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A longitudinal study of dominant *E. coli* lineages and antimicrobial resistance in the gut of children living in an upper middle-income country

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Abstract

Objectives: The gastrointestinal tract constitutes a complex and diverse ecosystem. *Escherichia coli* is one of the most frequently studied and characterised species in the gut ecosystem; nevertheless, there has been little research to determine their diversity and population dynamics in the intestines of children over time. We analysed the turnover or dominant *E. coli* isolates in children faecal matter during 1 year.

Methods: In this prospective study, a fresh faecal sample was obtained from children longitudinally over one year (30 faecal samples at sampling period 1 and 22 faecal samples at sampling periods 2 and 3). From each stool sample, five *E. coli* colonies were randomly selected (n = 405 *E. coli* isolates total) in order to characterize the genotype and phenotypic antimicrobial resistance patterns.

Results: We were unable to find same *E. coli* dominant clone in faecal matter from 30 children in different sampling periods. Whole-genome sequencing of three isolates belonging to ST131 found in one child during the sampling period I and II indicated that isolates were three different ST 131 clones that carried extended-spectrum β-lactamase (ESBL) genes.

Conclusion: We found that all numerically dominant *E. coli* lineages in children's intestines were transient colonisers, and antimicrobial resistance phenotypes of these strains varied significantly over time without any apparent selective force.

Keywords

Numerically dominant strains; E. coli, longitudinal; Antimicrobial resistance; Ecuador; ST131

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Declaration of Competing Interest

None declared.

1. Introduction

Escherichia coli is a minor component of the intestinal microbiota of warm-blooded animals [1,2]. Within the human gut and other warm-blooded animals, there may be more than 16 different lineages of *E. coli* at any time [3]; each of these lineages is present in different abundances and remains in the intestine for different periods. Intestinal *E. coli* could be classified as follows: resident when the lineage remains in the intestine for months and even years [4], transient when the lineage remains for days or weeks [4], dominant when the isolates make up a large proportion (>50%) [5] of the *E. coli* cells in the intestine, and minority when the pro-portion is smaller (<10%) [3].

The genetic dynamics of the *E. coli* population in the intestine have received little attention, although some are a source of problematic infections and antimicrobial resistance genes (ARGs) [6–8]. The high abundance and the persistence of certain *E. coli* pathogenic and antimicrobial-resistant lineages in the intestinal tract have been suggested to be a critical risk factor for disease and disease treatment [8–10]. Furthermore, these lineages could affect the dissemination of ARGs since *E. coli* is a species adept at horizontal gene transfer [1,2,11] and is likely the intestinal species that can be exchanged the most between different hosts [12].

Here we screened the dominant *E. coli* strains obtained from the faeces of children less than five years of age, and we also analysed the turnover of these strains in three sampling periods (SP I, II, and III) during one year; sampling periods were approximately 3 months apart. Dominant *E. coli* strains were defined as those colonies that grew on MacConkey agar at the highest proportion [5].

2. Methods

2.1. Study location

This one-year longitudinal study was carried out in six semi-rural communities belonging to the parishes of Yaruquí, Pifo, Tumbaco, Checa, Puembo, and Tababela; all of them located near Quito, Ecuador. For household enrolment, inclusion criteria included: (i) households have a child aged six months to four years, (ii) households have a childcare provider who was over 18 years, and (iii) informed consent was provided by the primary childcare provider. Sixty-one households were enrolled at the beginning of the study, but only 26 finished the full longitudinal study.

2.2. Ethical considerations

The study protocol was approved by the Committee for Protection of Human Subjects (CPHS) and the Office for Protection of Human Subjects (OPHS) at the University of California, Berkeley (Federalwide Assurance # 6252), the Human Research Ethics Committee at the Universidad San Francisco de Quito (no. 2017–178M), and the Ministry of Public Health, Ecuador (MSPCURI000243–3).

2.3. Sample collection

We collected a single faecal sample from each child during three sampling periods (SP): from October to December of 2018 (SP I), from January to May of 2019 (SP II), and from July to December of 2019 (SP III), obtaining a total of 120 stool samples. Each time a sample was collected, the childcare provider completed a survey related to the current family lifestyle and recent exposure-related factors relevant to AMR such as domestic animal, child antimicrobial use, accessibility to water, sanitation, and hygiene (WaSH) conditions (the results of this survey was published previously [7,13].

