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Mechanisms of Antibiotic Resistance in *Escherichia coli* Isolates Obtained from Healthy Children in Spain

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ABSTRACT

Antibiotic resistance and mechanisms involved were studied in *Escherichia coli* isolates from fecal samples of healthy children. Fifty fecal samples were analyzed, and one colony per sample was recovered and identified by biochemical and molecular tests. Forty-one *E. coli* isolates were obtained (82%). MIC testing was performed by agar dilution with 18 antibiotics, and the mechanisms of resistance were analyzed. Ampicillin resistance was detected in 24 isolates (58.5%), and bla_{TEM}, bla_{SHV}, and bla_{OXA} type genes were studied by PCR and sequencing. The following β-lactamases were detected (number of isolates): TEM (20), SHV-1 (1), and OXA-30 (1). The number of aminoglycoside-resistant isolates detected was as follows: streptomycin (15), tobramycin (1), gentamicin (1), and kanamycin (4). The *aac(3)-IV* gene was detected in the only gentamicin-resistant isolate. Nine (22%) and 2 (5%) isolates showed nalidixic acid (NAL^R) and ciprofloxacin resistance (CIP^R), respectively. Mutations in GyrA and ParC proteins were shown in both NAL^R-CIP^R isolates and were the following: (1) GyrA (S83L + D87N), ParC (S80I); and (2) GyrA (S83L + A84P), ParC (S80I + A108V). A single mutation in the S83 codon of the *gyrA* gene was found in the remaining seven NAL^R-CIP^S isolates. Tetracycline resistance was identified in 21 isolates (51%) and the following resistance genes were found (number of isolates): *tetA* (12), *tetB* (5), and *tetD* (1). Chloramphenicol resistance was detected in five isolates (12%). These results show that the intestinal tract of healthy children constitutes a reservoir of resistant bacteria and resistance genes.

INTRODUCTION

IN THE LAST FEW YEARS, a worrisome increase in antibiotic resistance of pathogenic bacteria has been observed.[18] *Escherichia coli* is one of the microorganisms most frequently found in human infections.[11] On the other hand, *E. coli* is a universal colonizer of the human and animal intestinal tract.[39] The use of antibiotics may be associated with the selection of antibiotic resistance in human pathogens as well as in bacteria of the intestinal tract, such as *E. coli*. The transfer of antibiotic resistance determinants can take place in the intestinal ecosystem among different microorganisms.[38,40]

The resistance to β-lactams in *E. coli* is primarily mediated by β-lactamase enzymes, and to date a large variety of them have been described and characterized.[5,6,19] Classical TEM or SHV β-lactamases, as well as OXA type β-lactamases, are frequently detected mechanisms of resistance in ampicillin-resistant (AMP^R) *E. coli* isolates.[19,23] The main mechanism of quinolone resistance in *E. coli* includes alterations of the molecular target of quinolone action, associated with mutations in the quinolone resistance-determining region (QRDR) of *gyrA* and *parC* genes.[42,45] Aminoglycoside resistance in *E. coli* is mainly due to the expression of aminoglycoside-modifying enzymes, and different genetic determinants have been identified in resistant *E. coli* isolates, such as *aac(6′)-I, aph(3′)-I, aac(3)-I, aac(3)-II, aac(3)-III, aac(3)-IV,* and *ant(2″)*, among others.[35] Different *tet* genes have been reported in tetracycline-resistant (TET^R) *E. coli* isolates, such as *tetA, tetB, tetC, tetD, tetE,* and *tetI*, although other genes have also been found in TET^R Gram-negative bacteria (*tetG, tetM,* and *tetO*, among others).[31]

A large variety of studies have focused on the analysis of antibiotic resistance rates and mechanisms in clinical *E. coli* isolates have been reported. Some studies have also been performed with *E. coli* isolates from healthy children using dif-

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ferent methodologies,[2,7,10,15,16,21,46] but the mechanisms of antibiotic resistance have not been determined in any of them. In the present work, antibiotic resistance mechanisms are analyzed in nonpathogenic *E. coli* isolates recovered from feces of healthy children in La Rioja, Spain.

