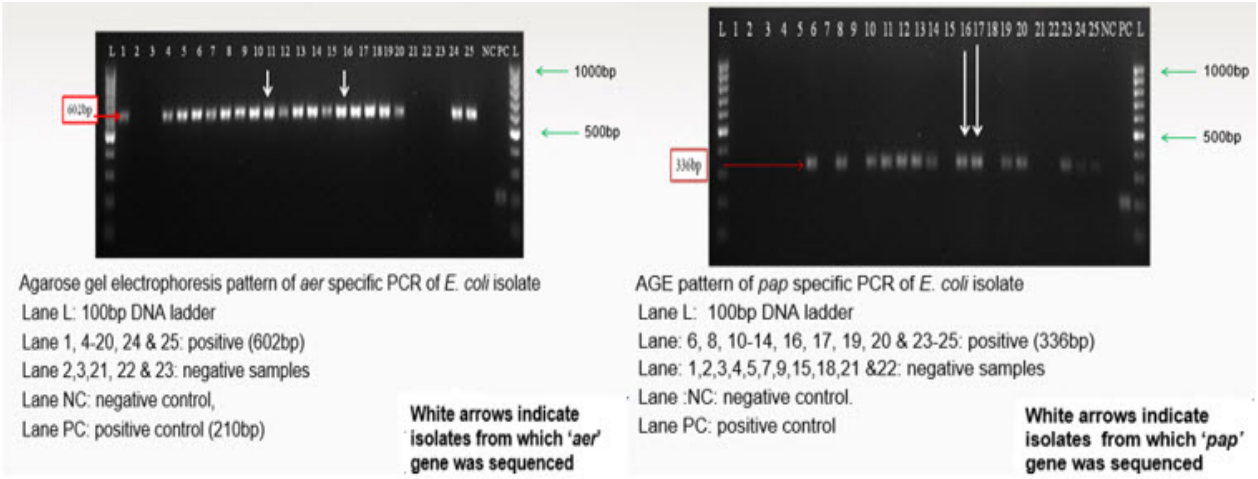

Figure 1. Agarose gel electrophoresis of *aer* and *pap*

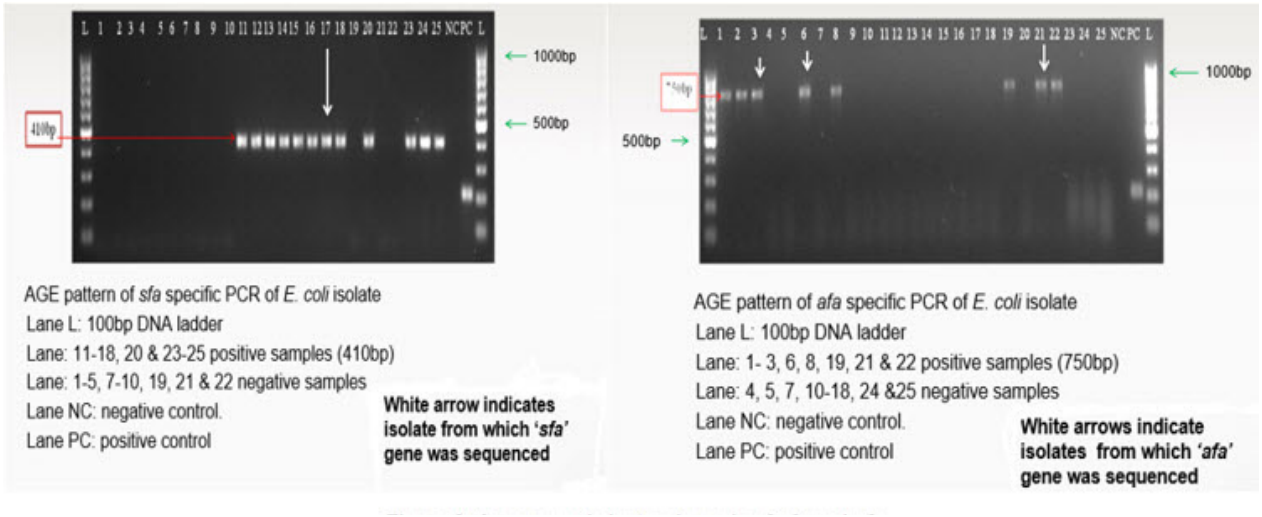

Figure 2. Agarose gel electrophoresis of *sfa* and *afa*

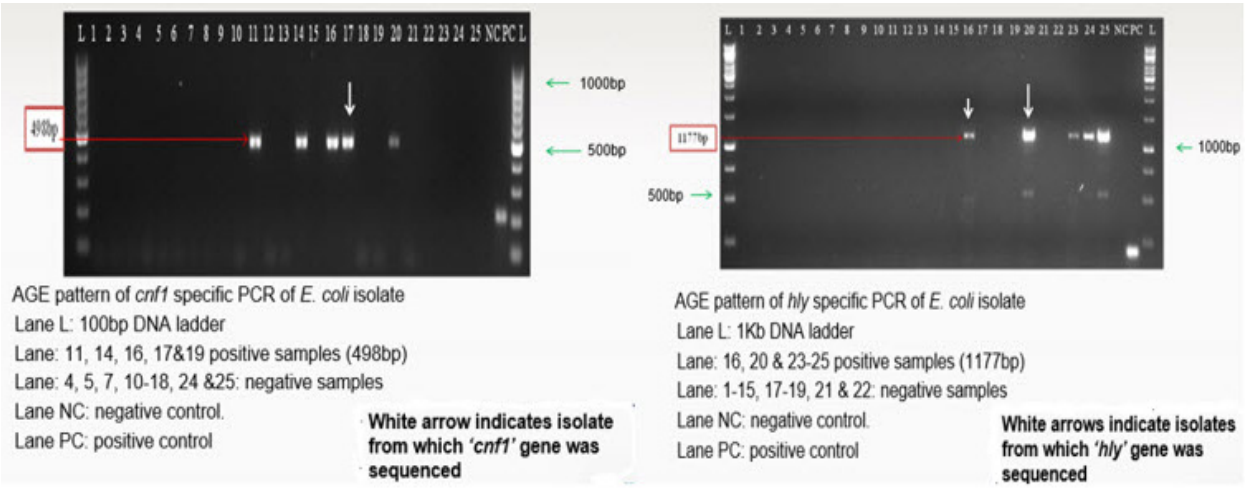

Figure 3. Agarose gel electrophoresis of *cnf1* and *hly*

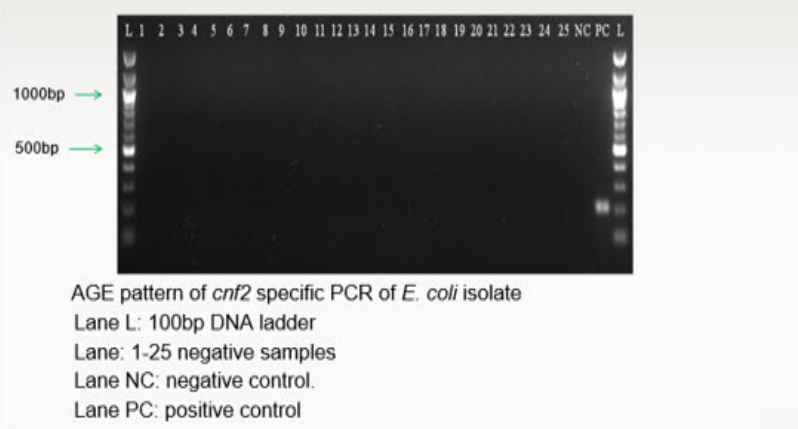

Figure 4. Agarose gel electrophoresis of *cnf2*

Discussion

Distribution of virulence factor gene associated with canine UTIs has not been previously determined and characterized in the study area (Hisar, India). The presence of the most common virulence genes such as a*er, pap, sfa, afa*, and *hly* detected in *E. coli* strains isolated in the present study established the pathogenic potentials of *E. coli* in dog's urogenital infections.

Aerobactic receptor gene, coded by *aer*, promotes bacterial growth in the limiting iron concentration encountered during infection and act as a virulence factor in the pathogenesis of UTIs (Mittals et al., 2014). However, iron is necessary for *E. coli* metabolism (Siqueira et al., 2009). Growth under iron-restricted conditions requires bacterial mechanisms that successfully compete with the host for the ion (Emody et al., 2003). *Escherichia coli* organism uses ion for oxygen transport and storage, DNA synthesis, electron transport, and peroxide metabolism (Siqueira et al., 2009). The *aer* virulent gene may be relevant during bacterial spreading from the urinary tract to the bloodstream (Garcia and Le Bouguenec, 1996). The *aer* virulent gene was seen in 80.0% of the isolates in the present study, this is because *aer* is a virulence factor in UPEC strains. Similarly, Slaychev et al. (2009) found comparable gene in their study as a virulent factor. In other studies, the detection of *aer* has been reported to be more than 30.0% and 56.0% (Firoozeh et al., 2014; Munkhdelger et al., 2017), which is in concordance with the current study. Furthermore, Drazenovich et al. (2004) also found *aer* in 30.0% from *E. coli* strains isolated with persistent UTIs in dogs. The present study demonstrated high occurrence of *aer* genes in uropathogenic *E. coli* strains suggesting that the frequencies of iron acquisition system genes vary according to the geographical locations.

