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Multi-drug resistant Escherichia coli isolated from canine feces in a public park in Quito, Ecuador

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Highlights

• First demonstration of the presence of MDR E. coli in canine feces from Ecuador.
• Presence of clinical relevant alleles of blaCTX-M, blaCMY and the mcr-1 gene.
• Older people is more conscious of collecting and disposing of dog’s feces in the park.
• Characterization of isolates from canine feces in public settings might be a sentinel method to study resistance.

ABSTRACT

Objectives: We focused on estimating the prevalence of extended-spectrum-β-lactamase (ESBL), pAmpC, carbapenemases, and mcr-1 producing Escherichia coli in canine feces from a public park in Quito, Ecuador.

Methods: We performed phenotypic and genotypic characterization of E. coli isolated from 50 canine feces samples recovered from a city park in Quito, Ecuador. Additionally, a multiple-choice survey was conducted among 50 dog owners.
Results: Twenty out of 50 samples (40%) presented E. coli resistant to ceftriaxone; 23 E. coli isolates were recovered for further analysis. All of the isolates showed the phenotype for multi-drug resistance (resistance to ≥ 3 antibiotic families). Resistance to carbapenems, tigecycline, and amikacin were not registered. No major clonal relatedness was observed among the resistant isolates. ESBL alleles bla\textsubscript{CTX-M-15}, bla\textsubscript{CTX-M-55}, and bla\textsubscript{CTX-M-65} were the most common. Two isolates harbor bla\textsubscript{cmy-2} gene and one isolate harbor both mcr-1 and bla\textsubscript{CTX-M-65} genes. Statistical analysis showed that older people were more conscious of collecting and disposing of dog’s feces than subjects younger than 35 years old (p < 0.05).

Conclusions: Our finding of multidrug resistant (MDR) E. coli in dog feces found in a city park illustrates the importance of analyzing canine feces in public settings (e.g., parks, playgrounds) as part of MDR E. coli surveillance programs. Additionally, our research might be a sentinel sampling method of study to gain a better understanding of community sources of antibiotic-resistant Enterobacteriaceae at human-animal-environment interfaces.

Keywords: pAmpC β-lactamases; Ecuador; Escherichia coli; ESBL; canine feces; mcr-1; multi-drug resistance; public park.
1. Introduction

Worldwide, the presence of animal feces in the ground of urban areas is a significant public health problem, due to the presence of different microorganisms that can be transmitted to humans [1]. This concern is increasing with the growing pet population and free roaming animals in large cities like Quito in Ecuador [2,3]. This increment has augmented the probability of contact between animals and humans, it has heightened the exposure risk to different pathogens, including multi-drug resistant (MDR) Escherichia coli [1]. MDR E. coli has been isolated from humans, animals, and the environment worldwide [4]. The most important resistance mechanisms in these strains are extended-spectrum-β-lactamase (ESBL), plasmid-mediated pAmpC β-lactamases, carbapenemases, and phosphoethanolamine-lipid A transferases (mobile colistin resistance, mcr), which lead to therapy failure.

Despite the studies on ESBL, pAmpC, carbapenemases, and mcr producing E. coli from different sources, the routes of transmission are missing, and the epidemiology of these strains is poorly understood. So far, the prevalence and the potential for transmission of MDR E. coli between companion animals and humans are unknown. The aim of this study was to estimate the prevalence of ESBL, pAmpC, bla\text{KPC}, and mcr-1 genes in ceftriaxone-resistant E. coli isolated from canine feces in a city park in Quito, Ecuador.

2. Materials and methods

2.1. Study design

We performed an exploratory, observational study to determine the prevalence of ceftriaxone-resistant E. coli in canine feces from a city park in our city, Quito, the capital city
of Ecuador, is located in the Andean Region of Ecuador (0°13′07″S and 78°30′35″W), at an altitude of 2,810 meters above sea level, with a population of 1’911.966 [5]. In this study, we establish a transect of 1 km² in a lineal park in the southern part of the city (-0.262678, -78.538390; -0.266270, -78.550295), where families walk their dogs, people and children play and do exercise, street food is sold, and where there are no facilities for the collection of canine feces. The collection of feces is regulated by two local ordinances [20, 21].

