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Extended Spectrum Beta-Lactamases and the Evolution of Uropathogenic *Escherichia coli*

Mecanismo de Titulación: Tesis en torno a una hipótesis o problema de investigación y su contrastación

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DEDICATION

A Jah, por permitirme conocer su basta otra.

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RESUMEN

La resistencia a los antibióticos es una grave amenaza para la salud pública porque limita las opciones para tratar infecciones, aumenta la mortalidad y el costo de tratamiento. *Escherichia coli* es una bacteria versátil que incluye tanto cepas patógenas como comensales. Uno de los patotipos de mayor relevancia es la *E. coli* uropatogenica (UPEC), ya que son la principal causa de infección en el tracto urinario (ITU). En este contexto, el rol de las cepas comensales de *E. coli* no es claro, puesto que algunos estudios indican que pueden actuar como un reservorio de genes de resistencia y se sugiere que existe un proceso de transición de cepa comensal a patógeno; que se encuentra mediado por la adquisición de factores de virulencia y genes de resistencia a antibióticos. Sin embargo, de ser así presentaran una tasa similar de resistencia tras ser sometidos a una presión antibiótica. Nuestros resultados muestran que las UPEC presentan una tasa de resistencia superior a las cepas comensales. Esto sugiere que las cepas UPEC divergieron como un grupo distinto de *E. coli* hace algún tiempo.

Por otro lado, las UPEC productoras de betalactamasas de espectro extendido (BLEE) como CTX-M, son de gran preocupación en el ámbito clínico debido a su capacidad de resistir antibióticos β-lactámicos, incluyendo cefalosporinas de tercera generación. Los mecanismos de dispersión de genes de resistencia son poco comprendidos debido a la complejidad de la transferencia horizontal de genes (THG) mediada por elementos genéticos móviles (EGM). Analizamos tres variantes alélicas *bla*$_{\text{CTX-M}}$ de E. *coli* comensal y UPEC, las cuales mostraron secuencias idénticas de nucleótidos flanqueantes a *bla*$_{\text{CTX-M}}$. Estos braquetes eran similares a los encontrados en otras partes del mundo; sin embargo, el análisis filogenético indicó que los entornos genéticos de los aislados ecuatorianos eran únicos; lo que sugiere que las secuencias de nucleótidos que flanquean los genes *bla*$_{\text{CTX-M}}$ pueden ser útiles para resolver las vías de transmisión del ARG.

El objetivo de la presente investigación fue comparar los fenotipos ESBL de cepas UPEC procedentes de infecciones comunitarias con cepas comensales de *E. coli*. Además, caracterizar los entornos genéticos de las variantes alélicas bla CTX-M (*bla*$_{\text{CTX-M-27,}}$ *bla*$_{\text{CTX-M-55}}$ y *bla*$_{\text{CTX-M-65}}$) mediante secuenciación del genoma completo (WGS) de cepas UPEC procedentes de infecciones comunitarias y las comparamos con las mismas variantes alélicas de *bla*$_{\text{CTX-M}}$ en otras partes del mundo.

Palabras clave: *E. coli* comensal, UPEC, ITU, presión selectiva, BLEE, *bla*$_{\text{CTX-M}}$, THG, EGM.

ABSTRACT

Antibiotic resistance is a serious threat to public health because it limits options for treating infections, increasing mortality and healthcare costs. *Escherichia coli* is a versatile bacterium that includes both pathogenic and commensal strains. One of the most relevant pathotypes is uropathogenic *E. coli* (UPEC), which is the leading cause of urinary tract infection (UTI). In this context, the role of commensal strains of *E. coli* is not clear since some studies indicate that they can act as a reservoir of resistance genes, and it is suggested that there is a transition process from commensal strain to pathogen, which is mediated by the acquisition of virulence factors and antibiotic resistance genes. However, if so, they will present a similar resistance rate after being subjected to antibiotic pressure. Our results show that UPEC shows a higher resistance rate than commensal strains. This suggests that UPEC strains diverged as a distinct group of *E. coli* some time ago.

On the other hand, extended-spectrum beta-lactamase (ESBL)-producing UPECs such as CTX-M are of great concern in the clinical setting due to their ability to resist β-lactam antibiotics, including third-generation cephalosporins. Resistance gene dispersal mechanisms are poorly understood due to the complexity of horizontal gene transfer (HGT) mediated by mobile genetic elements (MGEs). We analyzed three allelic *bla*$_{\text{CTX-M}}$ variants from commensal E. *coli* and UPEC, which showed identical flanking nucleotide sequences to *bla*$_{\text{CTX-M}}$. These genetic environments were similar to those found in other parts of the world; however, phylogenetic analysis indicated that the genetic environments of the Ecuadorian isolates were unique, suggesting that the nucleotide sequences flanking the *bla*$_{\text{CTX-M}}$ genes may help resolve ARG transmission pathways.

The present investigation aims to compare the ESBL phenotypes of UPEC strains from community infections with commensal strains of *E. coli*. In addition, to characterize the genetic environments of bla CTX-M allelic variants (*bla*$_{\text{CTX-M-27,}}$ *bla*$_{\text{CTX-M-55}}$ and *bla*$_{\text{CTX-M-65}}$) by whole genome sequencing (WGS) of UPEC strains from community infections and compare them with the same *bla*$_{\text{CTX-M}}$ allelic variants in other parts of the world.

Keywords: commensal *E. coli*, UPEC, ITU, selective pressure, ESBL, *bla*$_{\text{CTX-M}}$, HGT, MGE.

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General Introduction

Antimicrobial resistance (AMR) is one of the greatest threats to public health and global development, jeopardizing many of the advances of modern medicine (WHO, 2016). Infections caused by resistant bacteria hamper medical treatments, leading to higher morbidity rates, longer hospital stays, and increased costs for healthcare systems (CDC, 2019). In 2019, infections caused by antimicrobial-resistant bacteria caused approximately 1.27 million deaths (Murray et al., 2022a), and it is estimated that if left unchecked, these infections could cause up to 10 million deaths annually by 2050 (O'Neill, 2016).

AMR is a public health problem that has been exacerbated by the selective pressure created by the use of antibiotics in healthcare, agriculture, and the environment (Alvarez-Uria et al., 2016; Holmes et al., 2016). In the medical field, overuse, inappropriate prescribing, or unnecessary prophylactic use of antimicrobials are significant factors in the development of AMR (FDA, 2019; Ventola, 2015). In agriculture, using antimicrobials to promote growth and prevent infection in healthy animals can select resistant bacteria that can be transmitted to humans through the food chain (Marshall & Levy, 2011). In the environment, antibiotic residues from medical, animal, and industrial waste create reservoirs of resistance where bacteria adapt and pass on resistance genes to other bacteria (UNEP, 2023)(Wellington et al., 2013). On the other hand, the lack of strict regulatory systems also facilitates the unmonitored use of antibiotics, which increases the spread of resistance, especially in countries with free access to antibiotics (Getahun et al., 2020; O'Neill, 2016).

Among the bacteria of most significant concern are those that cause common infections, such as *Escherichia coli* and *Staphylococcus aureus*, which are increasingly resistant to first-line

treatments (CDC, 2019). Currently, *E. coli* is used as a sentinel bacterium for antimicrobial resistance surveillance (Nyirabahizi et al., 2020; Tadesse et al., 2012b), because it occurs in a wide range of hosts, it rapidly acquires resistance genes via mobile genetic elements (MGE) and transfers them to other pathogens (acting as a reservoir of antimicrobial resistance genes). It is considered an indicator of selective pressure for antibiotic use in food animals (Aarestrup et al., 1998), and is a possible predictor of resistance development in pathogenic bacteria in meat or animal products (Tadesse et al., 2012b).

E. coli is a highly diverse bacterium encompassing both commensal strains, which are integral to the normal gut microbiota, and uropathogenic *E. coli* (UPEC), the leading cause of urinary tract infections (UTIs). Commensal strains are generally non-pathogenic, although they appear to share many of the virulence genes found in UPEC, such as adhesins, hemolysins, and iron acquisition systems such as siderophores and iron receptors (Asadi Karam et al., 2018a) (Bunduki et al., 2021).

