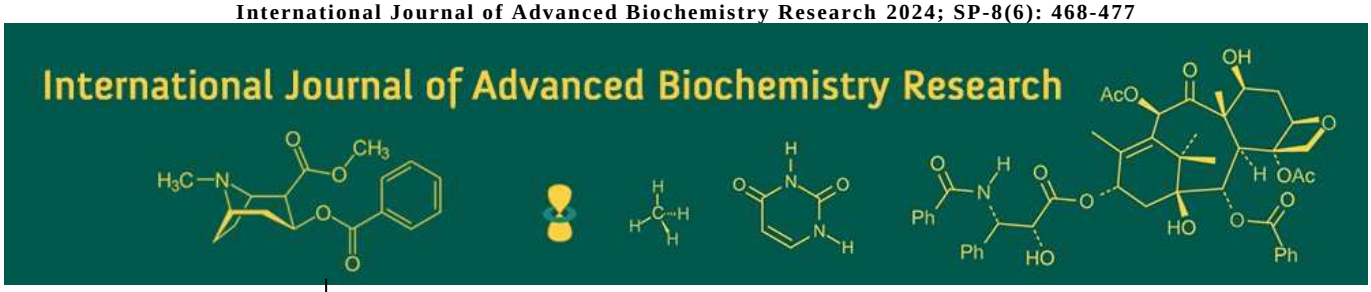

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Characterization of biofilm-forming *Escherichia coli* in canine urinary tract infections: Phenotypic and genotypic insights

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Abstract
Uro-pathogenic *Escherichia coli* (UPEC) is responsible for causing urinary tract infections in canines. The capacity to form biofilms and employ diverse virulence factors enables UPEC to endure within the uroepithelium, causing chronic and persistent infections. Unintended antimicrobial usage, whether for therapeutic purposes or otherwise, exposes bacterial pathogens to varying concentrations of antimicrobial drugs, promoting the emergence and proliferation of resistant bacteria. To characterize UPEC from urinary tract infections, samples (n=51) were collected and subjected to microbial isolation using MacConkey Lactose agar (MLA) and Eosin Methylene Blue agar (EMB), which led to the isolation of 19 *E. coli* isolates, confirmed using *usp*A gene amplification. These isolates were characterized using a set of three virulence genes namely *aer*, *pap* and *cnf1*. Furthermore, all the 19 isolates were subjected to microtiter plate assay for biofilm detection and the result showed that biofilm formation was observed in both LB and TSB media and at 72h incubation time indicated that in LB media, 2 (10.5%) isolates were strong biofilm producers, 3 (15.7%) isolates were moderate biofilm producers, 7 (36.8%) isolates were weak biofilm producers and 7 (36.8%) isolates had not produced any biofilm while in TSB media (72h), 4 (21%) isolates were strong biofilm producers, 9 (47.3%) isolates were moderate biofilm producers, 3 (15.7%) isolates were weak biofilm producers and 3 (15.7%) isolates had not produced any biofilm. Antimicrobial sensitivity test using Bauer-Kirby revealed that isolates were found maximum resistant to Cephalexin, Ciprofloxacin, Metronidazole, Ampicillin, Penicillin G, Tetracycline while susceptible to Chloramphenicol, Co-trimoxazole, Imipenem, Meropenem, Gentamicin and Amikacin. This study demonstrates the presence of UPEC capable of forming biofilms in canines suffering from urinary tract infections. Further research is essential to elucidate the role of biofilms in the pathogenesis of UPEC and their implications for antimicrobial resistance.

Keywords: Dogs, *Escherichia coli*, urinary tract infections, virulence genes, biofilm

Introduction
The bond between dogs and human's dates back to ancient times, with dogs continuing to be the most valued companion animals for people today. Among all infectious diseases that affect dogs, urinary tract infections (UTIs), which include conditions such as cystitis and pyelonephritis, are the most prevalent (Farshad *et al*., 2012; Xia *et al*., 2011) [11, 56]. On average, 14% of dogs will experience a urinary tract infection (UTI) at some point in their lives (Thompson *et al*., 2011) [52]. *Escherichia coli* (*E. coli*) is the leading cause of urinary tract infections (UTIs) in dogs, being isolated in over 80% of cases (Hilbert *et al*., 2008; Thompson *et al*., 2011) [18, 52]. *E. coli* strains that cause urinary tract infections (UTIs) are commonly referred to as uropathogenic *E. coli* (UPEC). These strains contain additional genetic material encoding various virulence genes, which are linked to severe or recurrent UTIs (Agarwal *et al*., 2013) [1]. Adhesins, which are surface virulence factors, are among the most significant virulence elements of uropathogenic *E. coli* (UPEC) (Nicole, 2008) [32]
*Aer*obactin, a low molecular weight siderophore encoded by the *aer* gene, exhibits strong Fe3+ binding capability in acidic conditions. Moreover, *aer*obactin production is enhanced in low pH environments (Tóth *et al*., 2003; Hagberg *et al*., 1981) [53, 16]. This siderophore scavenges Fe3+ from host iron-binding proteins and is internalized via an outer membrane TonB receptor protein (Tóth *et al*., 2003) [53].

