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RESEARCH ARTICLE

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Detection of Ciprofloxacin resistance genes in *Escherichia coli* isolated from dogs with urinary tract infections

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ABSTRACT

This research was performed on uropathogenic *Escherichia coli (E. coli)* isolates and established the genes of resistance to ciprofloxacin between the isolates. A total of one hundred and three urine samples were tested for uropathogenic *E. coli* which were obtained from dogs with urinary tract infections (UTIs) using cultural isolation, antimicrobial susceptibility test, and polymerase chain reaction (PCR). The results revealed that genes associated with ciprofloxacin resistance are 24.3% positive for *E. coli.* The *E. coli* isolates were resistant to both ciprofloxacin and ampicillin (100%), highly susceptible to chloramphenicol (84.0%), and less susceptible to gentamycin (44.0%) and amikacin (40.0%). The PCR tests showed the presence of the ParC (in 25 samples; 100%), GyrA (in 25 samples; 100%), and GyrB (in 4 samples; 16.0%) genes. The findings of the present study showed an upsetting rate of ciprofloxacin and ampicillin resistance among the *E. coli* isolates from dogs with UTIs.

Keywords

Dog, Uropathogenic E. coli, Antibiotic-resistant genes, Ciprofloxacin, PCR

Abbreviations

PCR: polymerase chain reaction

UTI: urinary tract infection

QRDR: quinolone-resistance determining region

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Introduction

Escherichia coli is the most common pathogen associated with urinary tract infections (UTIs) in dogs and cats, and many fluoroquinolones have been accepted for UTIs treatment in dogs and cats [1,2,3]. Resistance to fluoroquinolones is, however, increasing following their widespread clinical use in veterinary medicine [4]. Thus, treatment can be more complicated, particularly for those fluoroquinolones resistant *E. coli* isolates that show multidrug resistance (MDR) phenotypes [3, 5]. These resistances are both a medical problem for failure of treatment in veterinary medicine practice, and a public health worry for resistant *E. coli* transmission from companion animals to humans, and also in reverse order [2,6]. *E. coli* resistance to fluoroquinolones mainly results from the progressive accumulation of different point mutations in the genes that encode the target enzymes of fluoroquinolones, DNA gyrase (encoded by gyrA and gyrB) and topoisomerase IV (encoded by parC and parE). Such mutations are chiefly found in gyrA's quinolone-resistance deciding regions (QRDR) and its homologous parC region, which lead to multidrug resistance [7, 8]. However, decreased intracellular accumulation of fluoroquinolones in association with AcrAB-TolC system increased efflux pump activity [2]. Manipulation of the fluoroquinolone structure is among the approaches taken by pharmaceutical companies that could mitigate emerging resistance. Different molecular architectures may lead to a change in primacy targets for identical bacteria [9, 10]. Primacy is typically calculated using in vitro selection tests which classify the first mutation that confer resistance in the initial target protein, with mutations that subsequently occurred in the secondary target. Nevertheless, fluoroquinolones are commonly used in the therapy of UTIs due to a high concentration in the urinary tract and good concentrations on tissue [11]. However, there is comparatively less knowledge about the molecular mechanisms for resistance to ciprofloxacin in dog isolated uropathogenic *E. coli*.

The current study aims to use a molecular technique to identify the gene of resistance to ciprofloxacin and its distribution in *E. coli* associated with urinary tract infections.

Results

Assessment of a total of 103 urine samples from dogs of different breeds, genders, and age groups afflicted with urinary tract infections (UTI) divulged 25 (24.3%) of *Escherichia coli*.

Antimicrobial susceptibility profile of E. coli

The findings of antimicrobial susceptibility test showed that the isolates were resistant to ciprofloxacin (25 isolates; 100%), ampicillin (25 isolates; 100%), ceftriaxone (20 isolates; 80.0%), ceftizoxime (19 isolates; 76.0%), nitrofurantoin (14 isolates; 56.0%), streptomycin (13 isolates; 52.0%) and amoxyclav (12 isolates; 48.0%) while they were susceptible to chloramphenicol (21 isolates; 84.0%), gentamycin (11 isolates; 44.0%) and amikacin (10 isolates; 40.0%) as depicted in Table 2.

Ciprofloxacin resistance genes

PCR was used to explore the presence of the ParC, GyrA, and GyrB genes (Figures 1, 2, 3, and Table 1). In all 25 (100%) *E coli* isolates, ParC and GyrA were present, while only 4 (16.0 %) isolates harbored GyrB gene.