Phylogenetically, UPEC is predominantly classified within the B2 and D phylogroups, which are linked to higher frequency of virulent phenotypes. At the same time, commensal strains are primarily associated with the A and B1 groups, known for their lower pathogenic potential (Derakhshan et al., 2022a). This genetic divergence highlights the functional specialization of *E. coli* strains, allowing them to evolve as intestinal symbionts or as opportunistic pathogens in extraintestinal environments.

Antimicrobial resistance in *E. coli* has shown an increasing trend in recent decades, especially concerning multidrug-resistant isolates (resistance to more than three types of antimicrobials). A study conducted in the USA by Tadesse *et al.* (2012b) shows an increase in multidrug-resistant strains from 7.2% in 1950 to 63.6% in the 2000s. It also shows a high resistance

profile to drugs that have been on the market since 1936, such as tetracyclines, sulfonamides, aminoglycosides, and penicillins. However, antibiotics introduced since 1980, such as combining beta-lactams with inhibitors, third-generation cephalosporins (3GC), and fluoroquinolones, showed a much lower resistance profile.

Resistance to 3GC emerged in Germany in 1983 in *Klebsiella pneumoniae* strains resistant to third-generation cephalosporins such as cefotaxime (Cantón et al., 2007). However, following the release of the 3GC patent in the late 1990s, resistant strains shifted from almost exclusively hospital-acquired infections to community-acquired infections (Pitout et al., 2005; Woerther et al., 2013). Currently, the rise of 3GC resistance in *E. coli* is a growing public health concern due to the emergence of extended-spectrum beta-lactamases (ESBLs). These enzymes allow these bacteria to inactivate modern antibiotics such as ceftriaxone and ceftiofur. In particular, CTX-M enzymes have been the most widely used ESBLs since 2000 (Woerther et al., 2013), and have played a key role in *E. coli* resistance, particularly in UTIs and sepsis in community and hospital settings (Bevan et al., 2017). CTX-M are a group of enzymes derived from *Kluyvera* species, an environmental bacterium (Humeniuk et al., 2002). The transfer of resistance genes from *Kluyvera* to other Gram-negative pathogens through mobile genetic elements (MGE) has been decisive in the spread of CTX-M (Cantón & Coque, 2006). Likewise, using beta-lactam antibiotics has generated a positive selective pressure, generating more than 200 strains of this enzyme.

MGEs, such as insertion sequences (IS), transposons, and integrons, enable the capture, transfer, and expression of resistance genes between bacteria, accelerating their spread in clinical and community settings. Studies show that certain IS, such as IS*Ecp1* and IS*26*, facilitate the mobilization of the $bla_{\text{CTX-M}}$ gene on to plasmids and other mobile genetic elements, promoting

their spread among Gram-negative bacteria (Bonnet, 2004; Cantón et al., 2012a; Salinas et al., 2023).

Overall, our hypothesis is divided into two parts: 1) UPEC strains diverged from *E. coli* as a distinct group some time ago, and therefore, their ESBL production rate should be higher than that of commensal *E. coli* strains. And 2) that the flanking sequences forming a genetic environment with the same ISs are essential for the mobilization of $bla_{\text{CTX-M}}$ genes between different plasmids and that this could be used to detect potential spillover of these genes between bacterial populations from various geographical regions.

Therefore, the present investigation aims to compare the ESBL phenotypes of UPEC strains from community infections with commensal strains of *E. coli*. In addition, we characterized genetic environments of allelic variants $bla_{\text{CTX-M}}$ ($bla_{\text{CTX-M-27}}$, $bla_{\text{CTX-M-55}}$ and $bla_{\text{CTX-M-65}}$) by whole genome sequencing (WGS) of UPEC strains from community infections. We compared them with the same allelic variants of with the same $bla_{\text{CTX-M}}$ allelic variants in other parts of the world.

The results of this research are presented in two chapters:

- **Chapter 2:** Comparative Analysis of ESBL Phenotypes and Antimicrobial Resistance in Uropathogenic and Commensal *Escherichia coli*
- **Chapter 3:** Tracking Antimicrobial Resistance transmission through $bla_{CTX\text{-}M}$ Genes and Transposable Elements in Uropathogenic and Commensal *Escherichia coli*

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CHAPTER II

Comparative Analysis of ESBL Phenotypes and Antimicrobial Resistance in Uropathogenic and Commensal *Escherichia coli*

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Escherichia coli is a genetically versatile organism that easily adapts to different environments and can acquire antimicrobial resistance and virulence genes. It is thought that horizontal gene transfer and epistatic interactions contribute to the ability of commensal strains to develop resistance and possibly evolve into UPECs. This study compared the ESBL phenotype in UPECs and commensal E. coli strains isolated from humans during the same period. Among 1,739 *E. coli* isolates, 17.12% of UPEC strains and 5.33% of commensal strains were resistant to third-generation cephalosporins and sulfonamides, whereas UPECs showed higher resistance to multiple antibiotics. UPECs have a significantly higher prevalence of ESBL-producing strains and antimicrobial resistance than commensal strains, reflecting a higher selective pressure.
Keywords: UPEC, commensal, selective pressure, ESBL.

1. INTRODUCTION

Urinary tract infections (UTI) caused by uropathogenic *Escherichia coli* (UPEC) are one of the most common bacterial infections worldwide (Whelan et al., 2023). *E. coli* is a versatile bacterium that includes both pathogenic and commensal strains, UPECs, which are capable of both colonizing the gastrointestinal tract and, subsequently, the urinary tract (Bien et al., 2012) and causing urinary tract infections (Derakhshan et al., 2022b). Virulence factors allow UPECs to evade host defenses, adhere, invade, and persist in the urinary tract (Asadi Karam et al., 2018b). These strains are associated with infections ranging from cystitis to pyelonephritis (Derakhshan et al., 2022b).

The acquisition of genes coding for virulence is thought to play a critical role in the evolution of UPEC strains. However, it has been found that many commensal strains have these virulence genes (Sarowska et al., 2019), which suggests that epistatic interactions may be responsible for UPEC phenotype (Bien et al., 2012). Whether and how frequently commensal *E. coli* can become UPEC remains unanswered.

On the other hand, antimicrobial resistance is partly due to the high selective pressure caused by the use and abuse of antibiotics in hospitals and the community (Critchley et al., 2019). Commensal *E. coli* strains may serve as reservoirs for resistance genes that can be transferred to pathogenic strains, such as UPEC (Kim et al., 2017; Tawfick et al., 2022). Indeed, commensal strains that acquire antibiotic resistance can transfer these genes to UPEC and other pathogenic strains, facilitating the spread of multi-resistant strains in the community and in clinical settings. These genes are present in mobile genetic elements (MGE), including plasmids, transposons, and integrons, which facilitate horizontal gene transfer (HGT) between commensal and pathogenic bacteria. In the case of UPECs, the acquisition of these MGEs has conferred a significant evolutionary advantage, facilitating the emergence of multi-resistant strains (Pitout & Finn, 2020). Antimicrobial resistance to older antibiotics (such as ampicillin, tetracycline, chloramphenicol, and sulfonamides) is widespread among E. coli populations. It does not significantly differ between strains under selective pressure and those without. In contrast, resistance to newer antimicrobials, including third-generation cephalosporins, carbapenems, and colistin, is more prevalent in bacteria subjected to antibiotic pressure (Loayza et al., 2020).

If UPEC strains diverged as a distinct group of *E. coli* some time ago, their rate of ESBL production should be higher than that of commensal strains. Unlike commensal *E. coli*, UPEC and extraintestinal pathogenic *E. coli* (ExPEC) strains may co-express antimicrobial resistance and virulence traits due to differential selective pressures. For instance, strains belonging to sequence type ST131 are known ESBL producers and are frequently associated with urinary and other extraintestinal infections (Loayza et al., 2020). Conversely, if UPEC strains are continuously emerging from the commensal *E. coli* population, both UPEC and commensal strains likely exhibit similar levels of antimicrobial resistance. This study compared the ESBL phenotypes of UPEC strains from community infections with those of commensal *E. coli* strains isolated from humans during the same period.