MATERIALS AND METHODS

Bacterial isolates

Fifty fecal samples from 50 healthy children under 2 years of age (from 7 to 23 months) were collected from two nurseries in La Rioja (Spain) from November, 2000, to January, 2001. Approximately 0.2 gram of each sample were suspended in 3 ml of sterile saline solution. A volume of 50 μl of this suspension was seeded onto Levine agar plates and incubated at 37°C for 24 hr. One colony with typical *E. coli* morphology per sample was randomly picked out. All of the isolates were identified by biochemical tests (Gram stain, oxidase, TSI, indol, citrate, methyl red, and urea agar) and also by PCR amplification of the *uidA* gene.[14] Only the isolates identified as *E. coli* were considered for further studies.

Antibiotic susceptibility testing

MIC determination was performed by the agar dilution method with 18 antibiotics (ampicillin [AMP], amoxicillin-clavulanicacid [AMC], ticarcillin [TIC], cefazolin [CFZ], cefoxitin [FOX], cefotaxime [CTX], ceftazidime [CAZ], aztreonam [AZT], imipenem [IMP], gentamicin [GEN], kanamycin, streptomycin, tobramycin, amikacin, tetracycline [TET], nalidixic acid [NAL], ciprofloxacin [CIP], and chloramphenicol[CHL]) according to the NCCLS standard method.[24] Susceptibility testing was also carried out with three additional antibiotics (fosfomycin, apramycin, and trimethoprim-sulfametoxazole) by the disk diffusion method.[25] *E. coli* ATCC 25922 was included as susceptibility control strain.

Characterization of the antibiotic resistance mechanisms

Genomic DNA for the PCR analysis was obtained by the Instagene matrix system (Bio-Rad) according to the manufacturer's instructions. TEM, OXA, and SHV β-lactamase encoding genes were analyzed in AMP^R isolates by PCR amplification using the primers and conditions previously described.[3,29,37] Four of the β-lactamase amplicons obtained (2 bla_{TEM}, 1 bla_{OXA}, and 1 bla_{SHV}) were sequenced (both strands) using the Applied Biosystem sequencer (ABI 310).

PCR amplifications of *gyrA*[27] and *parC*[43] genes were carried out in the three NAL^R isolates of this study with CIP MICs of $\geq$1 μg/ml, and the amplicons obtained were sequenced. In the remaining six NAL^R *E. coli* isolates (with CIP MICs of $\leq$ 0.25 μg/ml) detected in this study, mutations of *gyrA* gene were analyzed by the PCR method proposed by Ozeki *et al.*,[28] which includes the introduction of an artificial restriction cleavage.

Genes related to the gentamicin and streptomycin resistance were analyzed by PCR using primers for the *aac(3)-IV*[41] and *ant(3″)(9)* genes,[9] respectively. The *tet* determinants, which confer resistance to tetracycline, were studied by PCR with specific primers for *tetA, tetB, tetC, tetD,* and *tetE* genes.[13] Positive and negative controls were included in all PCR reactions.

RESULTS

A total of 41 *E. coli* isolates were recovered from the 50 fecal samples of this study (one isolate per sample, 82% of recovery). Antibiotic susceptibility testing and the mechanisms of resistance were analyzed in the 41 *E. coli* isolates obtained in this study.