The *pap* operon encodes P fimbriae, which allow uropathogenic *E. coli* (UPEC) to penetrate the epithelial barrier and enter the bloodstream, leading to the hemagglutination of erythrocytes (Riegman *et al*., 1988) [40]. These fimbriae play a crucial role in the pathophysiology of pyelonephritis, as they enhance the early colonization of the tubular epithelium. Cytotoxic necrotizing factor 1 (*CNF1*), encoded by the *cnf1* gene, stimulates the formation of actin stress fibers and membrane ruffles in a Rho GTPase-dependent manner. This mechanism aids uropathogenic *E. coli* (UPEC) in entering the host's urinary tract cells (Connell *et al*., 1996) [3]. UPEC strains associated with pyelonephritis and kidney invasion typically carry the *cnf1* virulence factor. This factor can lead to the exfoliation of bladder cells, facilitating the deeper invasion of UPEC into the underlying tissues of the urinary tract (Hultgren *et al*., 1985; Hagberg *et al*., 1983) [19, 15].

The capacity to form biofilms and employ various virulence factors enables Uropathogenic *Escherichia coli* (UPEC) to persist within the uroepithelium, leading to chronic and persistent infections (Terlizzi *et al*., 2017) [51]. Biofilms consist of a sessile community of microbial cells that are firmly attached to surfaces or to each other, embedded in an extracellular matrix primarily composed of polysaccharides secreted by the bacteria (Donlan *et al*., 2002) [7]. The collective behavior of bacteria within biofilms optimizes nutrient utilization and enhances resistance to host defenses and antimicrobial agents compared to planktonic cells (Kostakioti *et al*., 2013) [23]. Additionally, bacteria within biofilms secrete a variety of antibiotic-inactivating enzymes, such as beta-lactamases, which further contribute to their resilience against antibiotic treatments.

The molecular characterization of *E. coli* virulence genes associated with UTIs in dogs has been extensively studied worldwide. However, there is relatively limited documented information on *E. coli* virulence genes and biofilm formation associated with UTIs in dogs specifically in India. Therefore, the present study aims to investigate the correlation between virulence genes, biofilm formation, and antibiotic resistance.

Materials and Methods

Sample and data collection

The present study was carried out in canines suspected to be suffering from urinary tract infections, admitted to Veterinary Clinical Complex (VCC), Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar. Dogs of varying ages, sexes, and breeds exhibiting clinical signs such as anorexia, urine dribbling, urinary retention, straining during urination, scant urine output, cloudy urine, increased water intake, frequent urination, excessive licking of the urinary opening, hematuria, lethargy, fever, proteinuria, painful urination, foul-smelling urine, and back arching (Indicative of cystitis) were diagnosed with urinary tract infections (UTIs). A total of 51 urine samples were aseptically collected in sterile containers, and information regarding the dogs' UTI symptoms was obtained through a self-designed questionnaire. Samples were promptly chilled and processed in the laboratory on the same day of collection.

Isolation and identification of *E. coli*

Aseptically collected fresh urine samples were inoculated and streaked onto 5% sheep blood agar (BA) and MacConkey lactose agar (MLA) plates using a 4 mm diameter platinum loop. The plates were then *aer*obically incubated at 37 °C for 24-48 hours.

Colonies displaying typical pink coloration indicative of fermentation on MLA plates were selected and subjected to Gram staining to identify other bacterial isolates. Subsequently, these colonies were purified by re-streaking on MLA and then plated on Eosin Methylene Blue (EMB) agar. Following another 24-hour incubation at 37 °C, bacterial colonies exhibiting pink to red fermentation on MLA and purple-bluish precipitation on EMB, with or without a metallic sheen, were tentatively identified as *E. coli* and assigned isolate numbers accordingly

The identification of these *E. coli* isolates was confirmed using the Vitek 2 Compact system. Gram-negative (GN) reagent cards were employed for the identification of *E. coli*, following the manufacturer's recommendations and the method outlined in a previous study (Mittal *et al*., 2014) [28].

PCR amplification for *E. coli:* The genomic DNA extraction from *E. coli* isolates confirmed by Vitek 2 was performed using a heat lysis/snap-chill method (Englen and Kelley, 2000) [9]. Briefly, 2-3 pure bacterial colonies were suspended in 150 µl of nuclease-free water, heated in a dry bath for 10 minutes, and immediately snap-chilled on ice for 5 minutes. After centrifugation at 8, 000×g, the supernatant containing DNA was collected and stored at -20 °C for further use. Genomic DNA from ATCC 25922 served as a positive control for *E. coli*. PCR amplification targeted the universal stress protein (uspA gene) using specific primer pairs. PCR reactions were conducted in 25 µl volumes in an Applied Biosystems thermocycler under the following conditions: initial denaturation at 95 °C for 2 minutes; followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 1 minute, extension at 72 °C for 1 minute; final extension at 72 °C for 5 minutes, followed by a hold at 4 °C. The amplified PCR products were analyzed by agarose gel electrophoresis at a constant voltage of 90V using 2% agarose in TRIS acetate EDTA buffer (TAE) stained with 0.5 µl/ml ethidium bromide. Subsequently, the amplified products were visualized under UV transillumination using a gel documentation system.

UPEC characterization

PCR primers targeting virulence genes specific to Uropathogenic *Escherichia coli* (UPEC) were employed to detect three virulence genes (Table 1). Each PCR assay was conducted separately for the *aer*, *pap*, and *cnf1* genes in a total volume of 25 µl, consisting of 12.5 µl Master Mix (2x), 3 µl DNA template, 1 µl forward primer, 1 µl reverse primer, and 7.5 µl nuclease-free water. The amplification conditions are detailed in Table 2.