2. METHODS

2.1.Escherichia coli isolates

1739 *Escherichia coli* isolates were collected from October 2018 to December 2019. The UPECs were obtained by analyzing urine samples from patients suspected of having a UTI. These samples were submitted to the Clinical Microbiology Laboratory (LABOMIC) at San Francisco de Quito University (USFQ). The urine samples came from people from different parishes in the city of Quito, Ecuador. In contrast, the commensal strains came from healthy children's fecal samples in semirural communities explicitly located in the parishes close to Quito's airport (Calderón et al., 2022). These samples were collected also in 2018 and 2019 (Calderón et al., 2022). In clinical samples, TBX CHROMagarTM was used to detect E. coli colonies. Each isolate was stored in cryovials containing Brain Heart Infusion (BHI) medium + glycerol (25%), which were subsequently incubated at 37°C for 18 hours and stored at - 80°C.

2.2.Identification of ESBL-producing strains

Strains were reactivated on nutrient agar supplemented with ceftriaxone (CRO; 2µg/ml) and incubated at 37°C for 18 - 24 hours. Beta-lactamase-producing strains were detected by the double-disc synergy technique using Mueller-Hinton agar and inoculating a bacterial suspension with turbidity adjusted to a McFarland standard of 0.5. Discs were used with third generation cephalosporins, alone and in combination with clavulanic acid; cefotaxime (CTX; 30 µg), cefotaxime + clavulanic acid (30/10 µg) and ceftazidime (CAZ; 30 µg), ceftazidime + clavulanic acid (30/10 µg) (CLSI, 2024).

2.3.Antimicrobial susceptibility tests

Using the Kirby Bauer technique (disc diffusion on Mueller-Hinton agar) the antimicrobial susceptibility profile of the strains was determined using the following antibiotic discs: cefotaxime (CTX; 30 µg), ceftazidime (CAZ; 30 µg), cefazolin (CZ; 30 µg), ciprofloxacin (CIP; 5 µg), trimethoprim-sulfamethoxazole (SXT; 1.25/23.75 µg), imipenem (IMP; 10 µg) and amoxicillin + clavulanic acid (AMC; 20/10 µg). Resistance or susceptibility was determined based on Clinical and Laboratory Standards Institute guidelines (CLSI, 2024).

2.4.Statistical analysis

To determine statistically significant differences between the antimicrobial resistance profile to the antimicrobial of the strains based on their origin (uropathogenic or commensal), a Fisher test was used. Data were considered statistically significant with a $p \leq 0.05$. All statistical analyses and graphs were performed in GraphPad Prism 10.

3. RESULTS

A total of 1,739 *E. coli* isolates were analyzed, of which 1,139 (65.50%) were UPECs, while 600 (34.50%) were of commensal origin. Among the isolates, 195 UPECs (17.12%) and 32 commensal strains (5.33%) were resistant to third-generation cephalosporins. 193 UPECs (16.94%) were identified as ESBL, while 29 commensal strains (4.83%) were ESBL producers. Table S1 (supplementary table) shows other antimicrobial resistances in UPECs and commensals.

Table 1 shows the antimicrobial characterization of E. coli strains ESBL producers. It is observed that the highest percentages of resistance are presented with 1st generation cephalosporins such as cefazolin (CZ), 97.93% and 100%; together with 3rd generation cephalosporins such as cefotaxime (CTX), 91.19% and 100%; sulfonamides such as sulfamethoxazole/trimethoprim (SXT), 69.95% and 96.55%; and with fluoroquinolones such as ciprofloxacin (CIP), 82.38% and 55.17%, corresponding to UPEC and commensal strains, respectively. On the other hand, it is observed that most strains are sensitive to carbapenems such as imipenem (IMP), with 96.37% for uropathogenic strains and 100% for commensal strains.

Table 1. Resistance rates of *Escherichia coli* strains, producers of ESBL, differentiated on the basis of strain origin (uropathogenic or commensal). Numbers in parenthesis are percentages.

Antimicrobial	**Cefotaxime (CTX)**	**Ceftazidime (CAZ)**	**Amoxicilin+ clavulanic acid (AMC)**	**Cefazoline (CZ)**	**Ciprofloxacin (CIP)**	**Imipenem (IMP)**	**Sulfamethoxazole/ Trimethoprim (SXT)**
Uropathogenic (n=193)	176(91.19)	107(55.44)	100(51.81)	189(97.93)	159(82.38)	7(3.63)	135(69.95)
Commensal (n=29)	29(100)	5(17.24)	10(34.48)	29(100)	16(55.17)	0	28(96.55)

Statistical analysis showed statistical significant differences between UPEC and commensal in the resistance to cefotaxime (CTX), ceftazidime (CAZ), cefazolin (CZ), ciprofloxacin (CIP), Amoxicillin + Clavulanic acid and Sulfamethoxazole/Trimethoprim and ESBL phenotype (Table 2) (Figure 1). On the other hand, it was found that there are no significant differences with resistance to imipenem (IMP).

Table 2. Percentages of phenotypic resistance of commensal E. coli strains vs. UPECs with their p value, obtained by the Fisher test. Values in parenthesis are percentages.

Antimicrobial	**Commensal n=600**	**Uropathogenic n=1139**	**p - value**
Cefotaxime (CTX)	29(4.83)	176(15.45)	<0.0001
Ceftazidime (CAZ)	5(0.83)	107(6.15)	<0.0001
Amoxicilin + clavulanic acid (AMC)	10(1.67)	100(8.78)	<0.0001
Cefazoline (CZ)	29(4.83)	189(16.59)	<0.0001
Ciprofloxacin (CIP)	16(2.67)	159(13.96)	<0.0001
Imipenem (IMP)	0(0)	7(0.61)	0.1034
Sulfamethoxazol/Trimethoprim (SXT)	28(4.67)	135 (11.85)	<0.0001
ESBL	29(4.83)	193(16.94)	<0.0001

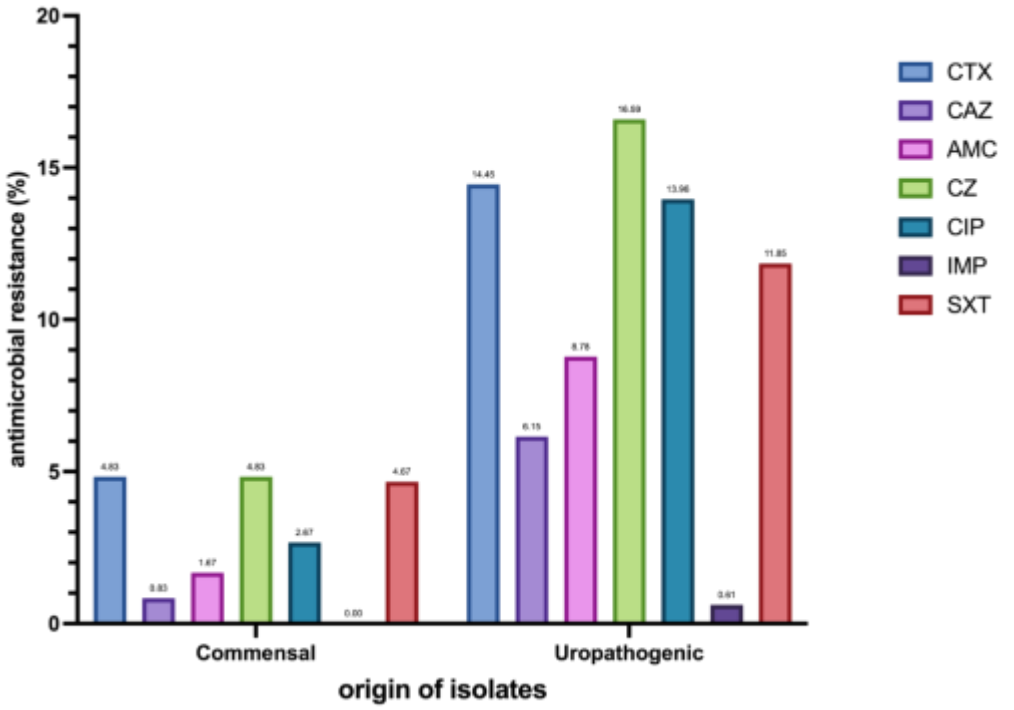

Figure 1. Resistance profile of Cefotaxime (CTX), Ceftazidime (CAZ), Cefazoline (CZ), Ciprofloxacine (CIP), Amoxicilin + clavulanic acid (AMC), imipemen (IMP) and Sulfamethoxazol/Trimethoprim (SXT); of uropathogenic vs. commensal E. coli strains.