2.4. E. coli isolation

Each faecal sample was plated on MacConkey agar and incubated at 37 °C for 18 hours. To ensure the selection of the dominant *E. coli* strains, we collected five colonies (with same morphology), which provides more than 95% likelihood of obtaining a dominant strain (comprising ≥50% of all *E. coli* colonies that grow in a Petri dish from a faecal sample) [14]. Additionally, each colony was transferred to Chromocult® coliform agar for the identification of *E. coli* through its β-D-glucuronidase activity. Those strains were incubated in Brain Heart Infusion (BHI) medium + glycerol (15%) at 37 °C for 18 hours to perform the antimicrobial susceptibility test. After that, the tubes were stored at −80 °C [15].

2.5. Antimicrobial susceptibility test

We used the Kirby Bauer technique (disc diffusion in Mueller Hinton agar) to determine the strains antimicrobial susceptibility using the following 12 antimicrobial discs: cefazolin (CZ; 30 μg), ciprofioxacin (CIP; 5 μg), ampicillin (AM; 10 μg), chloramphenicol (C; 30 μg), imipenem (IPM; 10 μg), trimethoprim-sulfamethoxazole (SXT; 1.25/23.75 μg), gentamicin (GM; 10 μg), ceftazidime (CAZ; 30 μg), cefepime (FEP; 30 μg), cefotaxime (CTX; 30 μg), tetracycline (TE; 30 μg), and amoxicillin + clavulanic acid (AMC; 20/10 μg). Resistance or susceptibility was determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines [16].

2.6. DNA extraction

Each isolate was grown on MacConkey agar at 37 °C for 18 hours, and five to six colonies were placed into Eppendorf® tubes with 500 μl of sterile distilled water; DNA was released by boiling cells suspensions for 1 minute. The quality of the DNA was monitored by gel electrophoresis [7].

2.7. Strains genotyping

The clonal relationship of the isolates was determined by amplifying and sequencing the *fumC* gene [17] using the Master Mix GoTaq. Those isolates coming from the same individual and sharing the same *fumC* allele were subjected to full multi-locus sequence-typing (MLST). Briefiy, PCR conditions were: 180 seconds at 95 °C, 30 cycles of 30 seconds at 94 °C, 30 seconds at the annealing temperature of each primer (*adk*: 52 °C; *fumC*: 55 °C; *gyrB* and *mdh*: 58 °C; *icd* and *recA*: 54 °C; *purA*: 50 °C) and 60 seconds at 72 °C (*fumC* and *icd*) or 45 seconds at 72 °C (*adk, gyrB, mdh, purA*, and *recA*), and a final extension of 7 min at 72 °C.

2.8. DNA sequencing

All PCR products were sequenced at Macrogen, Inc. Seoul, South Korea, using the Sanger sequencing method. The sequences were analysed using the program Geneious Prime 2020 and were screened using the Enterobase database [14].

2.9. Whole genome sequencing

Genomic DNA was extracted and purified from nine isolates (two from child 20, 26, and 30; and three from child 23) using the Wizard® Genomic DNA Purification according to the manufacturer's instructions. Whole-genome sequencing (WGS) was carried out using the Oxford Nanopore Technology (ONT) Rapid Bar-coding SQK-RBK004 Sequencing protocol. Raw data was demultiplexed and removed from adapters using Porechop (https://github.com/rrwick/Porechop version 0.2.3_seqan2.1.1). Genome assembly was performed using the Flye assembler (version 0.2.3_seqan2.1.1) [15], while the genome annotation was carried out using Prokka (version 1.14.6) [18]. Acquired AMR genes were identified using ABRicate (version 1.0.1) [19]. ARG-ANNOT, Resfinder [20] and CARD databases [21] were used for the identification of resistance genes.

2.10. Phylogenetic analysis

Pan-genome analysis was carried out using Roary, and a maximum-likelihood phylogenetic tree with 1000 bootstrap replicates based on core genomes of isolates was created using RaxML-NG (version 0.9.0) [22]. Sequence data from additional strains were included in this analysis and were obtained from the Enterobase database [14].