The amplified PCR products underwent analysis by agarose gel electrophoresis using 2% agarose in Tris-acetate EDTA buffer (TAE), stained with 0.5 µl/ml ethidium bromide, at a constant 80V. Subsequently, the products were visualized using UV transillumination in a gel documentation system.

Table 1: Primer pairs used for characterization of *E. coli* virulence genes

Target Genes	Primer sequence (5'-3')	Amplified Product (bp)	Reference
aer	F: TACCGGATTGTCATATGCAGACCGT R: AATATCTTCCTCCAGTCCGGAGAAG	602	Herrero *et al.*, 1988 [17]
pap	F: GCAACAGCAACGCTGGTTGCATCAT R: GAGAGAGCCACTCTTATACGGACA	336	Yamamoto *et al.*, 1995 [57]
cnf1	F: AAGATGGAGTTTCCTATGCAGGAG R: CATTCAGAGTCCTGCCCTCATTATT	498	Falbo *et al.*, 1992 [10]

Table 2: PCR timetable program for *aer*, *pap* and *cnf1* genes in UPEC strain

Genes	Initial denaturation	Denaturation	Annealing	Extension	Final cycle of extension
aer					
Time	3m	1m	1m	1m	7m
Temperature	94 °C	94 °C	59 °C	72 °C	72 °C
No. of cycle	1	35	35	35	1
pap					
Time	5m	1m	1m	1m	7m
Temperature	94 °C	94 °C	60 °C	72 °C	72 °C
No. of cycle	1	35	35	35	1
Cnf1					
Time	5m	1m	1m	1m	7m
Temperature	94 °C	94 °C	56 °C	72 °C	72 °C
No. of cycle	1	35	35	35	1

Biofilm formation and quantification

To assess the biofilm-forming ability of *E. coli* isolates, biofilm formation and quantification were performed in 96-well polystyrene microtiter plates according to methods described by Skyberg *et al.* (2006) [47] and Grakh *et al.* (2019) [14]. Initially, *E. coli* colonies were inoculated into 5 ml of Luria-Bertani (LB) broth and incubated overnight at 37 °C. The overnight culture turbidity was adjusted to OD 600 = 0.05 in both Tryptic Soy Broth (TSB) and LB broth separately. Next, 200 µl aliquots of these dilutions were dispensed into triplicate wells of a microtiter plate and incubated at 37 °C for 72 hours. After incubation, the contents of the plates were discarded, and the plates were washed with sterile double distilled water. The washed plates were then stained with 200 µl of 0.1% Crystal Violet solution for 30 minutes. Subsequently, the microtiter plates were washed three times with sterile double distilled water to remove excess stain, followed by air-drying for 30 minutes. To resolubilize adherent cells, 250 µl of a solution containing ethanol and acetone (80:20) was added to each well. A volume of 150 µl of the resolubilized solution was transferred to a new microtiter plate, and the optical density (OD) of each well was measured at 570 nm using an automated microplate reader. Uninoculated TSB and LB broths served as negative controls, while ATCC 25922 was used as a positive control for biofilm production. All tests were performed in triplicate, and the average value of the results was recorded. The cutoff OD (ODc) was calculated as: ODc= average OD of negative control + 3 (SD of negative control) where, SD= Standard deviation.

Based on the OD produced by bacterial biofilms, bacterial isolates were classified into the following categories as described by Stepanovic *et al.*, 2004 [49].

OD ≤ ODc = Isolates with no biofilm forming ability

ODc< OD < (2 x ODc) = Isolates with weak biofilm forming ability

(2 x ODc) < OD ≤ (4 x ODc) = Isolates with moderate biofilm forming ability

(4 x ODc) < OD = Isolates with strong biofilm forming ability

Antimicrobial susceptibility testing

The *in vitro* drug sensitivity testing of *E. coli* isolates was conducted following the Clinical Laboratory Standards Institute guidelines (CLSI) and Bauer-Kirby method (Bauer *et al.*, 1966) [60], using commercially prepared antibiotic discs (Hi-media, India) with predetermined concentrations. The diameter of the inhibition zones was measured in millimeters using a ruler, In this study, the following 24 antibiotics utilized against *E. coli* pathogens included Amikacin (AK 30), Amoxycillin (AMX 30), Amoxyclav (AMC 50/10), Ampicillin (AMP 10), Cephalothin(CF 22), Ceftizoxime (CZX 30), Ceftriaxone/sulbactum (CIS 30/15), Cephalexin (CN 30), Chloramphenicol (C30), Ciprofloxacin (CIP 10), Co-trimoxazole (COT 25), Doxycycline (DO 30), Enrofloxacin (EX 10), Gentamicin (GEN 10), Imipenem (IPM 10), Meropenem (MRP 10), Moxifloxacin (MO 5), Metronidazole (MT 5), Norfloxacin (NX10), Ofloxacin (OF 5), Oxytetracycline (O 30), Penicillin G (P10), Streptomycin (S 10), Tetracycline (TE 30).