4. DISCUSSION

In this study we observed that UPECs show a significantly higher prevalence of ESBL production (16.94%) than commensal strains (4.83%). Additionally, resistance to other antimicrobial classes—including sulfonamides, fluoroquinolones, and carbapenems—was markedly higher in UPECs than in commensals. Similar findings were reported in studies from China and Iran, where 52.3% of UPEC strains in China were ESBL producers compared to only 8.5% of commensal strains (Asadi Karam et al., 2018b; Qin et al., 2013). These results suggest that UPEC strains experience more substantial selective pressure from antibiotic use than commensal strains. Furthermore, the data imply that UPECs may represent a distinct lineage of *E. coli* that has evolved to cause urinary infections, diverging from commensal strains over time. This divergence suggests that UPECs may not frequently emerge from the general *E. coli* commensal population. Regarding the antimicrobial resistance profile, Table 1 shows a high resistance rate to cephalosporins and fluoroquinolones for both UPECs and commensal strains.

Our study also reveals a higher rate of sulfonamide resistance in commensal strains than in UPECs, and other studies obtained similar results with resistance rates above 75% for commensal strains(Tawfick et al., 2022) and 60% for UPECs(Derakhshan et al., 2022b). Higher commensal rates may be an artifact, as the resistance to this antimicrobial is widely distributed in commensal and pathogenic *E. coli* (Loayza et al., 2020). There was a low presence of carbapenem resistance in UPEC strains (3.63%). Carbapenems are the last available therapeutic resource(Asadi Karam et

al., 2018b; Sheu et al., 2019) UPECs are highly sensitive to carbapenems (Andrade et al., 2006; Derakhshan et al., 2022b; Lee et al., 2018).

One limitation of this study may be that antibiotics are sold over the counter in Ecuador, and many people with UTIs may use antimicrobials before consulting with a healthcare provider. This problem may select preferentially antimicrobial-resistant UPECS. However, the ESBL rates in UPECs in the United States (15.7%) were similar to ESBL carriage in Ecuadorian UPECs, which may indicate that purchasing antibiotics over the counter is not affecting this analysis.

In conclusion, UPEC strains have a higher prevalence of extended-spectrum beta-lactamase (ESBL) resistance than commensal strains, which is attributed to the higher selective pressure they are exposed to in clinical settings such as hospitals and UTI treatment. These environments favor the spread of resistant strains because they are more likely to acquire resistance genes from MGE, which are common in hospital environments and can be transferred between bacteria via HGT. This phenomenon highlights the need for more effective strategies to control antimicrobial resistance and limit the indiscriminate use of antibiotics. Finally, although carbapenems remain effective in most cases, the emergence of carbapenem-resistant UPEC strains represents a growing challenge for healthcare systems, as these antibiotics are the last available therapeutic resource for infections caused by multidrug-resistant bacteria.

Author's contributions

Denyss Guilcazo: samples reactivation and analysis, obtained metadata, selection of isolates, ESBL test, antibiotic susceptibility test, statical analysis, writing the manuscript. Lazaro Lopez: ESBL test, antibiotic susceptibility test and statistical analysis. Diana Calderon: collection of commensal samples and obtained metadata. Katherine Vasquez: collection of uropathogenic samples and obtained metadata. Cristina Chavez: collection of uropathogenic samples, obtained metadata and conceptualization. L Price: writing, reviewing and editing the manuscript. Jay Graham: writing, reviewing and editing the manuscript. Joseph Eisenberg: writing, reviewing and editing the manuscript. Gabriel Trueba: writing the manuscript, conceptualization, direction of the work in the laboratory.

Disclosure Statement

The authors declare that there are no conflicts of interest in relation to this study.

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CHAPTER III

Tracking Antimicrobial Resistance transmission through *bla*$_{CTX-M}$ Genes and Transposable Elements in Uropathogenic and Commensal *Escherichia coli*

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ABSTRACT

Aim: To investigate the nucleotide sequences associated with transposable elements carrying *bla*$_{CTX-M}$ allelic variants as potential markers for the transmission of antimicrobial resistance genes between domestic animals, humans and the environment.

Materials & Methods: We studied the nucleotide sequences of *bla*$_{CTX-M}$ allelic variants from commensal *E. coli* and uropathogenic *E. coli* (UPEC) from 9 clinics in Quito, Ecuador.

Results: The Ecuadorian commensal and UPEC displayed identical nucleotide sequences surrounding the *bla*$_{CTX-M}$ gene and the synteny was similar to those found in other parts of the world; however phylogenetic analysis indicated that the genetic environments in Ecuadorian isolates were unique.

Conclusion: These findings suggest that the nucleotide sequences flanking the *bla*$_{CTX-M}$ genes may be useful for resolving ARG transmission pathways.

PLAIN LANGUAGE SUMMARY

Bacterial transposable elements (TEs) capture random segments of DNA including those carrying genes coding adaptive functions, such as antimicrobial resistance. TEs containing antimicrobial resistance genes (ARGs) move among different plasmids and between plasmids and chromosomes, complicating efforts to resolve ARG transmission patterns among vertebrate hosts. We studied *bla*$_{CTX-M}$ allelic variants from commensal *E. coli* and uropathogenic *E. coli* (UPEC) from 9 clinics in Quito, Ecuador. Both commensal and UPEC displayed identical *bla*$_{CTX-M}$ neighboring nucleotide sequences. These configurations were similar to those found in other parts of the world; however phylogenetic analysis indicated that the genetic environments in Ecuadorian isolates were

unique. These findings suggest that the nucleotide sequences flanking the *bla*$_{\text{CTX-M}}$ genes may be useful for resolving ARG transmission pathways.

1. Background

Antimicrobial resistance (AMR) in bacteria was responsible for 1.27 million deaths worldwide in 2019 and the trend is increasing (Murray et al., 2022b). The AMR crisis has been fueled by the use and overuse of antimicrobials in both human medicine and food-animal production, augmenting the ability of bacteria to transmit antimicrobial resistance genes. To effectively control AMR, it is critical to understand the sources and transmission mechanisms of drug resistant bacteria and antimicrobial resistance genes (ARGs). There has been ongoing debate regarding the primary source of antimicrobial resistant bacteria and ARGs for the human population, with some experts implicating food-animal production, while others argue that food animals play a very limited role (Day et al., 2019; Ludden et al., 2019; Salinas et al., 2021).

The difficulties of determining sources and sinks of ARGs in bacterial populations within the environment could be attributed to the large diversity of: 1) antimicrobial resistance genes; 2) mobile genetic elements (MGEs) that carry and move these genes; and 3) drug resistant bacterial strains. This is further complicated by our lack of understanding about how these elements interplay. Most ARGs are transmitted among bacteria through horizontal gene transfer, where plasmids or bacteriophages carrying ARGs or naked DNA mobilize these genes to genetically related and unrelated bacteria (Partridge et al., 2018). Some of the most active carriers of ARGs are transposable elements. Transposable elements (TEs) are among the most active carriers of ARGs. These mobile genetic elements facilitate the transfer of ARGs across diverse genetic platforms, including plasmids, chromosomes, and bacteriophages (Partridge et al., 2018). In some cases, ARGs have been disseminating for a very long time (Tadesse et al., 2012a) and are probably more homogenously distributed in *E. coli* populations than ARGs that began to disseminate in the 1990s or 2000s, such as *bla*$_{\text{CTX-M}}$ or the *mcr-1* (Cantón et al., 2012b; Liu et al., 2016; Poirel et al., 2008). In this case, identifying ARGs that have recently started to disseminate may be better indicators of antibiotic selective pressure and the potential sources of these genes in an ecosystem (de Been et al., 2014; Trung et al., 2017).