2.11. Statistical analysis

Significant differences between phenotypic antimicrobial resistance prevalence of the individuals through time were tested using a χ^2 test [23].

3. Results

We analysed 405 *E. coli* isolates from 82 faecal samples: 30 children were sampled in SP I and SP II and 22 were sampled SP I through SP III. Clonal relationships were first assessed by sequencing the *fumC* alleles [24]: those isolates that carried a different *fumC* allele were classified as non-clonal. The isolates that were obtained from the same child in different SPs and showed that the same *fumC* allele was subjected to MLST analysis to determine the isolate's sequence type (ST). Isolates that showed a different ST number were also classified as non-clonal, while the isolates with the same ST number were analysed using WGS to confirm clonality (Supplementary Tables S1 and S2). All isolates were subjected to antimicrobial susceptibility tests following CLSI guidelines [16].

Among the 405 isolates, we found a total of 40 different *fumC* alleles where the most prevalent were *fumC* 11 (n = 108, 26.7%), *fumC* 35 (n = 40, 9.9%), and *fumC* 4 (n = 38, 9.4%). Among the dominant *E. coli* isolates randomly selected from each faecal sample, 47 of 82 faecal samples had the same *fumC* allele in all five colonies; 12 of the 47 faecal samples had isolates with the *fumC* 11 allele (14.6%), followed by five faecal samples that had isolates with the *fumC* 35 allele and five with the *fumC* 4 allele (6.1%, respectively).

We identified 14 cases (samples obtained from 12 children) in which we obtained isolates with the same *fumC* allele in different SPs from the same individual; however, when we carried out MLST, there were four cases where the isolates were the same ST (ST34 was recovered twice, 11 months apart in child 26; ST10 was recovered twice in children 20 and 30) at 7 and 5 months apart, respectively, and ST131 was recovered twice in child 23, 4 months apart) (Fig. 1). Whole-genome sequencing showed that none of the isolates belonging to the same ST were clonal. In SPI (child 23), we found *E. coli* isolates carrying *fumC* 40 in SPI and SPII. All *fumC* 40 isolates were resistant to imipenem except for one isolate (collected in SPI) that was sensitive to this antibiotic. MLST and WGS from two *fumC* 40 isolates (from SPI and SPII) and the *fumC* 40 isolate sensitive to imipenem showed that the three isolates belonged to ST131; however, none of the isolates was clonal (we found 589 SNPs among the ST131 isolates) (Fig. 2).

Among the 405 isolates recovered, 122 (30.1%) were susceptible to all the antimicrobials tested. The remaining fell into one of the 75 unique antibiotic resistance profiles: 50 isolates (12.3%) were resistant to only one antibiotic, 37 isolates (9.1%) were resistant to two antibiotics, and 196 (48.4%) were resistant to three or more antimicrobials. Phenotypic resistance to the combination of ampicillin (AM), trimethoprim-sulfamethoxazole (SXT), and tetra-cycline (TE) was the most common profile (n = 36, 8.9%), followed by tetracycline (TE) resistance (n=21, 5.2%) and by the combination of ampicillin (AM), trimethoprim-sulfamethoxazole (SXT), and amoxicillin-clavulanic acid (AMC) resistance (n = 15, 3.7%) (Table 1).

There were statistically significant differences in the resistance to chloramphenicol (C) between SP I and SP II, with a higher resistance in SP I (Table 1). Similarly, statistically significant differences in the resistance to ceftazidime (CAZ) were found, with a higher resistance in SP II vs. SP III (Table 1).