Statistical Analysis

Statistical analysis was conducted using SPSS software for Windows. Spearman's rank correlation was employed to assess the association between biofilm production in different media and the antimicrobial resistance of *E. coli* isolates. Additionally, to determine the relationship between the presence or absence of specific virulence genes and biofilm production, biofilm formation was categorized into two groups: moderate-strong biofilm and weak or no biofilm. Fischer's Exact test was applied using a 2x2 contingency table. A significance level of $p<0.05$ was considered statistically significant

Results

Out of 51 urine samples analyzed, bacterial growth was detected in 46 samples, while 5 samples showed no growth on blood agar, brain heart infusion (BHI) agar, and MacConkey lactose agar (MLA). Among the 46 isolates, 19 (37.25%) exhibited lactose-fermenting pink to red colonies on MLA and displayed a blue-green metallic sheen on Eosin-methylene blue (EMB) agar. Gram staining

confirmed these 19 isolates as gram-negative rods (Microscopic Examination). Biochemical tests indicated positive results for Indole and Methyl Red, and negative results for Voges-Proskauer and citrate utilization tests, confirming their identification as *E. coli*. These biochemically identified *E. coli* isolates were further confirmed using PCR targeting the *usp*A gene specific to *E. coli*. (Fig.1).

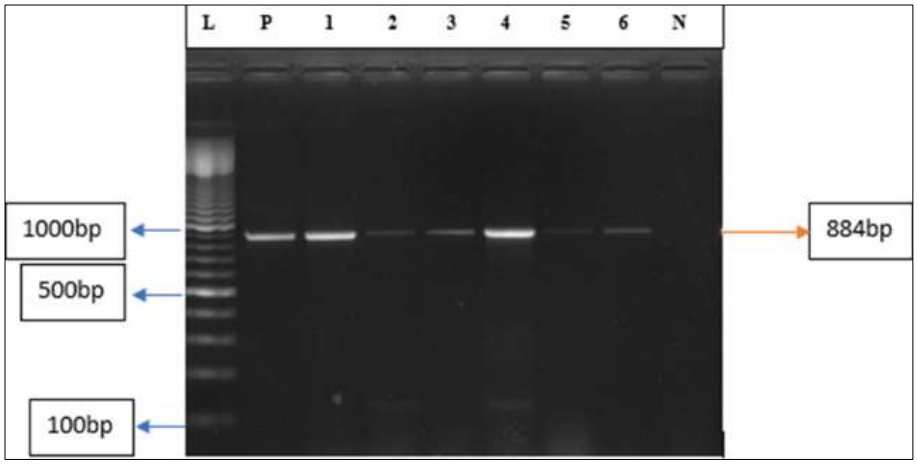

Fig 1: Agarose gel electrophoresis pattern of uspA specific PCR products of *E. coli* isolates. Lane L: 100 bp DNA ladder; Lane P: positive control (ATCC 25922), Lane 1-6: positive isolates (884 bp); Lane N: negative control

Characterization of UPEC using PCR

DNA extracted from colonies of confirmed *E. coli* isolates were subjected to PCR for each individual gene (*aer, pap* and *cnf1)* separately and distribution of these three virulence genes is shown in Fig. 2, Fig. 3 and Fig. 4. Out of 19 *E. coli* isolates, *aer*, *pap* and *cnf1* virulent genes were isolated from 12 (63.2%), 12 (63.2%) and 10 (52.6%) samples respectively. Distribution of these virulence gene is shown using heat map in Fig.5.

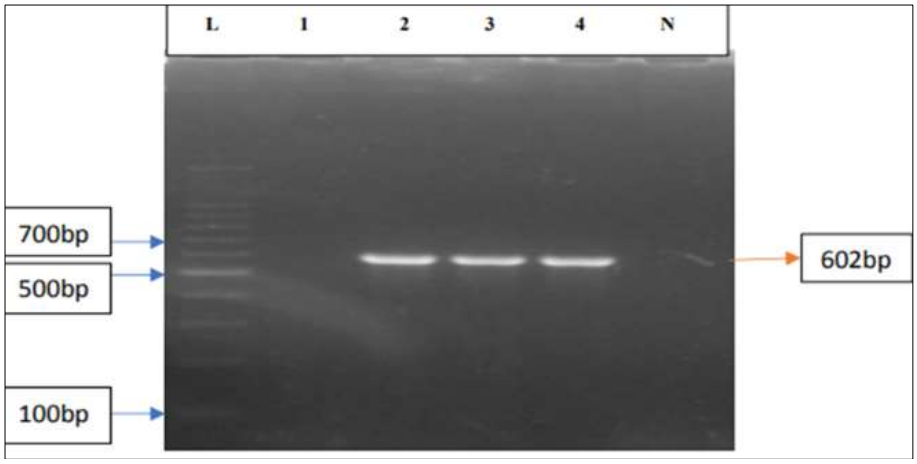

Fig 2: Agarose gel electrophoresis pattern of *aer* specific PCR products of *E. coli* isolates, Lane L: 100 bp DNA ladder, Lane 1: negative isolates, Lane 2, 3, 4: positive isolates (602 bp), Lane N: negative control