In communities near Quito, Ecuador, we have identified several distinct *E. coli* clones, each carrying the same *bla*$_{\text{CTX-M}}$ allelic variant with identical flanking sequences that are consistently genetic environment ed by the same TEs, but located on different plasmids or chromosomes (Salinas et al., 2024). This is a strong indication that these TEs are central to mobilizing *bla*$_{\text{CTX-M}}$ genes moving between different plasmids. Thus, prospectively analyzing *bla*$_{\text{CTX-M}}$ genes alongside associated sequences linked to TEs, could enhance data stratification, enabling the detection of potential spillover of these genes between bacterial populations of different ecosystems. In this study, we compared common *bla*$_{\text{CTX-M}}$ allelic variants *bla*$_{\text{CTX-M-27,}}$ *bla*$_{\text{CTX-M-55}}$ and *bla*$_{\text{CTX-M-65}}$ and their genetic environment within commensal *E. coli* and uropathogenic *E. coli* (UPEC) from Quito and compared them with *E. coli* with the same *bla*$_{\text{CTX-M}}$ allelic variants in other parts of the world.

2. Materials & Methods

2.1. *E. coli* isolates and DNA extraction

Ceftriaxone resistant *Escherichia coli* isolates (n=149) from urinary tract infections were collected over a period from May 2014 to May 2015 in the Diagnostic Microbiology Laboratory of the Microbiology Institute, Universidad San Francisco de Quito. Each bacterial isolate was cultured on Chromocult agar® to identify *E. coli* through β-D-glucuronidase activity and was stored at -80°C in Brain Heart Infusion (BHI) medium, supplemented with 20% glycerol. A single bacterial colony was selected and inoculated into 3 ml of trypticase soy broth (TSB) at 37°C for 18–24 hours. DNA from the bacterial isolates was extracted with the DNeasy Blood & Tissue kit (QIAGEN) according to the manufacturer's protocol. The concentrations and quality of bacterial DNA were measured using the NanoDrop Microvolume Spectrophotometer. All the protocols were approved by the USFQ bioethics committee (code 2015-167T). We also used 19 nucleotide sequences from ceftriaxone resistant commensal *E. coli* obtained in a previous study in the same communities (Salinas et al., 2021).

2.2. Whole genome sequencing

We used Illumina NextSeq 2000 and assembled the UPEC genomes using SPAdes v3.15.5 (https://ablab.github.io/spades/index.html). Contigs were joined into scaffold with a fixed gap size of 100 bp. (Prjibelski et al., 2020). The nucleotide sequences were submitted to GenBank and the accession number is SUB14757366.

2.3. Strain genotyping

The assembled UPEC genomes were analyzed using Multi-Locus Sequence Typing (MLST) (version 2.0.9) (Larsen et al., 2012) an online tool provided by the Center for Genomic Epidemiology (CGE). This tool identifies and classifies each bacterial isolate based on the sequences of seven housekeeping genes (*adk, fumC, gyrB, icd, mdh, purA and recA*). These genes were evaluated for their identity and coverage to assign a specific sequence type (ST) to each bacterial isolate (Supplementary information Table S1).

2.4. Antibiotic resistance genes

Antimicrobial resistance genes were identified using the ABRicate tool (version 1.0.1) with the Resfinder, CARD y NCBI databases. Each sequence carrying the $bla_{\text{CTX-M}}$ variant was analyzed and trimmed using Geneious Prime (version 2024.0.5) and Unipro UGENE 50.0 (Okonechnikov et al., 2012). Other ARGs are shown in the supplementary information Table S2.

2.5. Nucleotide sequences of $bla_{\text{CTX-M}}$ variants

Each sequence carrying the $bla_{\text{CTX-M}}$ variant (in UPECs) was annotated using the following tools: Prokka (version 1.14.6) (Seemann, 2014), Prokaryotic Genome Annotation Pipeline (PGAP) and the Pathosystems Resource Integration Center (PATRIC) (Wattam et al., 2017), and confirmed with BLAST.

The GenBank (gbk) files acquired were manually modified based on the data obtained from annotation tools for the identification of mobile genetic elements such as: MobileElementFinder (version 1.0.6) (Durrant et al., 2020), ISEScan (version 1.7.2.3) (Xie & Tang, 2017) and ISfinder (Siguier et al., 2006). A comparison of the genomic structure was performed with the nucleotide

sequences of bacterial isolates that had the same *bla*$_{\text{CTX-M}}$ allelic variant using Easyfig (version 3.0.0) (Sullivan et al., 2011).

2.6 Phylogenetic Analysis of Genetic Environment

Nucleotide sequences from the genetic environments (i.e. flanking region) of *bla*$_{\text{CTX-M-27}}$, *bla*$_{\text{CTX-M-55}}$, and *bla*$_{\text{CTX-M-65}}$ within the Ecuador sequences were subjected to BLAST and the highest hits were retrieved and used to construct phylogenetic trees. To construct the trees, we used Mega11 software and Maximum likelihood. We also analyzed the several configurations of these genes found around the world.

3. Results

3.1.Genetic environment of *bla*$_{\text{CTX-M}}$ genes

The genetic environment of the *bla*$_{\text{CTX-M-65}}$ allelic variant was formed by IS*26*-*flipA*-HP (hypothetical protein)-IS*Ecp1*-*bla*$_{\text{CTX-M-65}}$-IS*26* in 5 commensal *E. coli*; IS*26*-*flipA*-HP (hypothetical protein) -IS*Ecp1*-*bla*$_{\text{CTX-M-65}}$-IS*102*-*tonB*-PAS (methyl accepting protein sensor-IS*26*, in 4 commensal *E. coli* and 10 UPEC isolates; although the commensals had one additional nucleotide between IS*26* and *flipA* (Figure 3). The allelic variant *bla*$_{\text{CTX-M-55}}$ shows 3 different genetic environments: IS*26*-IS*Ecp1*- *bla*$_{\text{CTX-M-55}}$-*wbuC*-TnA-*bla*$_{\text{TEM}}$ *-IS26 in 9 commensal E. coli 4 UPECs;* IS*26*-IS*Ecp1*- *bla*$_{\text{CTX-M-55}}$-*wbuC*-TnA-*bla*$_{\text{TEM}}$ *-HP (hypothetical protein) in 1 UPEC; IS1380-HP- bla*$_{\text{CTX-M-55}}$-*wbuC* (fused to *yagA*)-HP in 2 UPECs. The *wbuC* gene encodes a cupin fold of metalloproteinase. The *yagA* gene encodes an integrase core domain-containing protein (Figure 2). The allelic variant *bla*$_{\text{CTX-M-27}}$, is found in 4.96% (n=7) of the strains, IS26-ISEcp1-*bla*$_{\text{CTX-M-27}}$-IS*102*-tonB-DUFF4158-IS*26* in 2 commensals and 4 UPECs (contigs of 2 additional UPECs showed the same configuration but incomplete due to the size of the contig); IS26-ISEcp1-*bla*$_{\text{CTX-M-27}}$-IS102-tonB-DUFF4158-phage integrase-TEM-IS6 in one UPEC. The DUF4158 is a gene coding for phage integrase (Figure 1).

3.2.Phylogenetic analysis of the *bla*$_{\text{CTX-M}}$ genetic environments

Nucleotide sequence alignments of Ecuadorian configurations of the genetic environments for the 3 *bla*$_{\text{CTX-M}}$ allelic variants showed 99-100 identity (0-4 SNPs) within each variant. A phylogenetic analysis of the Ecuadorian genetic environments with highest hits in Blast and identical configuration (synteny), revealed that sequences from different countries clustered in different groups (Figure 4).