In the current study, most of the uropathogenic *E. coli* was isolated from the urinary tract of dogs. This finding agrees with the findings of Siqueira et al. (2009), in which they documented that adhesion and colonization of the uroepithelium is the most important phenomenon involving uropathogenic *E. coli.* However, these adhesions promote bacterial adherence and are indispensable for the infection to be established as was reported by (Johnson, 1991; Emody et al., 2003; Marrs et al., 2005). P fimbria, encoded by *pap*, could probably contribute to the adhesion and colonization of the urinary tract because this virulence gene is always associ-

ated with pyelonephritis (Usein et al., 2001; Chen et al., 2003; Jacobsen et al., 2008).

In the present study, pap was found in 56.0% *E. coli* strains isolated from canine urinary tract infections cases indicating that *E .coli* isolates from the urine of dogs and cats have greater ability to colonize kidney and generate pyelonephritis (Antao et al., 2009). Shetty et al. (2014) reported that 60.87% of the uropathogenic strains of *E. coli* obtained from humans showed pap genes. Siquieira et al. (2009) found 23.5% *pap*C positive strains among the *E. coli* strains isolated from dogs suffering from UTIs. Whereas Tramuta et al. (2014) identified pap in 13.4% *E. coli* isolates obtained from dog urine.

The S fimbriae adhesion (*sfa*) was found in 48.0% of the isolates obtained from UTIs infected dogs. This result is consistent with the occurrence of sfa (45.2%) in clinical isolates of human origin (Rahman and Deka, 2014). Similar findings were recorded in previous studies in dogs (Coogan et al., 2004; Siqueira et al., 2009). In recent studies involving dogs (Tramuta et al., 2014) and human (Adib et al., 2014), sfa genes were found in more than 20.0% of UTI isolates studied. The demonstration of *sfa* gene in dog's urine was observed in 52.0% of the isolates in another study (Drazenovich et al., 2004). Likewise, more than 12.0% of the *E. coli* strains from cats show *sfa* genes. Further, Shetty et al. (2014) also observed the presence of *sfa* gene in 39.1% isolates obtained from human UTIs. Therefore, these findings highlight that *sfa* gene plays a vital role in both adhesions to the urogenital epithelium and the development of urinary tract infection in companion animal, especially dogs.

Afimbrial adhesion (*afa*) gene was found to be implicated in developing nephritis in human (Mulvey, 2002). In the current study, *afa* gene was found in high frequency in the *E. coli* isolates (32.0%) as compared with strains from another study in dogs with UTIs (6.0%) (Drazenovich et al., 2004), but found lower in frequency in *E. coli* isolates reported from humans, 39.1% and 64.0% (Yamamoto et al., 1995; Shetty et al., 2014). On the other hand, Ebadi et al. (2017) reported that 9.3% of the E .coli strains identified from human clinical samples showed fimbrial genes (*afa* BC). However, Dr adhesion family recognize human receptors on erythrocytes and other tissues (Mulvey, 2002), and this may explain the low incidence of this 'A' fimbrial adhesion factor observed in canine isolates as previously reported (Siqueira et al., 2009).

Uropathogenic *E. coli* strains isolated from a dog with UTI express the presence of cytotoxic necrotizing factor 1 (*cnf1*) and Alpha-haemolysin gene (*hly*). The *hly* gene is a pore-forming toxin that lyses not only erythrocytes of all mammals and even that of fishes (Johnson, 1991) but also leukocytes, endothelial and renal epithelial cells (Usein et al., 2001; Emody et al., 2003). Alpha-haemolysin gene (*hly*) was found present in 20.0% of the UTI isolates of the current study, similarly, Tramuta et al. (2014), Rahman and Deka (2014), Liu et al. (2017) and Rashki et al. (2017) also recorded 18.0%, 36.8%, 39.0% and 10.0% of hly gene in UTIs isolates in dogs and humans respectively. In another study, Drazenovich et al. (2004) demonstrated that the hly gene was found in 50.0% of the *E. coli* isolates recovered from the urine of dogs. Siqueira et al. (2009) also found that 33.3% of the canine isolates contained hly genes.

