Biofilm and fluoroquinolone resistance of canine *Escherichia coli* uropathogenic isolates

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**Abstract**

**Background:** *Escherichia coli* is the most common uropathogen involved in urinary tract infection (UTI). Virulence of strains may differ, and may be enhanced by antimicrobial resistance and biofilm formation, resulting in increased morbidity and recurrent infections. The aim of this study was to evaluate the *in vitro* biofilm forming capacity of *E. coli* isolates from dogs with UTI, by using fluorescent *in situ* hybridization, and its association with virulence genes and antimicrobial resistance.

**Findings:** The proportion of biofilm-producing isolates significantly increased with the length of incubation time ($P < 0.05$). Biofilm production was significantly associated with fluoroquinolone resistance at all incubation time points and was independent of the media used ($P < 0.05$). Biofilm production was not associated with *cnf1*, *hly*, *pap* and *sfa* genes ($P > 0.05$), but was significantly associated with *afa*, *aer* and the β-lactamase genes ($P < 0.05$).

**Conclusions:** To the best of our knowledge, this is the first report showing significant association between biofilm production and fluoroquinolone resistance in *E. coli* isolates from dogs with UTI. Biofilm formation may contribute to UTI treatment failure in dogs, through the development of bacterial reservoirs inside bladder cells, allowing them to overcome host immune defenses and to establish recurrent infections.

**Keywords:** Biofilm, Dogs, *Escherichia coli*, Fluoroquinolone resistance, Urinary tract infection

**Findings**

*Escherichia coli* is the most common uropathogen in urinary tract infections (UTI) of humans and animals, being responsible for high morbidity and increased health care costs [1-3]. These infections are usually considered acute and self-limiting, but recurrent clinical signs are often observed [3]. *E. coli* UTI pathogenesis is similar in dogs and humans, and dogs may serve as reservoirs of uropathogenic *E. coli* (UPEC) strains that can be transmitted to humans and other animals [2,4]. In fact, the human highly virulent O25:ST131 uropathogenic clone was recently found in a dog with chronic cystitis [5,6]. This fact suggests a possible human-to-animal transmission.

In humans, it is well established that UPEC are able to form biofilm structures within the bladder, forming bacterial reservoirs that allow infection persistence [7-10]. These structures are highly organized multicellular complexes, characterised by adherent colonies surrounded by a large exopolysaccharide matrix. Biofilm structures protect bacteria against high antimicrobial concentrations and phagocytosis, allowing their survival in hostile environments within the host [10]. Detection of biofilm-producer strains is therefore relevant for the design of adequate control measures for UPEC infections. Fluoroquinolones are extensively used for UTI treatment, due to the high concentration levels reached in the urinary tract and good tissue concentrations [11]. The aim of this study was to evaluate the *in vitro* biofilm-forming ability of *E. coli* isolates from dog urinary tract infections, and its association with virulence and β-lactamase antimicrobial resistance genes, and with 2nd generation quinolones resistance.

Sixty-six *E. coli* isolates were used, from a collection of bacterial isolates from dogs with UTI belonging to the Faculty of Veterinary Medicine, University of Lisbon. Isolates virulence factors had already been determined by multiplex PCR and described by us: 57.6% (n = 38) were positive for *S* fimbriae gene *sfa*; 1.5% (n = 1) for afimbrial adhesion I gene *afaI*; 42.4% (n = 28) for haemolysin gene *hly*; 42.4% (n = 28) for pyelonephritis-associated pil gene *pap*...
Table 1 Biofilm production and virulence and β-lactamase genes presence in 66 E. coli isolates from dogs with urinary infections*