4. Discussion

In this study we observed that nucleotide sequences of the genetic environment (comprising *bla*$_{\text{CTX-M-55,}}$ *bla*$_{\text{CTX-M-65}}$, and *bla*$_{\text{CTX-M-27,}}$ TEs and intervening sequences) are most often identical in Ecuadorian isolates but different from sequences from other countries showing identical synteny (Yang et al., 2023) (Figure 1). This similarity was also found in Korean isolates that demonstrated a similar genetic environment of *bla*$_{\text{CTX-M-27}}$ (Figure 3A). The results of this study suggest that

transposable elements carrying *bla*$_{CTX\text{-}M\text{-}55}$, *bla*$_{CTX\text{-}M\text{-}65}$, and *bla*$_{CTX\text{-}M\text{-}27}$ genes, are probably mobilizing frequently among different *E. coli* strains in different communities across the globe. The nucleotide sequences of transposable elements (and associated genetic sequences), even those showing identical configuration (synteny), seem to be geographically structured (Figure 3) and have a strong potential to be used as epidemiological tools to determine spillover of these important ARGs from different biotic or abiotic components of an ecosystem. Additionally, there were very different configurations in other countries.

The similarity of the genetic environment *bla*$_{CTX\text{-}M\text{-}55}$, *bla*$_{CTX\text{-}M\text{-}65}$, and *bla*$_{CTX\text{-}M\text{-}27}$ in *E. coli* commensal isolates from humans and from domestic animals (Sullivan et al., 2011) were identical to those found in UPECs in the same communities of Quito, Ecuador. These data suggest that *bla*$_{CTX\text{-}M\text{-}55}$, *bla*$_{CTX\text{-}M\text{-}65}$, and *bla*$_{CTX\text{-}M\text{-}27}$ genes from commensal (from human or domestic animal *E. coli*) are being mobilized to UPECs in humans, or vice versa. This is a strong indication that a One Health perspective (i.e. considering human-environment-animal) is critical for understanding transmission of AMR. The presence of the same genes in a similar configuration of other bacterial species such as *Salmonella, Klebsiella, Acinetobacter, Citrobacter,* and *Proteus* suggest that these structures are being mobilized among different bacterial taxa probably by means of transposable elements (Salinas et al., 2024).

The information about the genetic environment associated with transposable elements may be useful for other clinically important AMR genes. For instance Tn*125* has been shown to mobilize *bla*$_{NDM\text{-}1}$, a gene that confers resistance to carbapenem antibiotics from the potential origin *Acinetobacter* spp. (Bontron et al., 2016); an unknown TN mobilized the *bla*$_{KPC}$ gene from the potential original environmental bacteria *Chromobacterium spp*. (Gudeta et al., 2016). IS*Epec-1* mobilized the *mcr*-1 gene (causing resistance to colistin) from the potential original commensal bacteria *Moraxella* spp. (Kieffer et al., 2017). IS*Epec-1* was shown to likely mobilize *bla*$_{CTX\text{-}M}$ genes from *Kluyvera spp.*, (Lartigue et al., 2006), and the transposon IS*1999* may have moved the *bla*$_{OXA\text{-}48}$ gene from the environmental bacteria *Shewanella* spp. (Tacão et al., 2018). Once arriving at a new bacterial species, other transposable elements, such as IS26, Tn*4401*, may take a more dominant role in mobilization (Han et al., 2024; Weber et al., 2019; Zhang et al., 2023). Finally, the use and overuse of antimicrobials likely increases the mobility of TNs (Pribis et al., 2022).

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Competing interests disclosure

The authors have no competing interests.

Writing disclosure

We use ChatGPT to improve some sentences.

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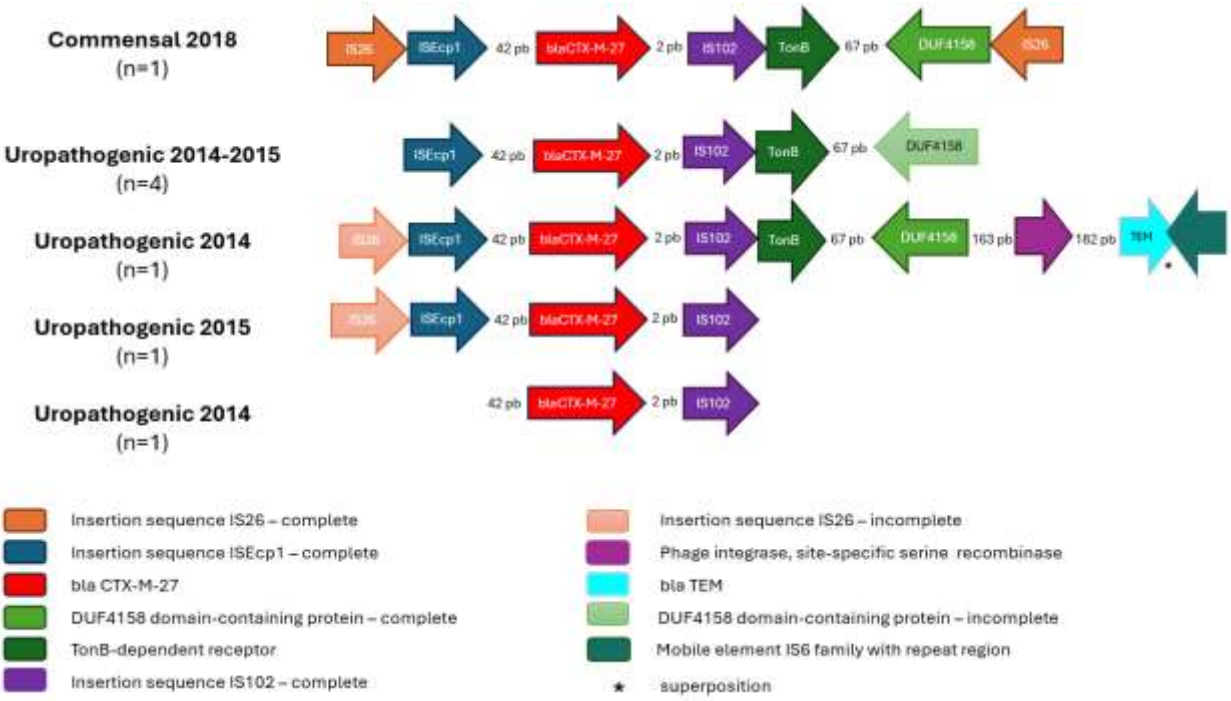

Figure 1. Genetic environment of *bla*$_{\text{CTX-M-27}}$ found in ceftriaxone resistant UPECs and commensal *E. coli* in Quito. Arrows indicate genes and transcriptional direction and numbers indicate the distance in base pairs. Tone down colors indicate incomplete genes (because of contig size).