2.2. Sampling and isolation

We collected 50 samples in August 2017. Samples were taken from the top of fresh feces in order to avoid cross contamination from environmental bacteria of the ground. Samples were collected in sterile plastic flasks, and sent to be processed in a laboratory, up to an hour after collection. Samples were plated, using a sterile swab, in MacConkey agar (Becton, Dickinson and Company, France) supplemented with 3 µg of ceftriaxone (CRO) and incubated at 35°C for 18-20 hours. Plates growing red/pink colonies were registered as positive. All different E. coli-like colonies were spiked and isolated in the same medium. A full loop of bacteria was mixed with trypticase soy broth (TSB, Becton, Dickinson and Company, France) + 40% glycerol (Himedia, France) and stored at -20°C for further analysis.

2.3. Identification and susceptibility testing

Species confirmation was performed using biochemical tests: triple sugar iron (TSI), Simmons citrate test, urease production, tryptophan reduction (indole), methyl-red test (RM) and Voges Proskauer (VP) test and confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) analysis in a Microflex LT MALDI-TOF spectrometer (Bruker Daltonics, Inc.).
To identify ESBL, pAmpC, or carbapenemase producing phenotypes, E. coli isolates were screened with double disk synergy using cefotaxime (30 µg), cefotaxime/clavulanic acid (30/10 µg), ceftazidime (30 µg), ceftazidime/clavulanic acid (30/10 µg), and susceptibility to cefepime (30 µg), imipenem (10 µg), and cefoxitin (30 µg). Antibiotic resistance profiles were carried out using the disk diffusion method with aztreonam (30 µg), imipenem (10 µg), tetracycline (30 µg), tigecycline (10 µg), doxycycline (30 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), norfloxacin (5 µg), levofloxacin (5 µg), gentamicin (10 µg), netilmicine (30 µg), amikacin (30 µg), fosfomycin (200 µg), nitrofurantoin (300 µg), chloramphenicol (30 µg), trimethoprim/sulfamethoxazole (1,25/23,75 µg), and azithromycin (15 µg) (Becton, Dickinson and Company, France). BD Phoenix™ Automated Microbiology System (France) was used for colistin. Results were interpreted following the Clinical and Laboratory Standards Institute (CLSI, 2017) recommendations.

2.4. Genotypic characterization

Resistance genes were identified using polymerase chain reaction (PCR) protocols described elsewhere: \( \text{bla}_{\text{CTX-M}} \) Group 1 [7], \( \text{bla}_{\text{CTX-M}} \) Group 2 [8], \( \text{bla}_{\text{CTX-M}} \) Group 8 [9], \( \text{bla}_{\text{CTX-M}} \) Group 9 [10], for \( \text{bla}_{\text{KPC}} \) [11], and colistin resistant \( \text{mcr-1} \) [12]. Positive and negative controls were included in each reaction. The phylogenetic group was also identified [13]. All amplicons were sequenced in both strains using the dideoxy sequencing method. Sequences were edited and analyzed in Geneious R10 using the database of ResFinder-3.0 [14]. Clonal analysis was carried out using the BOX-PCR method described elsewhere [15], and cluster analysis was completed in GelCompar II version 6.6.11 (Applied Maths). Fragments between
200 bp and 1,500 bp in size were included in the analysis using the UPGMA model with DICE coefficient.

2.5. Survey

Multiple-choice questionnaires were completed with face-to-face interviews conducted with 50 dog owners who were walking their dogs in the park (after observing whether they pick up after their dogs or not). Dog owners were asked several questions with the intention of assessing their dog-owner habits, in particular their attitudes concerning the disposal of their dog’s feces. The questionnaire included a total of 3 questions focused on establishing the participant’s attitude and behavior with respect to dog fouling in the park and what factors (e.g., availability of bags and bins, fines, and reasons) influence the decision to clean up their dog’s waste. In addition, participants were asked to rate the importance of stated factors that have influenced their behavior in relation to clearing up their dog feces (e.g., claims from a member of the public). We also gathered qualitative information from the participants’ points of view regarding waste disposal and personal opinions about the reasons for not collecting feces in the park. We did not collect information about whether the dogs present in the parks live in a home/dwelling with at least one other animal, the frequency of visiting the park, or regarding animal healthcare. The survey was adapted from the Dog Park Survey made by the Parks Advisory Commission of the City of Ann Arbor, Michigan [16]

2.6. Statistical Analysis
We calculated absolute and relative frequencies for qualitative variables of the survey. Fisher's exact test was used to compare proportions, and p value <0.05 was considered as statistically significant. Confidence interval analysis was carried out using R, V. 3.2.5.