β-lactams

A high percentage of the isolates showed AMP resistance (58.5%, 24 isolates), but none of them showed AMC resistance. An amoxicillin-clavulanicacid MIC of 16 μg/ml (included in the intermediate category according to the NCCLS standard) was detected in two isolates. None of the *E. coli* isolates showed resistance to cefoxitine or third-generation cephalosporins (Table 1). PCR reactions for the detection of bla_{TEM}, bla_{OXA}, and bla_{SHV} genes were carried out in all the 24 AMP^R isolates (Table 2). A bla_{TEM} amplicon was obtained in 20 of these isolates that were negative for bla_{OXA} and bla_{SHV} PCR reactions (MIC AMP, >128 μg/ml; AMC, $\leq$0.5–16 μg/ml). One AMP^R isolate gave a positive bla_{OXA} result being negative for the bla_{TEM} or bla_{SHV} genes (MIC for AMC of 16 μg/ml). One additional AMP^R isolate showed a positive bla_{SHV} PCR reaction (MIC for AMC of $\leq$0.5 μg/ml). Two of the 24 AMP^R isolates gave negative PCR reactions for the three β-lactamase genes studied. Four of the amplicons obtained were sequenced and they corresponded to the following genes: (1) two bla_{TEM} amplicons were obtained from the two *E. coli* isolates with the phenotype AMP^R-AMC^I and the sequences corresponded to a $bla_{TEM1\text{-}b}$ gene with a P3 weak promoter; (2) the bla_{SHV} amplicon, whose sequence corresponded to that of the $bla_{SHV\text{-}1}$ gene; (3) the bla_{OXA} amplicon showed a sequence identical to that of $bla_{OXA\text{-}30}$, which codifies the OXA-30 β-lactamase (Table 2).

Quinolones

Nine isolates were NAL^R (22%) and two isolates were CIP^R (4.9%). Sequences of the *gyrA* and *parC* genes from the two NAL^R-CIP^R *E. coli* isolates were studied by PCR and sequencing (Table 3). The *parC* and *gyrA* sequences were also studied in one *E. coli* isolate that showed a CIP MIC of 1 μg/ml. *E. coli* Co356 (MIC for CIP, 8 μg/ml) showed a double mutation in GyrA (S83L + A84P) and a double change in ParC (S80I + A108V). The *E. coli* isolate Co354 (MIC for CIP, 4 μg/ml) showed a double mutation in GyrA (S83L + D87N) and a single substitution in ParC (S80I). The *E. coli* isolate Co364 (MIC for CIP, 1 μg/ml) showed a single mutation in GyrA (S83L) and in ParC (S80I). Six additional NAL^R isolates showed a CIP MIC $\leq$ 0.25 μg/ml. In all these isolates, a single mutation in the S83 codon was detected when *gyrA* genes were analyzed by the PCR method described by Ozeki *et al.*[28]

Tetracycline

Twenty-one isolates (51%) showed TET resistance (MIC > 64 μg/ml). The *tet* genes were analyzed by PCR in all TET^R isolates, and the genes detected were the following: *tetA* (14

TABLE 1. MINIMAL INHIBITORY CONCENTRATION (MIC IN μG/ML) OF DIFFERENT ANTIBIOTICS IN 41 *E. COLI* ISOLATES RECOVERED FROM FECAL SAMPLES OF HEALTHY CHILDREN

Antibiotic	*MIC range*	MIC_{50}	MIC_{90}	*%R*
Ampicillin	≤0.5–>128	>128	>128	58.5
AMC[a]	≤0.5–16	2	8	0
Ticarcillin	1–>256	>256	>256	59.1
Cefazoline	1–32	2	4	4.9
Cefoxitin	≤1–8	2	8	0
Cefotaxime	≤0.125	≤0.125	0.125	0
Ceftazidime	≤0.125–0.25	≤0.125	≤0.125	0
Aztreonam	≤0.125	≤0.125	≤0.125	0
Imipenem	≤0.125–0.25	≤0.125	0.25	0
Nalidixic acid	≤1–>128	4	>128	22
Ciprofloxacin	≤0.25–8	≤0.25	≤0.25	4.9
Gentamicin	0.25–16	2	2	2.4
Tobramycin	0.5–64	1	2	2.4
Apramycin[b]	—	—	—	2.4
Kanamycin	1–>64	4	8	9.7
Streptomycin	2–>64	8	>64	36.6
Amikacin	≤0.5–8	2	4	0
Tetracycline	0.5–>64	64	>64	51.2
Chloramphenicol	≤2–>64	8	>64	12.2
SXT[a,b]	—	—	—	24.4
Fosfomycin[b]	—	—	—	7.3

[a]AMC, amoxicillin-clavulanic acid; SXT, trimethoprim-sulfamethoxazole.
[b]The susceptibility testing was performed by the disk diffusion method.

isolates), *tetB* (5 isolates), and *tetD* (1 isolate) (Table 4). Three PCR amplicons were confirmed by sequencing reactions (one *tetA*, one *tetB*, and one *tetD*). The *tetC* and *tetE* genes were not identified in any of the isolates studied. No *tet* genes were found in one of the TET^R isolates.