| Isolate | Biofilm production | Virulence genes | Multiplex-PCR |
|---------|--------------------|-----------------|---------------|
|         | TSB, 24 hours      | pap  sfa  afa  hly  cnf1  aer | TEM  SHV  OXA  AMP C  CTX-M |
| 5       | Negative           | -    +    -    -    -    - | -    -    -    -    - |
| 13      | Negative           | -    +    -    -    -    - | -    -    -    -    - |
| 21      | Negative           | +    +    +    +    +    - | -    -    -    -    - |
| 34      | Negative           | -    -    -    -    -    - | -    -    -    -    - |
| 36      | Positive           | -    -    -    -    -    - | -    -    -    -    - |
| 43      | Negative           | -    -    -    -    -    - | -    -    -    -    - |
| 78      | Positive           | +    -    -    -    -    - | +    -    -    -    - |
| 84      | Positive           | -    +    +    +    +    - | -    -    -    -    - |
| 88      | Negative           | +    +    +    +    +    - | -    -    -    -    - |
| 91      | Negative           | -    -    -    -    -    - | +    -    -    -    - |
| 95      | Negative           | -    -    -    -    -    - | +    -    -    -    - |
| 99      | Positive           | -    -    -    -    -    - | -    -    -    -    - |
| 109     | Positive           | +    +    -    -    -    - | -    -    -    -    - |
| 115     | Positive           | -    +    -    -    -    - | -    -    -    -    - |
| 125     | Positive           | -    -    -    -    -    - | -    -    -    -    - |
| 128     | Negative           | -    -    -    -    -    - | -    -    -    -    - |
| 133     | Positive           | -    +    -    -    -    - | -    -    -    -    - |
| 134     | Positive           | -    +    -    -    -    - | -    -    -    -    - |
| 138     | Positive           | +    -    -    -    -    - | -    -    -    -    - |
| 174     | Negative           | -    -    -    -    -    - | -    -    -    -    - |
| 179     | Negative           | -    +    -    -    -    - | -    -    -    -    - |
| 188     | Negative           | -    -    -    -    -    - | -    -    -    -    - |
| 194     | Negative           | -    -    -    -    -    - | -    -    -    -    - |
| 207     | Negative           | +    +    -    -    -    - | -    -    -    -    - |
| 209     | Negative           | +    +    -    -    -    - | -    -    -    -    - |
| 224     | Negative           | -    -    -    -    -    - | -    -    -    -    - |
| 226     | Negative           | -    -    -    -    -    - | -    -    -    -    - |
| 227     | Positive           | -    +    -    -    -    - | -    -    -    -    - |
| 237     | Negative           | +    +    -    -    -    - | -    -    -    -    - |
| 238     | Positive           | +    +    -    -    -    - | -    -    -    -    - |
| 239     | Positive           | +    +    -    -    -    - | -    -    -    -    - |
| 250     | Negative           | +    -    -    -    -    - | -    -    -    -    - |
| 251     | Positive           | +    +    -    -    -    - | -    -    -    -    - |
| 257     | Negative           | +    +    -    -    -    - | -    -    -    -    - |
| 258     | Negative           | -    -    -    -    -    - | +    -    -    -    - |
| 271     | Positive           | -    -    -    -    -    - | -    -    -    -    - |
| 274     | Positive           | -    -    -    -    -    - | -    -    -    -    - |
| 291     | Positive           | +    +    -    -    -    - | -    -    -    -    - |
| 304     | Positive           | -    +    -    -    -    - | -    -    -    -    - |
| 320     | Negative           | -    -    -    -    -    - | +    -    -    -    - |
| 325     | Positive           | +    +    -    -    -    - | -    -    -    -    - |
| 327     | Positive           | +    -    -    -    -    - | -    -    -    -    - |
Detection of genes related with β-lactamase resistance has also been previously described by us: 19 isolates were positive for \( \text{bla}^{\text{TEM}} \) (28.8%), three for \( \text{bla}^{\text{SHV}} \) (4.5%), two for \( \text{bla}^{\text{OXA-1}} \) (3.0%) and six for \( \text{ampC} \) (9.1%) [13].

Minimum inhibitory concentrations (MIC) of ciprofloxacin (CIP, Laboratório Atral-Cipan, Portugal), enrofloxacin (ENR, Bayer, Germany), marbofloxacin (MAR, Vétoquinol, France) and orbifloxacin (OBX, Schering-Plough, USA) were determined by broth microdilution, following Clinical and Laboratory Standards Institute guidelines [14,15]. E. coli ATCC 25922 was used as a reference control for MIC testing. Dilution range for all antimicrobial compounds tested was from 256 to 0.00003 \( \mu \text{g/mL} \).

Biofilm production was tested by fluorescent in situ hybridization, as previously described [16], in two broth media, TSB (Tryptic Soy Broth, Oxoid, CM0129B) and BHIB (Brain Heart Infusion Broth, Oxoid, CM0225), using the universal bacterial probe, Eub338, labelled with fluorescein (Stabvida, Portugal). Wilcoxon signed ranks test was applied for statistical purposes.