4. Discussion

In this one-year prospective study, we followed 30 children for three sampling periods to screen the predominant *E. coli* strains present in fresh faecal samples. We found that all numerically dominant *E. coli* strains were transient colonisers, which suggests a high turnover rate of this species' lineages in the gut of children (Fig. 1). This observation agrees with previous reports showing a high diversity and high turnover rate of *E. coli* lineages in the human intestines [25–28]. It remains unknown what causes this high strain turnover; however, bacteriophage infection, bacterial bacteriocins, protozoal predation, immune mechanisms, and diet are probably key factors [1,25,29]. We also found that at least three of the five selected colonies in every sample showed the same antimicrobial resistance profile as well as the same *fumC* allele, which suggests that the large proportion of the isolates obtained at a specific time, from one individual, were the same clone; this is consistent with the idea that many of these colonies represent dominant strains [5]. We found three different clones of ST131 colonising one child (child 23) collected during two different sampling periods; the isolates showed phenotypic resistance to the AM-SXT-FEP-CZ-CIP-CTX-AMC antimicrobials, and two of them (isolated during two different periods) showed resistance to imipenem (IMP). ST 131 is a known human extraintestinal pathogen,

characterised by its virulence [30] and by its resistance to extended-spectrum cephalosporins and fiuoroquinolones [17,30,]. In the same way, it is strongly related to extraintestinal infections, mainly urinary tract, which have been reported as dominant in human intestines [31], domestic animal intestines, and the environment [17,30,31]; our isolates belonged to a different cluster from those associated with humans or domestic animals (Fig. 2). It is unclear why this child was repeatedly colonised by ST131; however, the child had a diarrhoea episode and was exposed to antibiotics in SP I and visited a private clinic before SP I and SP III; it is possible that this child acquired these ST131 clones in the first visit to the healthcare facility. Our results also corroborated the notion that ST131 is a successful epidemic clone [31].

Among the 405 isolates analysed, 30.1% (122 of 405) were susceptible to all of the antimicrobials tested. Resistance to ampicillin (AM), trimethoprim-sulfamethoxazole (SXT), and tetracycline (TE) were the most prevalent, while gentamicin (GM) resistance was the least common; only six strains showed resistance to GM. We found 24 (5.9%) *E. coli* isolates (including one ST131 isolate) were resistant to IPM and 40 isolates (9.8%) to cefepime (FEP); these antibiotics are used uniquely in humans and the resistances should be associated with hospitals and not community isolates. In the IMP-resistant ST131 isolates we found $bla_{\text{OXA-1}}$, $bla_{\text{OXA-368}}$, and $bla_{\text{CTX-M15}}$ genes, whereas in the IMP-sensitive ST 131 isolate, the $bla_{\text{OXA-368}}$ was missing; this suggests that the $bla_{\text{OXA-368}}$ in combination with another ESBL gene or a porin mutation may be responsible for the IMP resistance phenotype [32].

There were significant different resistances to ampicillin (AM), ceftazidime (CAZ), ciprofioxacin (CIP), and amoxicillin + clavulanic acid (AMC) among SP I, SP II, and SP III (Table 1). Our household survey that was designed to capture antibiotic use in the house-hold, as well as other risk factors, did not detect any potential cause for this difference in antimicrobial susceptibility. For example, six children (20%) were treated with antimicrobials in SP I, five (16.6%) children received antimicrobials in SPII, and five (22.7%) of the children received antimicrobials in SPIII. Interestingly, one child (child 23) received antimicrobials repeatedly in SP I and SP III and was found colonised with three multidrug-resistant ST131 isolates. All 30 households reported access to uninterrupted potable water, and 28 (93.3%) had functional sewerage systems. During the sampling periods, eight children (26.7%) changed their dietary habits (stopped breastfeeding) in SP I, 14 (46.7%) reported having diarrhoea in the last seven days prior to sampling, and 16 (53.3%). Our findings suggest that *E. coli* antimicrobial resistant-prevalence surveys are potentially not that useful because the phenotypic resistance profiles change rapidly over time, without any identifiable driving factors. These results may be in line with some antimicrobial resistance reports that show similar levels of some antimicrobial resistance in animals treated with antimicrobials and those not given antimicrobials [33].

Understanding the factors involved in lineage turnover is critically important for understanding antimicrobial resistance and virulence gene carriage in commensal strains. Our research shows another level of complexity on the understanding of the gut microbiome, which is important as many strains of the same species carry different metabolic, virulence, or antibiotic resistance genes. Our study reinforces the idea of the constant transition of

microbiome members over time [1,2,26,34] and the need for more research in the dynamics of strain populations in the intestine.