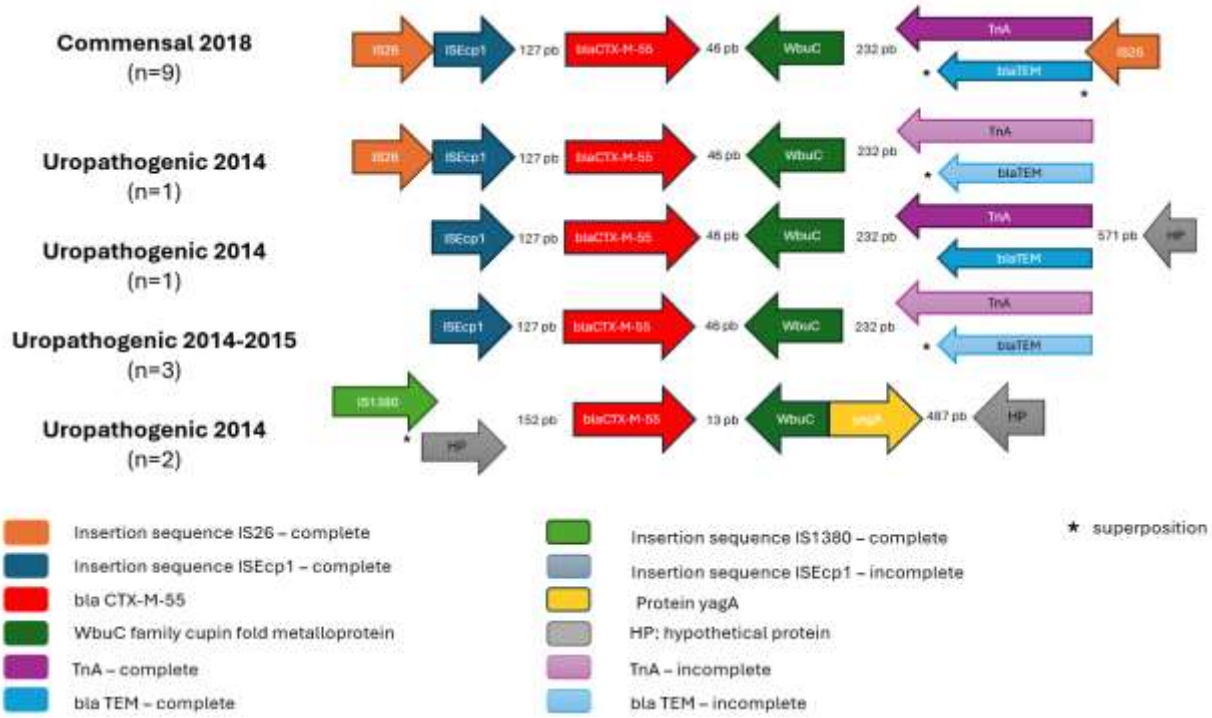

Figure 2. Genetic environment of *bla*$_{\text{CTX-M-55}}$ found in ceftriaxone resistant UPECs and commensal *E. coli* in Quito. Arrows indicate genes and transcriptional direction, and numbers indicate the distance in base pairs. Tone down colors indicate incomplete genes (because of contig size).

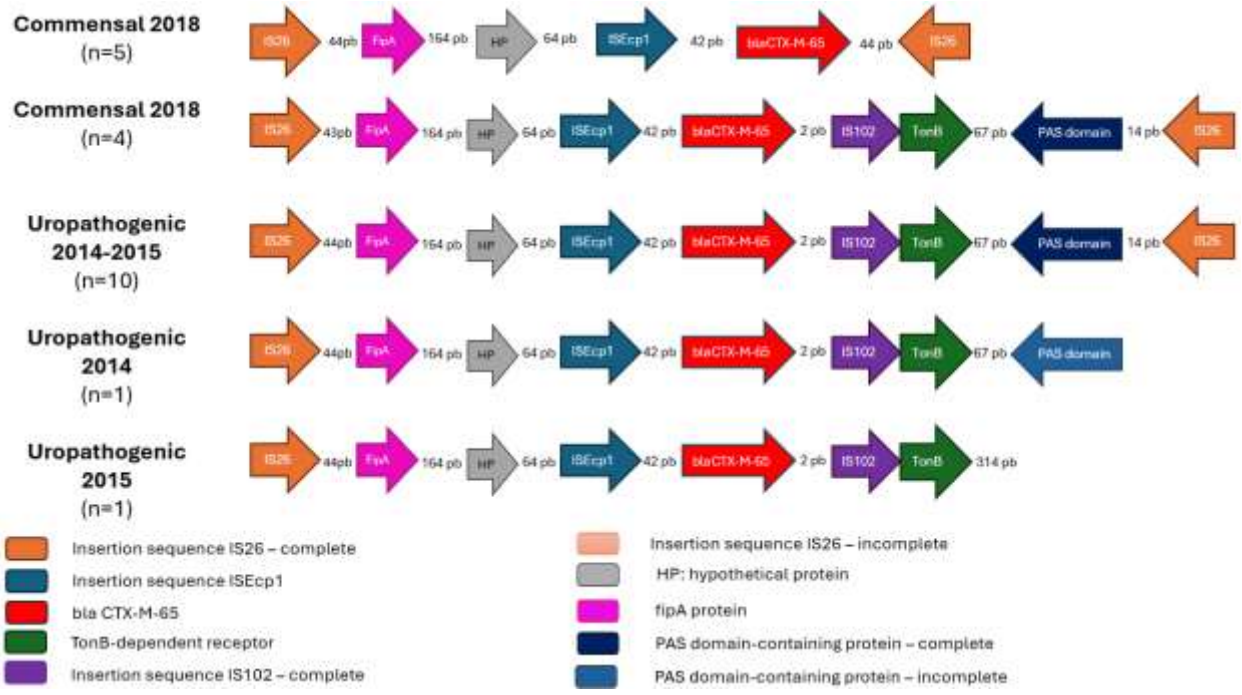

Figure 3. Genetic environment of *bla*$_{\text{CTX-M-65}}$ found in ceftriaxone resistant UPECs and commensal *E. coli* in Quito. Arrows indicate genes and transcriptional direction, numbers indicate the distance in base pairs. Toned down colors indicate the presence of incomplete genes (because of contig size).

