ACCESS MICROBIOLOGY

RESEARCH ARTICLE
Mota *et al.*, *Access Microbiology*
DOI 10.1099/acmi.0.000182

MICROBIOLOGY SOCIETY
OPEN ACCESS

Multidrug-resistant bacteria as intestinal colonizers and evolution of intestinal colonization in healthy university students in Portugal

Raquel Mota[1,2], Marisa Pinto[1,2], Josman Palmeira[1,2], Daniela Gonçalves[1,2,3] and Helena Ferreira[1,2,*]

Abstract

Multidrug-resistant bacteria have been increasingly described in healthcare institutions, however community resistance also seems to be emerging. *Escherichia coli* an intestinal commensal bacteria, is also a pathogen and represents an important intestinal reservoir of resistance. Our aim was the study of the intestinal colonization and of the persistence of antibiotic resistant intestinal bacteria in healthy university students of Porto, in the north of Portugal. Samples from 30 university students were collected and analysed. Two *E. coli* isolates were randomly obtained from each student and Gram-negative bacilli resistant to antibiotics were studied. In addition, we evaluated changes in the Gram-negative intestinal colonization of ten university students in a short period of time. Molecular characterization showed a high presence of bla_{TEM} in commensal *E. coli*. Gram-negative bacteria with intrinsic and extrinsic resistance were isolated, namely *Pseudomonas* spp., *Enterobacter* spp. and *Pantoea* spp. We isolated three ESBL-producing *E. coli* from two students. These isolates showed bla_{CTX-M} group 1 (*n*=1), bla_{CTX-M} group 9 (*n*=2), bla_{TEM} (*n*=2), bla_{SHV} (*n*=1) and *tetA* (*n*=2) genes. Additionally, they showed specific virulence factors and conjugational transfer of antibiotic resistance and virulence genes. One *Pseudomonas* spp. isolate resistant to carbapenems was detected colonizing one student. Our results confirm that healthy young adults may be colonized with commensals showing clinically relevant antibiotic resistance mechanisms, creating a risk of silent spread of these bacteria in the community.

INTRODUCTION

Composition of human intestinal microbiota is reflected, albeit incompletely, in faeces, with anaerobic bacteria representing the largest quantity, but *Enterobacteriaceae* are the main type of facultative anaerobic pathogens associated with antibiotic resistance [1]. This Gram-negative bacilli resistant to antibiotics have been associated with health institutions, but community acquired infections are increasing worldwide [2]. Consequently, multidrug-resistant (MDR) bacteria are also increasing in the intestinal microbiota of healthy humans [3, 4]. There is evidence that the human gut is an important reservoir of MDR bacteria and that infections caused by this type of bacteria are associated with intestinal colonization, *E. coli* being the main carrier of antibiotic resistance genes in the faecal microbiota and an important opportunistic pathogen related with urinary tract and bloodstream infections [2, 5, 6]. Knowledge of silent faecal carriage is important once asymptomatic humans can be a reservoir for transmission through different niches, representing a public health threat [7]. Antibiotic resistant *E. coli* silent carriage has been described, with rates varying between 6 and 63% in different parts of the world [8]. Particularly in university students, colonization by extended-spectrum beta lactamase (ESBL)-producing bacteria have been described in Hungary (2,6%), Sweden (7%) and ESBL and/or AmpC in Mozambique (20%) [6, 9, 10]. Previous antibiotic consumption and recent travel to countries with high antibiotic resistance rates have been associated with colonization, although the association with food, contact with animals and human patients, is not well known [2, 11, 12]. Some studies had investigated the current state of human intestinal colonization by MDR *E. coli* and *Klebsiella pneumoniae* in vulnerable populations, like the

Received 24 July 2020; Accepted 02 November 2020; Published 25 November 2020
Author affiliations: [1]UCIBIO, Microbiology, Faculty of Pharmacy of University of Porto, Portugal; [2]Microbiology, Faculty of Pharmacy of University of Porto, Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal; [3]Instituto Superior de Saúde, Rua Castelo de Almourol, 4720-155 Amares, Portugal.
***Correspondence:** Helena Ferreira, hferr@ff.up.pt
Keywords: Multidrug-resistant *E.coli*; Microbiota; Healthy young adults; Colonization.
Abbreviations: CLSI, Clinical and Laboratory Standards Institute; ESBL, extended-spectrum beta-lactamase; FFUP, Faculty of Pharmacy of University of Porto; ICBAS, Institute of Biomedical Sciences Abel Salazar- Veterinary Medicine; KESC, *Klebsiella* spp., *Enterobacter* spp., *Serratia* spp., *Citrobacter* spp.; MDR, multidrug resistant; UP, University of Porto.
Supplementary material is available with the online version of this article.