Discussion

Urinary tract infections in companion animals, particularly dogs, are life threatening. Nonetheless, if detected sooner, the disease is curable with antibiotics. This may happen at any age, but the highest incidence is observed in adult dogs. Infection occurs mainly in a dog's urinary tract as a result of bacterial colonization. *Escherichia coli* is by far the most important cause of urinary tract diseases in dogs and other pets [16,

Table 1.
Distribution of ciprofloxacin resistance genes among 25 *E. coli* isolates from dogs with UTI

Antibiotics	No. of resistant isolates (%)	No. of positive isolates (%)
Ciprofloxacin	ParC	25 (100)
	GyrA	25 (100)
	GyrB	4 (16)

17]. The prevalence of bacterial infections in dogs with urinary tract is not completely known. In the present study out of 103 urine samples of dogs with UTIs symptoms, *Escherichia coli* was isolated from 25. Chang et al. [18] reported that 114 dogs out of 201 dogs were positive for *E. coli.* Moyaert et al. [19] isolated *E. coli* from dogs with UTIs symptoms from 204 out of 437 urine samples. Kuan et al. [20] recorded that 146 out of 200 urinary specimens from dogs diagnosed with UTIs were cultivated with *E. coli*. Liu et al. [21] also confirmed *E. coli* growth detection in 106 out of 174 dogs investigated. Such data suggest that infections of the urinary tract caused by bacteria are mainly diagnosed by urinalysis, clinical tests, and culture. The pattern of antimicrobial susceptibility of the *E. coli* isolates reported in the current studies has identified a very high level of ciprofloxacin and ampicillin resistance. Fluoroquinolone/quinolone class ciprofloxacin exhibits its antimicrobial activity by inhibiting DNA gyrase and topoisomerase IV through alterations in target enzymes. The presence of mutations in the DNA gyrase enzyme's quinolone-resis-

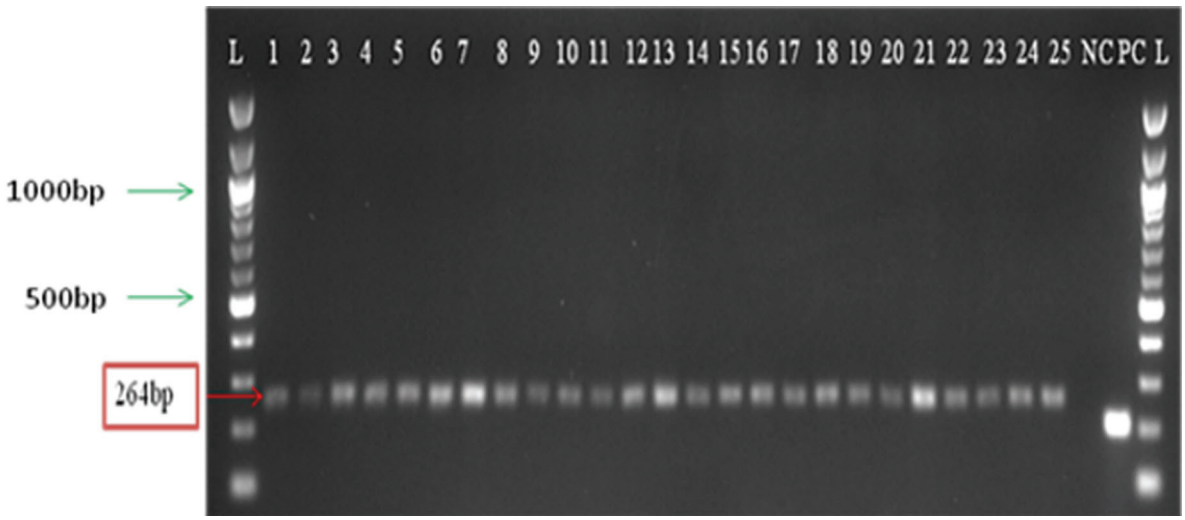

Figure 1.
Agarose gel electrophoresis showing PCR amplified products of ciprofloxacin resistance gene (ParC) of *E. coli* isolates
Lane L: 100bp DNA ladder
Lane: 1-25 positive samples (264bp)
Lane NC: negative control. Lane PC: positive control