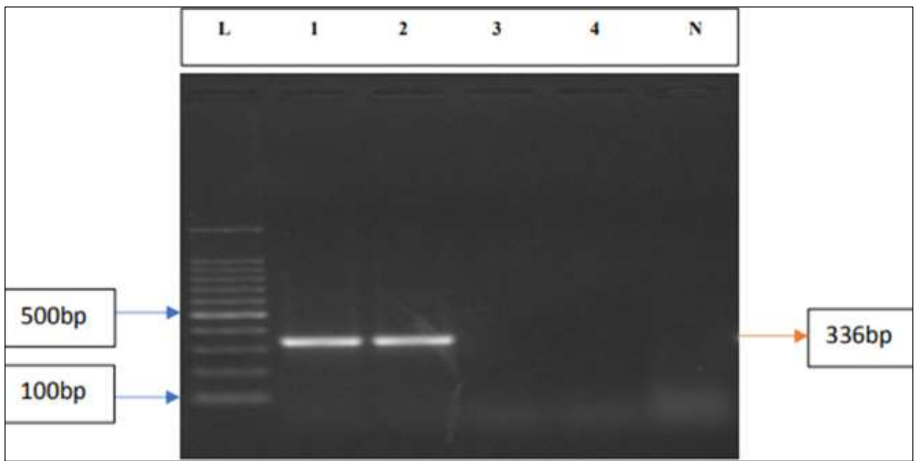

Fig 3: Agarose gel electrophoresis pattern of *pap* specific PCR products of *E. coli* isolates, Lane L: 100 bp DNA ladder, Lane 1, 2: positive isolates (336 bp), Lane 3, 4: negative isolates, Lane N: negative control

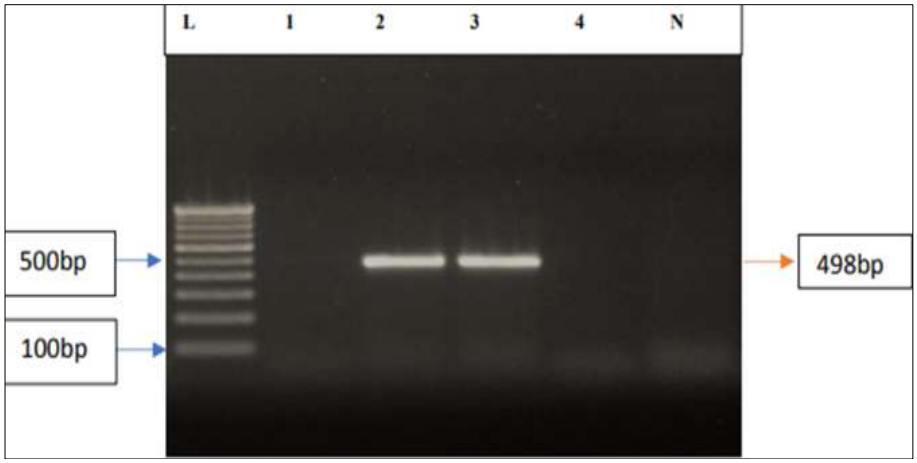

Fig 4: Agarose gel electrophoresis pattern of *cnf1* specific PCR products of *E. coli* isolates, Lane L: 100 bp DNA ladder, Lane 1, 4: negative isolates, Lane 2, 3: positive isolates (498 bp), Lane N: negative control

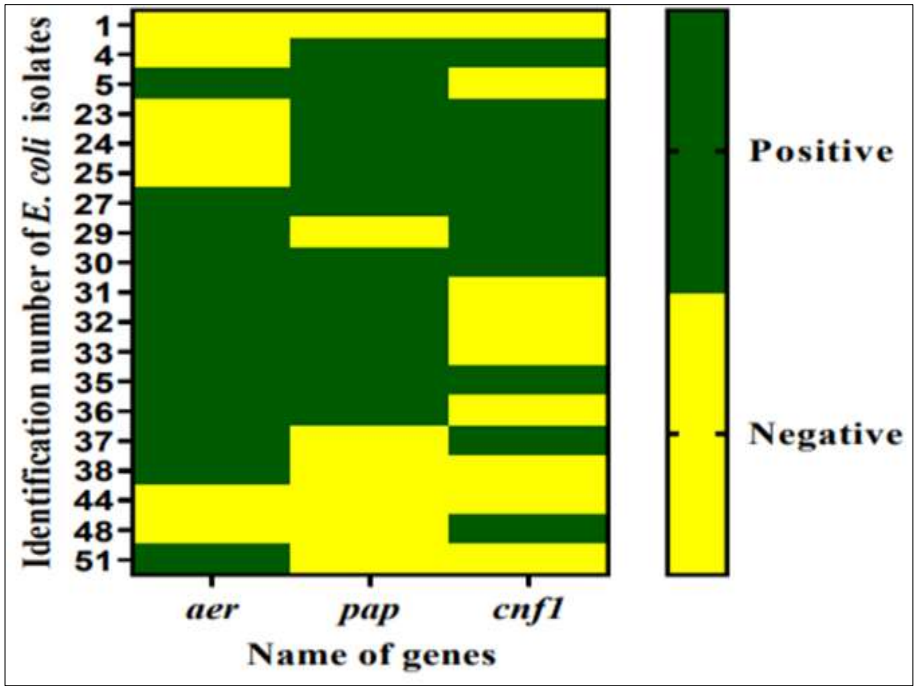

Fig 5: Distribution of virulence genes of *E. coli* isolates (n=19) (heat map)

Biofilm detection and quantification:
All 19 isolates were cultured in two different media, LB and TSB, to evaluate their ability to form biofilms after 72 hours of incubation. In LB medium, 2 isolates (10.5%) were classified as strong biofilm producers, 3 isolates (15.7%) as moderate producers, 7 isolates (36.8%) as weak producers, and 7 isolates (36.8%) did not produce any biofilm. In TSB medium, after 72 hours of incubation, 4 isolates (21%) were identified as strong biofilm producers, 9 isolates (47.3%) as moderate producers, 3 isolates (15.7%) as weak producers, and 3 isolates (15.7%) did not produce any biofilm (Table 3).