3. Results

3.1. Isolation and phenotypic characterization

Twenty out of 50 samples showed the growth of E. coli-like colonies on antibiotic-supplemented McConkey agar plates with a prevalence of 40% (CI: 39.74; 40.55). From the 20 samples, 23 isolates were selected and confirmed as E. coli, one per sample except for sample 26, in which colonies 26Apl, 26Bpl, and 26Cpl were included and sample 49, in which colonies 49Apl and 49Bpl were included). All 23 isolates were submitted to the screening for β-lactamase production. Twenty-one isolates were positive for ESBL production, two to pAmpC β-lactamases production, and there were no isolates suspicious to carbapenemases production. All isolates were resistant to cefotaxime and tetracycline. We registered a high percentage of resistance to ceftazidime, aztreonam, cefepime, doxycycline, ciprofloxacin, nalidixic acid, norfloxacin, levofloxacin, and trimethoprim-sulfamethoxazole, less than 50% of resistance to gentamicin, netilmicin, fosfomycin, chloramphenicol, cefoxitin, and azithromycin, and none of the isolates were resistant to imipenem, tigecycline, or amikacin. All of the isolates were MDR (resistant to ≥ 3 families of antibiotics). One isolate presented the mcr-1 gene and was resistant to colistin MIC = 4; ampicillin MIC > 16, ceftriaxone MIC > 32, cefuroxime MIC > 16, ciprofloxacin MIC > 2 and levofloxacin MIC > 4 (Figures 2 and 3).

3.2. Genetic characterization
No major clonal relatedness was observed among the resistant isolates. The phylogroup B1 was the most prevalent among the isolates, followed by A, D, and B2. The bla_{CTX-M} Group 1 genes were the most prevalent (43.5%, 10 isolates), represented by the variants bla_{CTX-M-15} (17.4%, 4 isolates), bla_{CTX-M-55} (17.4%, 4 isolates), and bla_{CTX-M-3} (8.7%, 2 isolates), followed by bla_{CTX-M} Group 9 (26%, 6 isolates), with the variants bla_{CTX-M-65} (13%, 3 isolates), bla_{CTX-M-14} (4.3%, 1 isolate), bla_{CTX-M-27} (1 isolate) and bla_{CTX-M-106} (1 isolate), and bla_{CTX-M} Group 2, with the variant bla_{CTX-M-2} (8.7%, 2 isolate). Additionally, one isolate (32 pl) presented the hybrid bla_{CTX-M-123}. The bla_{CTX-M} Group 8 and bla_{KPC} were not detected. One isolate (19 pl) presented two bla_{CTX-M} genes (bla_{CTX-M-2} and bla_{CTX-M-3}). Two isolates (8.7%) presented the bla_{CMY-2} gene.

Finally, the isolate 4pl harbor bla_{CTX-M-65} and mcr-1 genes (Figure 2).

3.3. Owners survey results

The survey was carried out in Spanish, questionnaire in English is in supplementary data 1:

We collected answers from 50 dog owners. The mean age of owners was 36.6 years, with a standard deviation of 13.6 years. Fifty-eight percent of the interviewees were men. Most of the owners collected the fresh feces of their dogs (86%), a tendency that was more accentuated in men; however, we did not find any significant statistical difference between the habits of men and women in picking up feces.

The mean age of owners less than 35 years old was 25.6 years, with a standard deviation (SD) of 6.2 years, while older subjects had a mean age of 47.7 years and SD of 8.2 years. There are significant statistical differences between these two groups regarding collecting and disposing of feces (p<0.05). Older people were more conscious about these tasks than subjects younger than 35 years. From those to collect the waste of their dog, approximately
60% do it always, while the rest do it sometimes. \((p<0.05)\). There were no statistically significant differences in the rest of the variables explored.

There was a high percentage of participants who collected the feces because of hygienic reasons. Also, the possibility of getting a fine was stated by the majority as a good reason to collect the feces of their dogs. The leading excuse for not properly disposing of the feces was the lack of availability of bins and bags (Table 1).