From the 66 UPEC dog isolates evaluated, 31 isolates were biofilm-positive in BHIB at 24 hours, 51 at 48 hours, and 59 at 72 hours. In TSB, a higher number of biofilm-producing isolates was observed at all incubation times: 35 isolates at 24 hours; 52 at 48 hours; 62 at 72 hours.

No significant differences (\( P > 0.05 \)) were found between biofilm formation in the two culture media, but significant differences were found between biofilm production between 24 and 48 hours, 48 and 72 hours, and 24 and 72 hours (\( P < 0.05 \)).

Association between biofilm formation in TSB at 24 hours and the presence of \( \text{cnf1} \), \( \text{hly} \), \( \text{pap} \) and \( \text{sfa} \) was not significant (\( P > 0.05 \)), whilst there was a significant association between biofilm and \( \text{afa} \) and \( \text{aer} \) (\( P < 0.05 \)) (Table 1). Biofilm production was also associated to the

| Total (n=) | 35 | 28 | 38 | 1 | 28 | 27 | 23 | 20 | 3 | 2 | 25 | 0 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| % | 53.0 | 42.4 | 57.6 | 1.5 | 42.4 | 40.9 | 34.8 | 30.3 | 4.5 | 3.0 | 37.9 | 0 |

*Virulence and β-lactamase genes results are adapted from Féria et al. [12] and Pomba et al. [13]; + PCR positive result; − PCR negative result.
presence of the β-lactam genes blaTEM, blaOXA-1, blaSHV and ampC (P < 0.05) (Table 1).

Fluoroquinolones resistance is summarized in Table 2. Resistance was found in 13.6% of the uropathogenic isolates (n = 9) towards ciprofloxacin, enrofloxacin, marbofloxacin and orbifloxacin. One additional isolate was resistant to orbifloxacin. All E. coli isolates were simultaneously resistant to all the fluoroquinolones tested.

Biofilm formation has been described as an important E. coli virulence factor in human UTI. In this study, biofilm-forming ability of 66 UPEC dog isolates was evaluated. Previous works showed that isolates ability to form biofilm depends upon the medium used and time of observation [16-19]. In our study, no differences were found regarding biofilm production in BHIB and TSB. Almost half of the isolates were able to form biofilm at 24 hours in both media, and this percentage significantly increased with incubation time.

Association between biofilm and other virulence factors has already been studied [18]. In this work, biofilm was not associated to toxin production (hly and cnfI), or to filamentous adhesions involved in host specific adhesion (sfa and pap). Nevertheless, associations between biofilm and afa and aer were significant. These results may indicate that adhesive non-fimbrial adhesions are important for the initial steps of biofilm formation and that the aerotaxis receptor may be involved in the oxygenation of these structures. Biofilm production was also associated to the presence of the β-lactamase genes. Our results are not in accordance with previous works [18,20] that stated that E. coli strains that are β-lactamase producers may not be able to form biofilms.

Regarding fluoroquinolones resistance, compounds tested showed an in vitro efficacy of more than 80%, as already observed by other authors [11,21]. It is important to refer that although these broad-spectrum antibiotics are extensively used for treatment of animal related infections, their efficacy remains high [11]. Biofilm structures are believed to impair antimicrobial compounds action [10,22]. Association between biofilm and fluoroquinolone resistance was considered significant in all time points, independently of the media, which is in agreement with previous human UTI studies [9]. Biofilm formation by UPEC may contribute for UTI treatment failure in dogs, since these structures are responsible for the establishment of bacterial reservoirs inside the bladder cells, allowing them to overcome the host immune defences and to establish recurrent infections [9].

To our knowledge, this is the first report of the association between biofilm formation and fluoroquinolone resistance in E. coli dog UTI isolates, representing an important novelty. This fact is relevant for biofilm and antimicrobial resistance control in veterinary medicine and the establishment of more adequate therapeutic protocols.

Animal ethics
No experimental research on vertebrates or any regulated invertebrates were performed in this study.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
MO participated in the study conception and design, carried out the biofilm studies and drafted the manuscript. CP participated in the study conception and design, carried virulence and antimicrobial resistance genes studies and minimum inhibitory concentration determinations and helped to draft the manuscript. FRO participated in the biofilm studies. All authors read and approved the final manuscript.

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