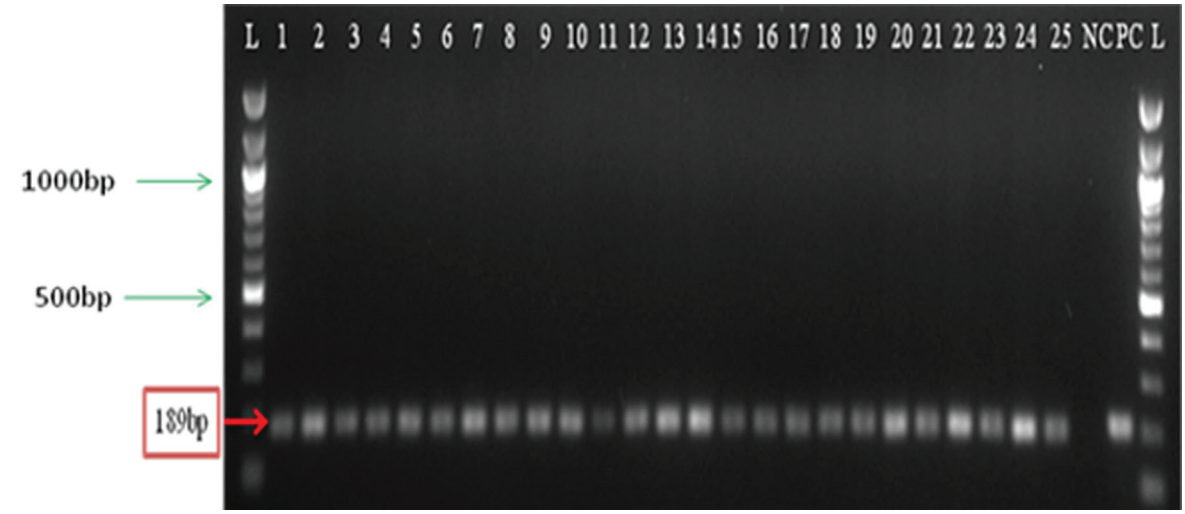

Figure2.
Agarose gel electrophoresis showing PCR amplified products of ciprofloxacin resistance gene (GyrA) of *E. coli* isolates
Lane L: 100bp DNA ladder
Lane: 1-25 positive samples (189bp)
Lane NC: negative control. Lane PC: positive control

Figure 3.
Agarose gel electrophoresis showing PCR amplified products of ciprofloxacin resistance gene (GyrB) of *E. coli* isolates
Lane L: 100bp DNA ladder
Lane: 3, 7, 21 & 23 positive samples (203bp)
Lane: 1-2, 4, 5, 6, 8-20, 22, 24 & 25 negative samples
Lane NC: negative control.

Table 2.
Antibiotic susceptibility pattern of *E. coli* isolated from dogs (n = 25)

Antibiotics	No. of resistant isolates (%)	No. of intermediate resistance isolates (%)	No. of susceptible isolates (%)
Amikacin	9 (36.0)	6 (24.0)	10 (40.0)
Amoxyclav	12 (48.0)	3 (12.0)	10 (40.0)
Ampicillin	25 (100)	0 (0.0)	0 (0.0)
Ceftizoxime	19 (76.0)	0 (0.0)	6 (24.0)
Ceftriaxone	20 (80.0)	0 (0.0)	5 (20.0)
Chloramphenicol	3 (12.0)	1 (4.0)	21 (84.0)
Ciprofloxacin	25 (100)	0 (0.0)	0 (0.0)
Gentamicin	9 (36.0)	5 (20.0)	11 (44.0)
Nitrofurantoin	14 (56.0)	5 (20.0)	6 (24.0)
Streptomycin	13 (52.0)	3 (12.0)	9 (36.0)

tance determining region (QRDR) is the main cause of high-fluoroquinolone resistance in gram-negative bacteria such as *E. coli* [22]. This high degree of resistance observed against ciprofloxacin is possibly explained by cross-resistance with the other members of quinolones such as enrofloxacin, nalidixic acid, and norfloxacin [23].This finding is close to that observed in a previous research [21]. However, Oliveira et al. [24], and Thungrat et al. [25] published previously on fluoroquinolone tolerance in companion animals.