From those who do not collect the feces of their dogs we found that 71.4% did not collect because of disgust or lack of bags or bins. (Figure 3). Forty-two percent do not care whether they pick up the dog’s feces or not.

Regarding qualitative information, some participants expressed not having knowledge about ordinances \([20, 21]\) related to feces disposal, while others think that there is a lack of personnel to enforce ordinances, and they also expressed a need for more educational campaigns related to management of animal feces disposals.

4. Discussion

This is the first study performed to assess canine fecal contamination and resistance in an urban park and dogs frequenting such a park in Ecuador. In most countries, the overall number of antimicrobials used for companion animals is not consistently measured. However, antimicrobials used in human and veterinary hospitals are almost identical. In Ecuador, there is no data of veterinary prescription of antibiotics. Nevertheless, in our experience, the most commonly prescribed antimicrobials in small animals are amoxicillin, fluoroquinolones, third-generation cephalosporins, and tetracyclines. These prescribing
trends have been described elsewhere [17, 18]. Antimicrobial prescription has received little attention in veterinary clinical settings, regarding the augmented bacterial resistance worldwide. The role of pets in the dissemination of antimicrobial resistance has been given little attention when compared with that of food animals [19]. Companion animals are thought to be a reservoir of resistant bacteria and the risk of spread to humans is facilitated through their feces. In a study performed by Ferreira et al. (2017) [20], they found that almost all dog owners (94.1%), claimed to collect their dog's feces. In our study, this figure was smaller (83.3%). This percentage may be overestimated since the survey was not anonymous.

Although there were dog feces bag dispensers at the park, all of them were without plastic bags. Dog owners who picked up the feces carried their own plastic bags. The collection of pet waste is regulated in Quito by two ordinances. The ordinance of the Metropolitan Council of Quito # 48 in articles 6 and 30 of the Municipal Code For The Metropolitan District Of Quito, Municipal Ordinance 1, specifically the article 357.2 [21, 22], provides a penalty for not picking up the dogs' feces. Nevertheless, these ordinances are not well known by the owners of animals in the city and poorly implemented by the local police. The fact that older people are more aware of hygiene than younger people (Table 1) suggests that more educational campaigns are needed. These educational campaigns should be related to management of animal feces disposal in order to avoid the spread of MDR E. coli.

To date, the prevalence of third generation cephalosporin-resistant E. coli in dog feces from public parks has been poorly described. In our work, we reported a 40% (20/50) prevalence, which is much higher than the prevalence reported for other public spaces (1.9% - 4/209),
such as public gardens in Denmark [23]. On the other hand, there are several studies on ESBL-E. coli prevalence in healthy dogs that have been reported prevalence from 45% (9/20) in the Netherlands [24], 17% (9/53) in Mexico [25], to 7.2% (5/102) in Algeria [26]. To the best of our knowledge, there are no reports of the Andean region in the indexed literature. Despite using direct plating on the selective MacConkey agar plate method, without an enrichment step, which could lead to the loss of positive samples with low counts, as reported elsewhere [24]. However, the prevalence (40%) of samples carrying E. coli resistant to third-generation cephalosporins in our study is worrisome for a public area.

Genes belonging to the bla<sub>CTX-M</sub> family are the most frequently reported in human, animal, and environment ESBL-E coli isolates [27], and bla<sub>CMY-2</sub> is the most frequently identified pAmpC gene worldwide [28]. The same pattern has been identified in canine fecal samples, where bla<sub>CTX-M-1</sub>, bla<sub>CTX-M-15</sub> (bla<sub>CTX-M-group 1</sub>), and bla<sub>CMY-2</sub> have been frequently described [24, 25, 26].

In this study, we identified bla<sub>CTX-M</sub> genes belonging to groups 1, 2, and 9. In the bla<sub>CTX-M-group 1</sub>, variants bla<sub>CTX-M-15</sub> and bla<sub>CTX-M-55</sub> were the most prevalent. bla<sub>CTX-M-15</sub> is the most prevalent worldwide, and bla<sub>CTX-M-55</sub> is increasing in prevalence in several locations [29, 30]. The most prevalent bla<sub>CTX-M-group 9</sub> was bla<sub>CTX-M-65</sub>, previously described in our location [31].