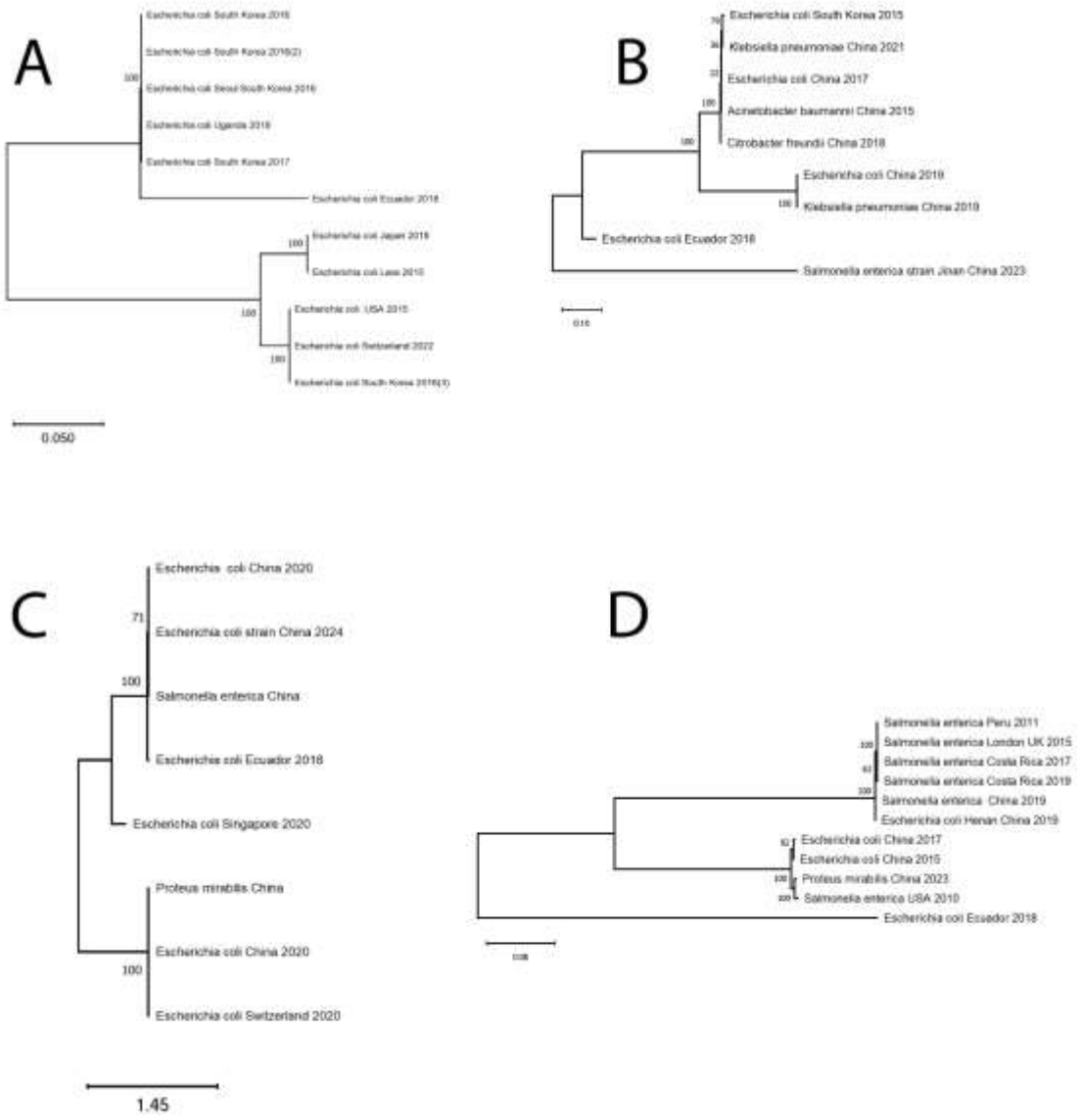

Figure 4. Phylogenetic tree using Maximum Likelihood of genetic environment of *bla*$_{\text{CTX-M}}$ genes found in Ecuadorian *E. coli* isolates and sequences from different parts of the world that showed the highest hits in blast. A) is *bla*$_{\text{CTX-M-27,}}$ B) is *bla*$_{\text{CTX-M-55,}}$ C) is *bla*$_{\text{CTX-M-65}}$ configuration IS*26*-*flipA*-HP (hypothetical protein)-IS*Ecp1*-*bla*$_{\text{CTX-M-65}}$-IS*26* and, D) *bla*$_{\text{CTX-M-65}}$ configuration IS*26*-*flipA*-HP (hypothetical protein)-IS*Ecp1*-*bla*$_{\text{CTX-M-65}}$-IS*102*-*tonB*-PAS (methyl accepting protein sensor)-IS*26*. Numbers indicate bootstrap values after 100 pseudo-replicates.

6. Supplementary information

Table S1. Variety of sequence type (ST) obtained in the analyzed bacterial isolates of Escherichia coli UPEC.

Number of isolates	Sequence type (ST)	Loci						
		adk	fumC	gyrB	icd	mdh	purA	recA
63 (44,68%)	131	53	40	47	13	36	28	29
9 (6,38%)	38	4	26	2	25	5	5	19
7 (4,96%)	44	10	11	4	8	8	8	7
6 (4,26%)	617	10	11	4	8	8	13	73
5 (3,55%)	167	10	11	4	8	8	13	2
4 (2,84%)	58	6	4	4	16	24	8	14
4 (2,84%)	648	92	4	87	96	70	58	2
4 (2,84%)	1193	14	14	10	200	17	7	10
3 (2,13%)	57	6	31	5	28	1	1	2
3 (2,13%)	117	20	45	41	43	5	32	2
3 (2,13%)	457	101	88	97	108	26	79	2
3 (2,13%)	744	10	11	135	8	8	8	2
2 (1,42%)	69	21	35	27	6	5	5	4
2 (1,42%)	1049	6	4	14	16	24	5	14
2 (1,42%)	1196	6	6	33	26	11	8	2
2 (1,42%)	1290	10	189	4	8	8	2	2
1 (0,71%)	10	10	11	4	8	8	8	2
1 (0,71%)	48	6	11	4	8	8	8	2
1 (0,71%)	73	36	24	9	13	17	11	25
1 (0,71%)	90	6	4	12	1	20	8	7
1 (0,71%)	93	6	11	4	10	7	8	6
1 (0,71%)	101	43	41	15	18	11	7	6
1 (0,71%)	155	6	4	14	16	24	8	14
1 (0,71%)	224	6	4	33	16	11	8	6
1 (0,71%)	354	85	88	78	29	59	58	62
1 (0,71%)	393	18	106	17	6	5	5	4
1 (0,71%)	394	21	35	61	52	5	5	4
1 (0,71%)	405	35	37	29	25	4	5	73
1 (0,71%)	410	6	4	12	1	20	18	7
1 (0,71%)	569	13	38	84	13	17	64	34
1 (0,71%)	770	52	116	55	101	113	40	38
1 (0,71%)	2063	6	4	4	18	9	26	7
1 (0,71%)	4204	24	11	4	8	8	8	73
1 (0,71%)	6253	565	36	207	87	67	16	288
1 (0,71%)	7358	692	37	29	25	4	5	73

Table S2. Summary of antimicrobial resistance genes found in uropathogenic Escherichia coli isolates.

number of isolates	%	genes	antibiotic to which it confers resistance
141	100	*blaEC (19,18,15,13)* *blaCMY-2* *blaSHV-5* *blaTEM-1, blaTEM-150* *blaOXA-1* *blaCTX-M* *(1,3,8,9,14,15,27,55,65)*	β-lactams
131	92,91	*aac(3)-IIe, aac(3)-IId, aac(3)-Iva, aac(6')-Ib-D181Y, aph(2'')-Ia, aph(3'')-Ia, aph(3'')-Ib, aph(3'')-Ila, aph(4)-la, aph(6)-Id, aadA1, aadA2, aadA5, aadA16, ant(2'')-la*	aminoglucosides
122	86,52	*sul1, sul2, sul3*	sulfonamides
116	82,27	*tet(A), tet(B), tet(D)*	tetracyclines
109	77,30	*drfA17, drfA14, drfA12, drfA8drfA5, drfA1*	trimetroprim
87	61,70	*mph(A), mef(B)*	macrolides
27	19,15	*fosA3, fosA6*	fosfomycin
14	9,93	*catA1, catB3*	chloramphenicol
9	6,38	*erm(B)*	MLSB (macrolides, lincosamides and streptogramins B)
9	6,38	*floR*	florfenicol
8	5,67	*cmlA1, cmlA6*	chloramphenicol
7	4,96	*qnrB19*	quinolones
3	2,13	*arr-3*	rifamycins
2	1,42	*oqxA, oqxB*	quinolones
2	1,42	*qepA4*	quinolones
2	1,42	*sat2_fam*	streptomycin
1	0,71	*ble_Tn5*	bleomycins

CHAPTER IV

General Conclusions

This research focused on the evolution of uropathogenic *Escherichia coli* and its ESBL phenotype. We first evaluated the ESBL production rate in UPEC and commensal isolates collected during the same time period. The UPEC strains showed a significantly higher ESBL production rate than the ESBL production rate of the commensal strains. These results suggest that UPECs are under stronger antimicrobial selective pressure compared to commensals. Furthermore, this may imply that UPECs may represent a distinct lineage of *E. coli*, diverging from commensals over time. And that UPECs are not continuously emerging from the commensal *E. coli* population.

Secondly, genetic environments formed with the same flanking sequences (ISs) of UPEC and commensal strains with the same allelic variant of $bla_{\text{CTX-M}}$ ($bla_{\text{CTX-M-55}}$, $bla_{\text{CTX-M-65}}$ and $bla_{\text{CTX-M-27}}$), were analyzed by WGS; and subsequently compared with other genetic environments with the same allelic variants of $bla_{\text{CTX-M}}$ from other parts of the world. The genetic configurations found in both UPEC, and commensals were similar to those found in other parts of the world, however the phylogenetic analysis of these regions was different in Ecuador as compared with similar regions in other parts of the world. These results suggest that the $bla_{\text{CTX-M}}$ genes are mobilized from commensal *E. coli* strains (from humans and domestic animals) to UPEC strains (from humans) or vice versa. On the other hand, the detection of the same genes in the same configuration but in other bacterial species suggests that these structures are being mobilized between different bacterial taxa, possibly by mobile genetic elements, such as transposons, integrons or insertion sequences. Finally, the flanking sequences of these genetic environments of the $bla_{\text{CTX-M}}$ genes may be useful in elucidating the pathways of ARG transmission.

Information on the genetic environment associated with transposable elements may be useful for other clinically important AMR genes. These findings suggest that the One Health perspective (i.e., human-environment-animal) is critical to understanding AMR transmission.

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