Aminoglycosides

Seventeen isolates (41.5%) showed resistance to at least one of the aminoglycoside antibiotics tested in this study. The percentage of isolates resistant to this class of antibiotic were as follows: gentamicin (2.4%), tobramycin (2.4%), apramycin (2.4%), streptomycin (36.6%), kanamycin (9.8%), and amikacin (0%). The *aac(3)-IV* gene was detected by PCR in the only *E. coli* isolate that showed gentamicin, apramycin, and tobramycin resistance. The *ant(3″)(9)* gene was analyzed by PCR in all the streptomycin-resistant isolates, but negative results were obtained in all of the cases.

Other antibiotics

Chloramphenicol resistance was detected in five isolates (MIC > 64 μg/ml). Trimethoprim-sulfamethoxazole and fosfomycin resistances (detected by the disk diffusion method) were found in 10 and 3 isolates (24.4% and 7.3%), respectively.

The different phenotypes of antibiotic resistance detected in our *E. coli* isolates, as well as the mechanisms of resistance

TABLE 2. SUSCEPTIBILITY TO β-LACTAMS AND MECHANISMS OF RESISTANCE IN *41 E. COLI* ISOLATES RECOVERED FROM HEALTHY CHILDREN

	MIC (μg/ml) range[a]						
Isolates (number)	*AMP*	*AMC*	*CFZ*	*CTX*	*CAZ*	*FOX*	bla *genes detected*[b]
17	≤0.5–4	≤0.5–4	1–2	≤0.125	≤0.125–0.25	2–8	—
1	32	≤0.5	2	≤0.125	≤0.125	2	Negative
1	>128	4	2	≤0.125	≤0.125	2	Negative
18	>128	≤0.5–8	1–4	≤0.125	≤0.125–0.25	≤1–8	bla_{TEM}
2	>128	16	32	≤0.125	0.25	2,4	bla_{TEM-1b}
1	>128	≤0.5	2	≤0.125	≤0.125	2	bla_{SHV-1}
1	>128	16	2	≤0.125	≤0.125	2	bla_{OXA-30}

[a]AMP, ampicillin; AMC, amoxicillin-clavulanic acid; CFZ, cefazolin; CTX, cefotaxime; CAZ, ceftazidime; FOX, cefoxitin.
[b]The genes were detected by PCR analysis (bla_{TEM}) or by PCR and amplicon sequencing (bla_{TEM-1b}, bla_{SHV-1}, bla_{OXA-30}). Negative: PCR results were negative for bla_{TEM}, bla_{SHV}, and bla_{OXA} genes.

TABLE 3. MUTATIONS IN THE QRDR OF GYRA AND PARC PROTEINS OF 9 NALIDIXIC ACID-RESISTANT *E. COLI* ISOLATES (MIC > 128 μG/ML)

Number of isolates (name)	*Ciprofloxacin MIC (μg/ml)*	*Mutations*	
		GyrA	*ParC*
1 (Co356)	8	S83L + A84P	S80I + A108V
1 (Co354)	4	S83L + D87N	S80I
1 (Co364)	1	S83L	S80I
6 isolates	≤0.25	S83[a]	ND[b]

[a]Mutations in *gyrA* were analyzed using the method proposed by Ozeki *et al.*[28]
[b]ND, Not determined.

found, are summarized in Table 4. Thirteen of the 41 *E. coli* isolates (32%) showed resistance to five or more of the antibiotics tested, which corresponded to at least four different structural groups. Quinolone resistance with multiple mutations in *gyrA* and *parC* was detected in two isolates that, additionally, showed multiresistance, being resistant to 8 or 10 of the antibiotics tested in this study. Almost a half of the isolates (46%) showed resistance to at least three of the antibiotics, and only five of the isolates (12%) were susceptible to all the antibiotics tested (Table 4).