Table 3: Biofilm produced by UPEC isolates in LB and TSB media (72h)

Group	Strong (%)	Moderate (%)	Weak (%)	None (%)	Total
LB 72h	2 (10.5%)	3 (15.7%)	7 (36.8%)	7 (36.8%)	19
TSB 72h	4 (21.0%)	9 (47.4%)	3 (15.7%)	3 (15.7%)	19

Antimicrobial susceptibility testing: The result of antimicrobial susceptibility test for 19 *E. coli* isolates indicated the maximum sensitivity towards Chloramphenicol (78.9%), Co-trimoxazole (78.9%), Imipenem (68.4%), Meropenem (63.1%), Gentamicin (57.8%) and Amikacin (52.6%) whereas maximum resistance towards Cephalexin (100%), Ciprofloxacin (100%), Metronidazole (100%), Ampicillin (100%), Penicillin G (100%), Tetracycline (100%), Cephalothin (94.7%), Enrofloxacin (94.7%), Norfloxacin (94.7%), Ofloxacin (94.7%), Doxycycline (94.7%), Oxytetracycline (89.4%), Amoxycillin (89.4%), Moxifloxacin (89.4%), Moxifloxacin (89.4%), Ceftizoxime (73.6%) and Ceftriaxone/sulbactum (57.8%) as shown in Fig 6.
Using ranked correlation, Biofilm formation is significantly associated ($p<0.05$) with antimicrobial resistance. Of the two media, more significantly associated with TSB media than LB media.
Using Fisher's exact test, non-significant relationship of *aer* and *cnf1* virulence genes with biofilm formation was found. The absence of *pap* gene found significant ($p<0.05$) with Moderate-Strong biofilm in LB media not in TSB media.

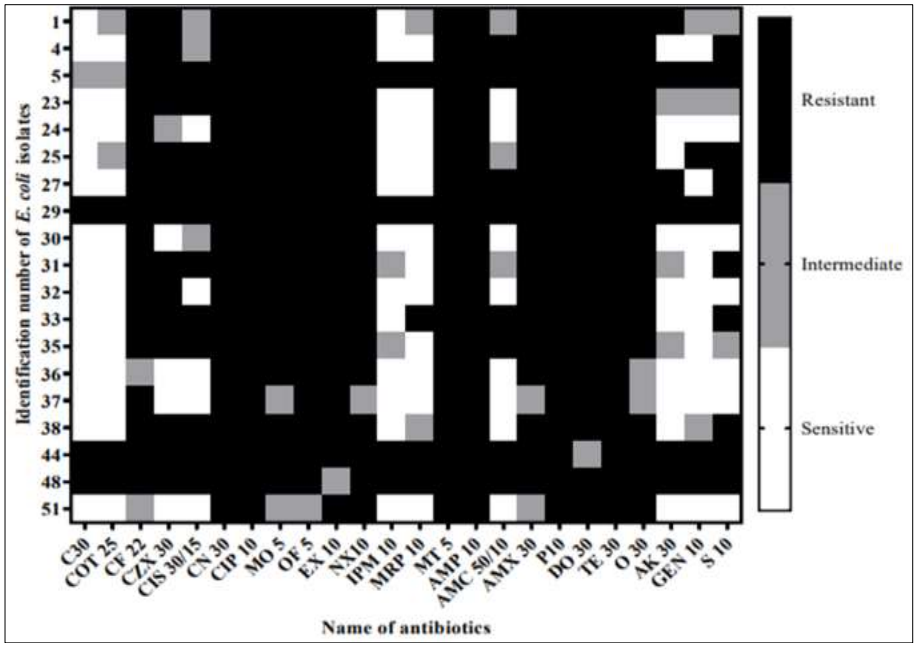

Fig 6: Antibiotic Susceptibility Pattern of *E. coli* isolated from dogs (n=19)

Discussion

Dogs and humans have shared an unparalleled bond for ages, with dogs often considered man's best companion. In dogs, urinary tract infections (UTIs) are very common and can be life-threatening if not treated promptly. *Escherichia coli* remains the most frequent pathogen causing UTIs in dogs (Johnson, 2003; Brolund, 2014) [21, 2]. Consequently, there is a pressing need for thorough investigations into Uropathogenic *Escherichia coli* (UPEC). While numerous studies on UPEC in dogs have been conducted globally and in India, significant gaps remain in our understanding of the emergence of UPEC in dogs.

In the present study, out of 51 collected samples, 19 isolates (37.2%) were identified as *E. coli* based on conventional methods such as colony morphology on MLA and EMB, and Gram staining. All 19 isolates were confirmed as *E. coli* using PCR. These findings are consistent with a study by Kuan *et al*. (2016) [24], who reported that 35.6% (146/289) of canine urine samples were positive for *E. coli*. Other studies by Qekwana *et al*. (2018) [37], Punia *et al*. (2018) [36], and Mustapha *et al*. (2020) [30] reported lower proportions of *E. coli*-positive canine urine samples, with rates of 22.3% (168/755), 22.7% (5/22), and 24.3% (25/103), respectively, in urine samples from dogs with UTIs. Conversely, higher proportions of *E. coli* in canine urine samples affected by UTIs were reported by Liu *et al*. (2017) [26] and Roopali *et al*. (2018) [12], documenting positivity rates of 60.9% (106/174) and 80.0% (8/10), respectively.