Cytotoxic necrotizing factor 1 (*cnf1*) is a toxin that can cause reorganization of actin microfilament in eukaryotic cells. The cytotoxin may facilitate the bacterial invasion

of the bloodstream as a result of interference with polymorpho-nuclear phagocytosis and even evokes apoptotic death of epithelial cells in the bladder (Oelschlaeger et al., 2002; Emody et al., 2003). In the present study, *cnf1* was found in 20.0% of the UTIs strains. This was less than what was reported by Rahman and Deka (2014) who found 61.9% of the isolates from humans UTIs produce *cnf1* genes. Drazonavich et al. (2004) reported *cnf1* genes in 50. 0% of the *E. coli* isolates from dogs.

Furthermore, Johnson et al. (2003) reported that 41.0% of *E. coli* isolates obtained from canine UTIs produce *cnf1*. Liu et al. (2017) found 46.6% of the isolates contained *cnf1*. However, the results of the current study show less occurrence of *cnf1* in the *E. coli* isolates. This could probably be attributed to the mutation of this gene or could be due to variation in the geographical distribution of these genes.

In the present study, none of these *E. coli* isolates harbored the gene that encodes cytotoxic necrotizing factor 2 (*cnf2*), which is also related to UTIs. In contrast, Rahman and Deka (2014) found *cnf2* in *E. coli* isolates obtained from humans suffering from UTIs. These results indicate that *cnf2* may probably be of less significant in the uropathogenic *E. coli* pathogenesis in canine.

The phylogenetic analysis revealed that *afa, aer, sfa, cnf1, hly*, and *pap* genes exhibited 99% identity with the different variants in several geographical regions of the world. The *E. coli* falls into four leading phylogenetic groups: A, B1, B2, and D (Clermont et al., (2000). In the present study, the phylogenetic comparisons of virulence gene sequences show that *sfa, cnf1, hly* and *pap* genes matched group B2 and, these results are consistent with the findings noted by other investigators (Tramuta et al., 2011; Munkhdelger et al., 2017; Coura et al., 2018). However, aer virulence gene was found to match both B2 and D phylogenetic group. This is consistent with the previous study that demonstrated that aer sequence belongs to B2 and D phylogenetic group (ZhuGe et al., 2014; Liu et al., 2015). Moreover, only *afa* virulence gene sequence was found to fall within phylogenetic group A. This is also in accordance to recent studies, where it has been shown that *afa* gene sequence was observed to be associated to phylogenetic group A (Marti et al., 2017; Munkhdelger et al., 2017).

In the current study, majority of the virulence gene sequences were found to be associated with the phylogenetic group B2, suggesting that these strains were more virulent than others. Similar outcomes were observed in a recent study conducted by Piatti et al. (2008) who found an association between virulence factors and phylogenetic group in urinary *E. coli* strains isolated in humans. Furthermore, most of the extraintestinal pathogenic *E. coli* (ExPEC), including those with the most robust virulence factors repertories and those which are best able to infect non-compromised hosts, are derived from phylogenetic group B2 (Johnson, 2002). Group D contains the second-highest number of ExPEC, extraintestinal isolates. This group typically have somewhat fewer virulence factors and a different mix of virulence factors than group B2 isolates. *E. coli* strains belonging to group A and B1 do not frequently cause extraintestinal infection. These strains that are not highly virulent cause disease only in immunocompromised hosts, and could be pathogenic in healthy hosts only if they were to acquire sufficient extraintestinal virulence factors (Johnson, 2002).

In conclusion, the results of the present study demonstrated a high occurrence of virulence genes. The phylogenetic comparisons of these gene sequences detected in UPEC isolated from dogs showed high similarity to those present in the urine of humans with urinary tract infection. Therefore, the present study supports the assertion that dogs may serve as a reservoir for human infection with pathogenic *E. coli*. The phylogenetic comparison showed that *hly, sfa, cnf1*, and *pap* virulence genes matched to group B2, *afa* to A and *aer* to group B2 and D, indicating B2 as the dominant phylogenetic group among UPEC strains in Hisar, Haryana and virulence-associated genes were mostly distributed in this group. Further studies are required on virulence genes and their specific phylogenetic background with a large number of samples to advance our understanding of uropathogenic *E. coli* in companion animals especially dogs, and this may help in controlling urinary tract infection in future.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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