DISCUSSION

As expected, one *E. coli* isolate was recovered in most of the fecal samples of the healthy children of this study (82%)

TABLE 4. ANTIBIOTIC RESISTANCE PHENOTYPES AND RESISTANCE GENES FOUND AMONG 41 *E. COLI* ISOLATES RECOVERED FROM HEALTHY CHILDREN

Number of E. coli	*Phenotype of resistance*[a]	*Detected mechanisms of resistance*		
		Resistance genes	*Mutations*	
			GyrA	*ParC*
1	AMP-TIC-GEN-TOB-APR-STR-STX-NAL-CIP-TET	bla_{TEM}, *aac(3)-IV, tetA*	S83L + D87N	S80I
1	AMP-TIC-STR-STX-CHL-NAL-CIP-TET	bla_{TEM}, *tetB*	S83L + A84P	S80I + A108V
1	AMP-TIC-KAN-STR-NAL-TET	bla_{TEM}, *tetA*	S83L	S80I[c]
1	AMP-TIC-STR-CHL-NAL-TET	bla_{TEM}, *tetA*	S83	
1	AMP-TIC-AMC[b]-CFZ-STR-TET	bla_{TEM-1b}, *tetA*		
5	AMP-TIC-STR-SXT-TET	bla_{TEM}, *tetA*		
1	AMP-TIC-CFZ-STR-TET	bla_{TEM-1b}, *tetA*		
1	AMP-TIC-STR-SXT-TET	bla_{TEM}, *tetA*		
1	AMP-TIC-KAN-SXT-TET	*tetB*		
1	KAN-STX-CHL-TET	*tetB*		
1	AMP-TIC-TET	bla_{TEM}, *tetA*		
1	NAL-CHL-TET	*tetA*	S83	
1	AMP-TIC-STR	bla_{SHV-1}		
1	AMP-TIC-STR	bla_{TEM}		
1	KAN-STR-TET	*tetB*		
1	AMP-AMC[b]-TET	bla_{OXA-30}, *tetB*		
1	TET-CHL	*tetD*		
6	AMP-TIC	bla_{TEM}		
1	NAL-TET	*tetA*	S83	
1	AMP-TIC			
3	NAL		S83	
1	TET			
3	FOS			
5	Susceptible			

[a]AMP, ampicillin; TIC, ticarcillin; AMC, amoxicillin-clavulanic acid; CFZ, cefazolin; CTX, cefotaxime; CAZ, ceftazidime; GEN, gentamicin; TOB, tobramycin; APR, apramycin; KAN, kanamycin; STR, streptomycin; STX, trimethoprim-sulfamethoxazole; NAL, nalidixic acid; CIP, ciprofloxacin; TET, tetracycline; FOS, fosfomycin; CHL, chloramphenicol.
[b]AMC, amoxicillin-clavulanic acid in the intermediate category (MIC, 16 μg/ml).
[c]This isolate showed a MIC for CIP of 1 μg/ml.

and similar *E. coli* fecal carriage rates had been previously reported.[10] It is well documented that *E. coli* colonizes the human intestinal tract and constitutes a prominent species of normal intestinal microflora.[39]

High percentages of antibiotic resistance were detected in this study in the nonpathogenic fecal *E. coli* isolates from healthy children. More than a half of the isolates showed ampicillin, ticarcillin, and tetracycline resistance and a high percentage of resistance to streptomycin (36.6%), nalidixic acid (22%), trimethoprim-sulfamethoxazole (24.4%), and ciprofloxacin (5%) was identified. The percentages of antibiotic resistance found in a previous study performed by our group,[32] which focused on *E. coli* isolates recovered during 1997–1999 from healthy adults, were lower than the percentages of resistance found in this study in isolates from healthy children (2000–2001). Zhang *et al.*[46] carried out a study to determine the carriage of antibiotic-resistant *E. coli* in healthy population of Shanghai; they found that all of the healthy people carried resistant *E. coli* isolates in their intestine, and that the younger the population was, the higher the level of resistance to the antibiotics of the fecal *E. coli* was. Nevertheless, no statistically significant differences between the bacterial resistance carriage rates and the age were reported in another study performed in Finland.[15]