Several studies (Munkhdelger *et al*., 2017; Tramuta *et al*., 2014; Liu *et al*., 2017; Siqueira *et al*., 2009) [29, 54, 26, 46] have investigated and characterized the distribution of virulence genes associated with UTIs caused by UPEC in canines worldwide. Specifically, Mustapha *et al*. (2020) documented the distribution and characterization of seven UPEC virulence genes, namely *aer*, *pap*, *cnf1*, cnf2, afa, sfa, and hly, in the study area. In the present study, the distribution of three common UPEC genes-*aer*, *pap*, and *cnf1*-was examined. The *aer*obactin receptor gene, encoded by *aer*, facilitates bacterial growth in iron-limited conditions and contributes significantly to the pathogenesis of urinary tract infections, making it a crucial virulence factor for UPEC (Mittal *et al*., 2014) [28]. In this study, the *aer* virulence gene was detected in 63.20% of *E. coli* strains isolated from canine urinary tract infection cases. Similarly, Munkhdelger *et al*. (2017) [29] reported the presence of the *aer* gene in over 56% of *E. coli* strains from urine samples of dogs with persistent UTIs. Other studies by Drazenovich *et al*. (2004) [8], Firoozeh *et al*. (2014) [12], and Oliveira *et al*. (2014) [34] found the *aer* gene in about 30% of *E. coli* strains from dogs with UTIs. In contrast, a study by Mustapha *et al*. (2020) [30] in the same region as the present study reported an even higher prevalence (80%) of the *aer* gene in *E. coli* isolates from dogs suspected of having UTIs. These findings suggest that the prevalence of iron acquisition system genes may vary geographically, warranting further research to confirm this observation

In uropathogenic *E. coli*, adhesion and colonization of the uroepithelium are critical processes (Siqueira *et al*., 2009) [45]. The *pap* virulence gene is consistently associated with pyelonephritis and is essential for the pathogenesis of UPEC (Johnson, 1991; Marrs *et al*., 2005) [20, 27]. In the current study, the *pap* gene was found in 63.20% of *E. coli* strains isolated from dogs with urinary tract infections. This finding is in line with studies by Mustapha *et al*. (2020) [30] and Oliveira *et al*. (2014) [34], which observed the *pap* gene in 56.0% and 42.4% of *E. coli* strains, respectively. Conversely, studies by Siqueira *et al*. (2009) [45] and Tramuta *et al*. (2014) [54] reported a lower prevalence of the *pap* gene, with only 23.5% and 13.4% of *E. coli* strains from dogs with UTIs testing positive for this gene.

Several studies have reported that cytotoxic necrotizing factor 1 (*CNF1*), encoded by the *cnf1* gene, is expressed by UPEC strains isolated from dogs. This virulence factor enables the bacteria to evade host defense mechanisms such as phagocytosis by the polymorphonuclear system, thus facilitating bacterial invasion into the bloodstream (Oelschlaeger *et al*., 2002) [33]. In the present study, the *cnf1* gene was found in 52.60% of *E. coli* strains isolated from dogs with UTI symptoms. Similar findings have been reported by Johnson *et al*. (2003) [21], Drazonavich *et al*. (2004) [8], Rahman and Deka (2014) [38], and Liu *et al*. (2017) [26], who documented *cnf1* gene presence in 41%,

50%, 61.90%, and 46.90% of *E. coli* isolates from dogs with UTIs, respectively. In contrast, Mustapha *et al*. (2020) [30] found the *cnf1* gene in only 20.0% of UTI-affected *E. coli* strains. These variations suggest that the distribution of virulence genes among UPEC can vary even within the same geographical regions, underscoring the need for larger-scale studies to better define the distribution of these genes among UPEC.

Antimicrobial susceptibility pattern of the *Escherichia coli* isolates recorded in the current study demonstrated a very high level of resistance to antibiotics of class Cephalosporins (Cephalexin [100%] and Cephalothin [94.73%]); Fluoroquinolones (Ciprofloxacin [100%], Norfloxacin [94.73%] and Enrofloxacin [94.73%]); Penicillins (Ampicillin [100%] and Penicillin G [100%]) and Tetracyclines (Tetracycline [100%] and Doxycycline [94.73%]). Such high level of resistance for antibiotics commonly used for treating canine UTI can be attributed to the natural selection of *E. coli* isolates competent of evading these antibiotics through different mechanisms (Sunde and Sorum, 1999; Weese *et al*., 2011) [50, 55]. The high levels of resistance to both newer and commonly used antibiotics observed in this study may be attributed to the fact that the samples were collected from the Veterinary Clinical Complex (VCC) hospital. As a referral hospital, VCC receives cases from numerous small animal clinics and veterinary practitioners, where dogs are often treated before being referred to VCC. Due to the lack of infrastructure at these smaller clinics, different classes of antibiotics are administered to dogs without prior laboratory assessment. Furthermore, the presence of multi-drug resistant *E. coli* in dogs poses a significant public health risk, given the zoonotic potential of *E. coli* as established by various studies. The role of livestock as a source of pathogen transmission to humans is well recognized, with food-borne exposure and direct contact being the two major means of transmission (Dechet *et al*., 2006; Cummings *et al*., 2012) [6, 4].