5. Conclusion

In this study we found that numerically dominant *E. coli* lineages in children's intestines change in less than 3 months; we did not find evidence of resident and dominant *E. coli* in these children. Additionally, we found that some antimicrobial resistance could change significantly overtime without any apparent selective force.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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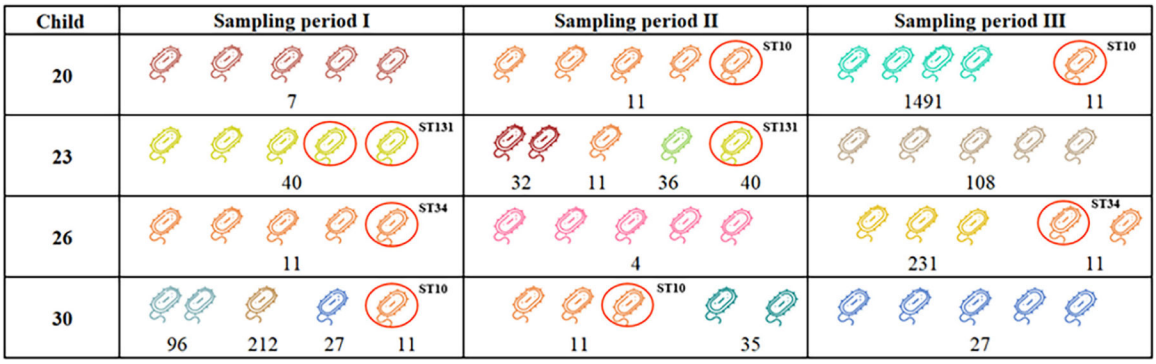

Fig. 1.

Numerically dominant *Escherichia coli* isolates showing evidence of permanence in children's intestines for 3 months. The figure showed five isolates obtained from four children during three periods (approximately 3 months apart). The numbers indicate different alleles of the *fumC* gene. The isolates showing same *fumC* allele during three periods were subjected to MLST and represented by sequence types (ST).

Fig. 2.

Maximum likelihood tree of whole genome sequences of *Escherichia coli* isolates belonging to ST131. The analysis shows our sequences from Ecuador compared with sequences of *E. coli* from different sources in North America, Europe, and Oceania.

Table 1

Prevalence of phenotypical resistance of *Escherichia coli* numerically dominant isolates obtained from 22 children during SP I, SP II, and SP III

Antimicrobials	SPIOct.–Dec. 2018	SPIIJan.–May 2019	SPIIIJul.–Dec 2019	χ^2 test	*P* value
Ampicillin (AM)	64 (58.2 %)	60 (54.5 %)	46 (41.8 %)	**6.502**	**0.039**
Chloramphenicol (C)	5 (4.5 %)	12 (10.9 %)	9 (8.2 %)	3.090	0.213
Imipenem (IPM)	12 (10.9 %)	1 (0.9 %)	7 (6.4 %)	**9.687**	**0.008**
Trim-sulfamet (SXT)	58 (52.7 %)	58 (52.7 %)	50 (45.4 %)	1.552	0.460
Gentamicin (GM)	0 (0 %)	6 (5.4 %)	0 (0 %)		
Ceftazidime (CAZ)	2 (1.8 %)	14 (12.7 %)	10 (9.1 %)	**9.352**	**0.009**
Cefepime (FEP)	13 (11.8 %)	15 (13.6 %)	9 (8.2 %)	1.705	0.426
Cefazolin (CZ)	18 (16.4 %)	14 (12.7 %)	15 (13.6 %)	0.645	0.724
Ciprofioxacin (CIP)	22 (20 %)	21 (19.1 %)	9 (8.2 %)	**7.168**	**0.028**
Cefotaxime (CTX)	14 (12.7 %)	18 (16.4 %)	14 (12.7 %)	0.808	0.668
Amoxicillin + Clav. Ac. (AMC)	35 (31.8 %)	28 (25.4 %)	15 (13.6 %)	**10.376**	**0.006**
Tetracycline (TE)	55 (50 %)	52 (47.3 %)	50 (45.4 %)	0.462	0.794

NOTE: Bold numbers indicate statistical significance (*P* value <0.05).