Other studies have nevertheless reported a higher degree of canine resistance to ciprofloxacin in *E. coli* [26, 27]. Besides, increased resistance to fluoroquinolone in companion animals might restrict the treatment of uropathogenic *E. coli* infections in humans who might develop *E. coli* infection from their dogs [21]. This also demonstrates that the widespread use of ampicillin and other Beta lactams antibiotics may be related to the selection of antibiotic resistance mechanisms in pathogenic and non-pathogenic *E.*

coli isolates [28]. Beta-lactam antimicrobial resistance in *E. coli* is primarily mediated by B lactamases that mostly hydrolyze the beta-lactam ring and hence deactivate the antibiotics [29]. Ampicillin resistance found in this research was higher than the previously recorded studies of the companion animals. [30], while the results are consistent with those of Gilliver et al. [31], Wedley et al. [32], Wong et al. [33], Nhung et al. [34], Liu et al. [35], Cavalho et al. [36] and Liu et al. [21]. Furthermore, Allen et al. [37] reported high ampicillin resistance. The high degree of antibiotic resistance among the isolates of *E. coli* reported in this study is directly due to the colossal number of cases referred to the Veterinary Clinical Complex (VCC) hospital from primary health care centers and private practitioners with complicated diseases and repeated cases where dogs have already been exposed to various groups of antibiotics without any prior laboratory evaluation as previously stated by Mustapha et al. [38]. The presence of drug-resistant *E. coli* in dogs presents a possible public health threat. The role of livestock as a source of pathogen transmission to humans was well established, mainly through nutritional exposure, but also through direct contact [39, 40]. In Hisar, though, dogs usually share the entire home setting with their owners and are considered as family members [38]. Therefore, close interaction with dogs is typical among humans. It can serve as an essential route of transmission of *E. coli* to humans. Indeed, an increasing body of evidence suggests that resistant bacteria or mobile resistance determinants can be passed between dogs and humans through direct contact. [41, 42]. The isolates of *E. coli* are particularly susceptible to chloramphenicol, gentamicin, and amikacin. This indicated that chloramphenicol has broad-spectrum activity against gram-positive and gram-negative organisms while also providing protection from anaerobic infection. Moreover, gentamicin and amikacin of the same aminoglycoside class have exhibited high activity against uropathogenic *E. coli,* which could be the product of aminoglycoside complexity and possibly due to the route of administration [43].

The genes ParC, GyrA, and GyrB were found to be associated with the resistance of fluoroquinolones to all the *E. coli* urinary tract isolates tested in this study. The genes ParC and GyrA were found in all isolates, while only four isolates had GyrB genes. In addition, the high-level resistance isolates found in this study that contain multiple mutations within the *E. coli* isolates at different stages. This was consistent with the previous research which showed that enrofloxacin-resistant uropathogenic *E. coli* isolates had a mutation of two points, one in ParC and the other in GyrA [18]. In addition, several researchers have documented over-expression of the ACrAB –TolC system causing multiple drug resistance like fluoroquinolone [44].

In conclusion, the study clearly reported a large number of pathogenic *E. coli* strains in the urine of dogs with high levels of resistance to ciprofloxacin and ampicillin. The molecular study shows that the genes responsible for the resistance of ciprofloxacin in clinical isolates of *E. coli* were ParC, GyrA, and GyrB. Monitoring the patterns of antibiotic prescription and resistance in companion animal medicine, serves as an early indicator for the changes in the susceptibility of clinical isolates to antibiotics via cultural isolation, antimicrobial susceptibility, and PCR-based amplification of antibiotic resistance genes that provide useful data to the veterinarians. These are more effective therapeutic protocols in the control of chronic or recurrent UTIs in dogs. Prescription of antibiotics such as chloramphenicol, gentamicin, and amikacin may be more effective for treating UTIs in dogs.

Material and methods

Source of Animals

The present research was performed on dogs of various breeds, different age groups and of both sexes, presented at the Veterinary Clinical Complex (VCC), Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar with History / clinical symptoms of anorexia, vaginal discharge, failure to urinate or just dribbling a small amount of urine, dark urine, dysuria, increased water intake, constant licking of urinary opening, soiling in appropriate areas, hematuria, vomiting, lethargy, fever, proteinuria, weight loss, heavy urinal odor (smelly urine), back arching (cystitis), and oliguria.

Sample Collection

A maximum of 103 urine samples was collected aseptically from dogs diagnosed with UTIs in sterile test tubes between February 2017 and January 2018 using cystocentesis. Where each sample was classified with an identifier number and collection date. The samples were transported for evaluation immediately in the central laboratory at the college.