Also, studies by Sidjabat *et al*., (2006) [44] and Platell *et al*., (2010) [35] had established similarities between MDR strains of *E. coli* of human and animal origin and proposed that transfer of MDR *E. coli* between dogs and humans via the faecal-oral route likely had occurred.

Biofilm formation and quantification associated with canine urinary tract infections had not been previously investigated in the study area. Biofilm-forming organisms are a common cause of recurrent and complicated UTIs, and this ability is often associated with multi-drug resistant (MDR) bacteria (Flores-Mireles *et al*., 2015) [13]. Understanding the pathogenesis of biofilm formation and the factors contributing to it is essential for developing new therapies (Romling *et al*., 2012) [41]. This study aimed to elucidate biofilm formation among UPEC strains isolated from UTIs. In this study, biofilm formation in LB media (72h) showed that 2 (10.5%) isolates were strong biofilm producers, 3 (15.7%) were moderate biofilm producers, 7 (36.8%) were weak biofilm producers, and 7 (36.8%) did not produce any biofilm. In TSB media (72h), 4 (21%) isolates were strong biofilm producers, 9 (47.3%) were moderate biofilm producers, 3 (15.7%) were weak biofilm producers, and 3 (15.7%) did not produce any biofilm.

Zamani *et al*. (2018) [58] indicated that the majority of UPEC isolates (84%) were moderate to strong biofilm producers, while the remaining isolates either did not produce biofilm or exhibited weak biofilm formation potential. Similarly, Katongole *et al*. (2020) [22] demonstrated that among 200 *E. coli* isolates, 125 (62.5%) were biofilm formers. These studies suggest that a significant number of UPEC isolates are capable of forming biofilms, highlighting biofilm formation as an important virulence determinant. The association between UTIs and biofilm formation ability has been previously reported, indicating its critical role (Soto *et al*., 2007; Naves *et al*., 2008) [48, 31]. Additionally, Grakh *et al*. (2019) [14] observed that 30 (63.8%) APEC isolates formed moderate to strong biofilms in TSB media, 25 (53.2%) in 1/20 diluted TSB media, and 9 (19.1%) in LB media. In the present study, a significant positive correlation ($p<0.05$) was found between biofilm presence in either media and antimicrobial resistance. Similar findings have been reported by Oliveira *et al*. (2014) [34] and Shimizu and Harada (2017). Both studies reported a significant positive relationship ($p<0.05$) between biofilm production by UPEC and antimicrobial resistance in dogs. In our study, a significant association ($p<0.05$) was found between the absence of the *pap* gene and the presence of strong to moderate biofilm formation. This finding suggests that biofilm production is not associated with fimbrial adhesin. Similarly, Oliveira *et al*. (2014) [34] reported that fimbrial adhesins were not related to biofilm production by canine UPEC isolates. Furthermore, no significant association ($p<0.05$) was observed between the *aer* and *cnf1* genes and moderate to strong biofilm production by UPEC isolates. In contrast, Oliveira *et al*. (2014) [34] found a significant association between the *aer* gene and biofilm production.

In this study, once *E. coli* isolates were dispensed into the microtiter plates with media, no additional fresh media was added during the incubation period. As incubation time increased, the nutrient levels surrounding the bacteria decreased, creating a nutrient-deficient environment in both media types, which favored biofilm formation. During *in vivo* biofilm production, environmental changes trigger the transition from planktonic growth to the biofilm stage, allowing bacteria to develop properties that promote survival under adverse conditions. The steps involved in *in vivo* biofilm production include reversible attachment to a surface followed by irreversible attachment. Once irreversibly attached, external matrix secretion occurs, leading to the maturation of biofilms and the development of a three-dimensional structure. Similarly, during *in vitro* biofilm production, dilution within the media or nutrient depletion triggers the activation of extracellular polymeric substance (EPS) production, converting planktonic bacterial cells into the biofilm phenotype (Reisner *et al*., 2006; Zhang *et al*., 2014) [39, 59]. The growth of biofilms *in vitro* depends on the composition of the media and the duration of incubation. In the present study, a higher number of isolates produced moderate to strong biofilms in TSB media compared to LB media. This could be attributed to the fact that TSB media contains sugars, whereas LB media contains salts. It has been previously reported that sugars enhance *in vitro* biofilm production by *E. coli* isolates (Leme *et al*., 2006) [25].

Our study underscores the prevalence of biofilm-producing and multidrug-resistant UPEC infections in canines. Further exploration and comparison of UPEC with ExPEC strains of human origin could unveil their zoonotic potential, thereby aiding in the development of preventive and control strategies.

Conclusion

In conclusion, our study provides comprehensive insights into uropathogenic *Escherichia coli* (UPEC) isolated from canine urinary tract infections (UTIs). We identified E. coli as the predominant pathogen in our sample set, confirming their presence using conventional methods and PCR. Analysis of virulence genes revealed significant prevalence of aer, pap, and cnf1, crucial in UPEC pathogenesis. Biofilm formation assays highlighted varying capabilities among isolates, with notable associations between biofilm production and antimicrobial resistance. High resistance to several antibiotics underscores the urgency for judicious use in veterinary practices. These findings contribute to understanding UPEC epidemiology in dogs, emphasizing implications for public health and necessitating further research into zoonotic risks and control strategies.

Reverences

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