Different studies evaluating the colonization rate by resistant *E. coli* isolates in fecal samples of healthy humans, using antibiotic-supplemented plates to recover antibiotic resistant isolates,[1,4,7,10,12,15–17,20–22,26,34,46] have been published. Nevertheless, very few studies analyze, as our work, the percentage of antibiotic resistance in randomly selected *E. coli* isolates from fecal samples of healthy volunteers[4,20,26] or healthy children.[16,46] In addition, the antibiotic resistance mechanisms are not determined in any of the above-mentioned papers.

In our study, a variety of *bla* genes were found in the AMP^R isolates, such as bla_{TEM}, bla_{SHV-1}, and bla_{OXA-30}. The rate of β-lactamases, different from the TEM type ones, in the nonpathogenic AMP^R *E. coli* isolates of our study was relatively high (2 of 24 isolates, 8.3%). The β-lactamase OXA-30 has been recently detected and characterized in clinical strains of *Shigella flexneri* isolated in Shanghai and in Hong Kong.[36] To our knowledge, this is the first time that the β-lactamase OXA-30 has been found in the species *E. coli.* Our strain with the β-lactamase OXA-30 showed the following resistance phenotype: AMP^R-AMC^I-TIC^R. The gene bla_{OXA-30} differs from the bla_{OXA-1}, since it has one mutation at the codon 131, AGA→GGA (arginine to glycine). The functional significance of this amino acid substitution seems to be minimal, because the V_{max} and IC_{50} values of the OXA-30 are similar to those of the OXA-1, as previously reported.[36]

A high percentage of NAL^R (22%) and CIP^R (5%) *E. coli* isolates were detected in this study. A double mutation in GyrA was identified in both NAL^R-CIP^R isolates (S83L + A84P and S83L + D87N, respectively). Both isolates also showed mutations in ParC (S80I + A108V and S80I, respectively). To our knowledge, this is the first time that the combination of GyrA (S83L + A84P) + ParC (S80I + A108V) substitutions is found in a single *E. coli* isolate. The A108V substitution in ParC had been previously reported in *E. coli* isolates, but always combined with S83L + D87N mutations in GyrA.[44]

Tetracycline resistance genes were found in 18 of 21 of the TET^R *E. coli* isolates. A variety of *tet* genes were identified in our isolates, such as *tetA, tetB,* and *tetD.* These three *tet* genes are associated with the tetracycline efflux in Gram-negative bacilli and have been previously detected in TET^R *E. coli* isolates.[31] The majority of the *tet* determinants are associated with either conjugative or mobilizable elements, and this fact may partially explain their wide distribution in the bacterial species. The Gram-negative efflux determinants are normally found in transposons inserted into a diverse group of plasmids from a variety of incompatibility groups. These plasmids may carry multiple antibiotic resistance genes, which confer resistance to a number of antibiotic families.[31] No resistance mechanisms were detected in three TET^R *E. coli* isolates from our collection. Other mechanisms of TET resistance could be involved in these isolates, such as enzymatic alteration or ribosomal protection.[30]

Only one of the isolates in this study showed apramycin, gentamicin, and tobramycin resistance and the *aac(3)-IV* gene was found in this isolate. The apramycin is an aminoglycoside structurally related to gentamicin and is used exclusively in the veterinary field. This antibiotic could have selected apramycin-resistant *E. coli* isolates (mediated by the AAC(3)-IV enzyme) in the animal environment,[8,33] and this type of resistant isolate could have been disseminated to the human environment.

To conclude, this study shows that 71% of the *E. coli* isolates were resistant to at least two of the 21 antibiotics tested and 26% of the isolates showed resistance to at least five of the antibiotics studied. It is demonstrated that the nonpathogenic *E. coli* isolates from the intestinal tract of healthy children constitute a reservoir of resistance genes.

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