Bacteriological Examination

The aseptically collected fresh urine samples 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 aid of a 4 mm diameter platinum loop. Over 24 - 48 hours, the plates were aerobically incubated at 37 °C and analyzed for the presence of any standard distinct purple / pink colonies. Positive isolates were streaked onto Eosine Methylene Blue Agar (EMB), (HiMedia, Mumbai, India), a selective medium for *E. coli* and the plates were aerobically incubated for 24 hours at 37 °C. The appearance of blue, green colonies with a metallic luster on EMB was presumptive to indicate the existence of *E. coli*. Biochemical techniques have further confirmed all positive *E. coli* isolates, including Indole, Methyl Red, Voges Proskauer, Glucuronidase, Nitrate reduction, ONPG, Lysine utilization, Lactose, Glucose, Sucrose, and Sorbitol) using commercially KB010 HiE.ColiTM identification Kit (HiMedia Mumbai, India) following the guidelines of the manufacturer.

Antimicrobial Susceptibility Testing

The drug sensitivity test of Escherichia coli isolates was assessed using the Bauer-Kirby method [12] by using commercially prepared disc (Himedia, Mumbai, India) with a well-known antibiotic concentration. Using a sterile platinum loop, small amounts of test culture were transferred to a brain heart infusion broth (BHI) tube and incubated at 37 °C for 2-5 hours to achieve turbidity. The broth culture was then uniformly distributed by smearing on the surface of Mueller Hinton agar plates with the aid of a sterile cotton swab. The antibiotic discs were placed on the agar and gently squeezed to provide a smooth, near contact with the medium with a sterile forceps. The inoculated plates were held 3-4 hours at low temperatures to allow the antibiotics to pre-diffuse. For 24 hours, the plates were then incubated at 37 °C. The sensitivity was observed based on the manufacturer's zones size definition map. Sensitive (S), intermediate (I), and resistant (R) results were reported. Amikacin (AK) 30mcg, ampicillin (AMP) 10mcg, amoxyclav (AMC) 30mcg, ceptriaxone (CTR) 10mcg, ceftizoxime (CZX) 30cmg, chloramphenicol (C) 30mcg, ciprofloxacin (CIP) 10mcg, gentamycin (GEN) 30mcg, nitrofurantoin (NIT) 300mcg and streptomycin (S) 25mcg (HiMedia, Mumbai, India) are the 10 antibiotics used in this study.

Bacterial DNA Insolation

Following the manufacturer's instructions, DNA of *E. coli* from all the positive isolates was extracted using commercially available PureLink Genomic DNA mini-kit (Invitrogen, USA). The extracted DNA was stored at -20 °C until further processing.

Detection of Ciprofloxacin resistance gene

The presence of resistance genes to ciprofloxacin in *E. coli* DNA extracts was determined by standard PCR. Table 3 gives the primers sequences, target genes, product size, and references. The standard PCR was conducted in a 25 µl volume reaction in Veriti thermo cycler (ABI, USA) containing 6 µl of template DNA, 1µl of each of the primers (10 pmoles concentration), 12.5 µl Phusion PCR Mastermix (2x) (High Fidelity, USA), 1µl DMSO and 2.5 µl nuclease-free water. The amplification process involved initial denaturation at 98 °C for 30 sec, followed by 35 denaturation cycles at 98 °C for 10 secs, annealing at 60 °C for 30 secs, extension at 72 °C for 30 secs, and a final extension at 72 °C for 5 mins. The PCR products were analyzed with 1.5 % agarose gel electrophoresis and visualized under the Gel Doc UV Trans illuminator XR 170-8171 (BIO-RAD, India).

Descriptive statistics were used to analyze the data obtained with JMP Version 11 (SAS, Cary, NC, USA).

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Author Contributions

The experiment was designed by MM and PG. The experiments were conducted by MM. The data were interpreted by MM and PG. The figures were prepared by MM and the manuscript was written by MM. The manuscript was reviewed by PG. The final manuscript was read and approved by all authors.

Conflict of Interest

The authors have no conflict of interest.

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Table 3.
Oligonucleotide primers used for amplification of ciprofloxacin resistance genes

Target Genes	Primer sequence (5'-3')	Amplified Product (bp)	Reference
GyrA	F:ACGTACTAGGCAATGACTGG	189	[13]
	R:AGAAGTCGCCGTCGATAGAAC		
GyrB	F: CAGACTGCCAGGAACGCGAT	203	[14]
	R:biotin AGCCAAGTGCGGTGATAAGA		
ParC	F: CAGACTGCCAGGAACGCGAT	264	[15]
	R:biotin-AGCCAAGTGCGGTGATAAGA		

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