Our screening for carbapenemase production was based in the sensitivity to imipenem. However, there have been reports of enterobacteriaceae strains harboring bla<sub>KPC</sub> genes sensitive to imipenem. Additionally, in Ecuador, bla<sub>KPC</sub> is the most prevalent gene associated with carbapenem-resistant in Enterobacteriaceae [11]. Therefore, we screen all the isolates for this gene [32]. However, we did not register isolates harboring bla<sub>KPC</sub>. Nevertheless,
carbapenemases-producing E. coli isolated from dog feces has been reported previously [33].

Colistin is the last resort antibiotic against extensively resistant Gram-negative bacteria. Plasmid-mediated colistin-resistant gene mcr-1 was described in E. coli isolates from food animals and humans in November 2015 [34]. In Ecuador, the mcr-1 gene was reported in 2016 in a human E. coli isolate from peritoneal fluid [12]. To date, the mcr-1 gene has been reported worldwide from a variety of sources [35]. However, there are few reports of E. coli harboring mcr-1 isolated from dogs. In this study, the isolation of E. coli harboring mcr-1/blaCTX-M-65 from canine feces in a city park, opens the possibility of further studies to determine whether there is a potential transmission of these strains to humans as it was reported previously [36, 37]. Additionally, the high genotypic diversity shown by the clonal analysis, suggests a variety of genetic backgrounds of resistant determinants present in MDR E. coli in dogs.

A variety of reports support the transmission of E. coli between dog feces, dogs, and humans. Schaufler et al. [38] reported dog feces around veterinary facilities as a non-point infection source of ESBL-producing E. coli for both animals and humans. Dog feces have been identified as a vector for dissemination of ESBL-E. coli within urban areas [23]. As mcr-1–producing E. coli in dogs can be transferred between companion animals and humans [36, 39], we wanted to know if dog feces in a Ecuadorian park could be a source of MDR bacteria for humans. Finally, cross-transmission of resistant E. coli can easily occur between owners and non-owners [40]. Based on this evidence, the dissemination of these bacteria from the dog feces in the park to humans is plausible. This hypothesis must be examined
more thoroughly to establish the real implication of dog feces in city parks over human and pet heath.

5. Conclusion

Although people consider public parks excellent recreational areas for their family and dogs, our results highlight the potential of urban parks as a reservoir and source of transmission of MDR bacteria. Additionally, actions like improving the availability of disposal bags, dog owner’s education, and fines could help to limit their dissemination. Further studies are needed to evaluate the role of dog feces contamination in public environments, as a dissemination pathway of bacterial resistance. The characterization of isolates from canine feces in public parks might be a sentinel sampling method to gain a better understanding of community sources of antibiotic-resistant enterobacteriaceae at human-animal-environment interfaces.

Declarations

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Ethical Approval: Not required

Competing Interests: None declared
Acknowledgments

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References


Figure 1. Resistance patterns and genetic characterization. CTX, cefotaxime; CAZ; ceftazidime; ATM, aztreonam, FEP; cefepime, FOX; cefoxitin, IMP; imipenem, TE; tetracycline, TGC; tigecycline, DO; doxycycline, CIP; ciprofloxacin, NA; nalidixic acid, NOR; norfloxacin, LEV; levofloxacin, GEN; gentamicin, NET; netilmicin, AK; amikacin (30 µg), fosfomycin (200 µg), nitrofurantoin (300 µg), chloramphenicol, SXT; trimethoprim/sulfamethoxazole, AZM; azithromycin.

Figure 2. Resistance profiles
CTX, cefotaxime; CAZ; ceftazidime; ATM, aztreonam, FEP; cefepime, FOX; cefoxitin, IMP; imipenem, TE; tetracycline, TGC; tigecycline, DO; doxycycline, CIP; ciprofloxacin, NA; nalidixic acid, NOR; norfloxacin, LEV; levofloxacin, GEN; gentamicin, NET; netilmicin, AK; amikacin (30 µg), fosfomycin (200 µg), nitrofurantoin (300 µg), chloramphenicol, SXT; trimethoprim/sulfamethoxazole, AZM; azithromycin.

Figure 3. Causes for not collecting dogs' feces.
Table 1. Statistical analysis of attitudes of dog owners regarding the disposal of dog feces survey.

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*Fisher exact Test*