Table 1. Background information distribution among participants in absolute number and percent

Background data		Percent/absolute no.
Sex	Female	76.7% (23/30)
	Male	23.3% (7/30)
Nationality	Portuguese	83.3% (25/30)
	Spanish	6.7% (2/30)
	Brazilian	6.7% (2/30)
	Georgian	3.3% (1/30)
Predominant alimentation*	Mediterranic	92.3% (24/26)
	Raw meat eater	26.9% (7/26)
	Vegetarian	7.7% (2/26)
Contact with animals*		65.4% (17/26)
International travel in last year*†		42.3% (11/26)
Gastrointestinal changes in last year*		23.1% (6/26)
Contact with human patients*		23.1% (6/26)

*Data were extracted from 26 out of 30 questionnaires.
†England, Italy, Spain, France, Brazil, Germany, Netherlands, Denmark, United States of America, Mexico, Hungary, Austria, Egypt.

elderly and healthcare-dependent populations, however silent carriage of MDR bacteria in human gut is still poorly studied, particularly the prevalence and intestinal colonization by these bacteria in healthy young individuals in community in Portugal [13, 14].

In this work we aimed to study the intestinal colonization and the persistence of intestinal bacteria in healthy young university students and the association with possible risk factors for colonization, in the north of Portugal.

METHODS

Recruitment of volunteer university students, faecal samples and study design

Faecal samples were collected from 30 volunteer students, of the University of Porto (UP), 24 students of the Faculty of Pharmacy (FFUP) and six students of Veterinary Medicine of Institute of Biomedical Sciences Abel Salazar (ICBAS), from October 2015 to March 2016. All students were clinically healthy young adults with ages between 18 and 25 years, without antibiotic consumption in the previous 6 months and from all course years. Epidemiological data for putative risk factors were assessed by questionnaire (Supplementary Material S1, available in the online version of the article). At a second approach, ten students were selected randomly for evaluation of intestinal colonization changes, carrying out a new detection after 3 months of the first detection and after 1 month of the second detection. In each detection, volunteers completed a follow-up questionnaire, to identify changes relatively to the previous questionnaire. Informed consent was obtained from all participants.

Faecal samples were stored in a refrigerator and analysed within 24 h after collection.

Selection of bacterial isolates

In total, 1 g of faeces was suspended in 10 ml of saline, and successive dilutions were performed of 1:10 and plated on MacConkey agar 100 µl of the dilution 1:100, 1:1000 and 1:10000. When necessary to perform colony counting (between 30 and 150 c.f.u., approximately) the dilution 1:100000 was plated. The dilution used for colony counts was plated in CHROMagar orientation (ChrOmagar). From the later medium, two *E. coli* colonies were randomly selected for further characterization in relation to resistance to β-lactam and non-β-lactam antibiotics.

To search Gram-negative bacilli resistant to antibiotics, 100 µl of suspension in saline was plated on MacConkey agar (Oxoid, Hampshire, UK) with cefotaxime, ceftazidime or meropenem in subinhibitory concentrations (2 mg l^{-1}) (Sigma-Aldrich, St Louis, MO, USA). At the same time, 1 g of faeces was suspended in 40 ml of Tryptic Soy Broth (TSB) (Sigma-Aldrich) and after incubation 200 µl were spread in the same culture media. Representative isolates were selected to confirm the identification using API 20E and ID32GN Biomérieux.

Antimicrobial susceptibility

Susceptibility to antibiotics test was carried out by agar diffusion method, according to Clinical and Laboratory Standards Institute (CLSI) [15]. To study the predominant antibiotic resistance profile in *E. coli* isolates from intestinal microbiota in this specific population susceptibility profile to β-lactam antibiotics amoxicillin (10 µg), amoxicillin/clavulanic acid (30 µg), cefotaxime (30 µg) and meropenem (10 µg), and non-β-lactam antibiotics ciprofloxacin (5 µg), gentamicin (5 µg), chloramphenicol (30 µg), trimethoprim/sulfamethoxazole (25 µg) and tetracycline (30 µg) was determined. In order to search other Gram-negative bacilli resistant to antibiotics, susceptibility profile to β-lactam antibiotics amoxicillin (10 µg), amoxicillin/clavulanic acid (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), aztreonam (30 µg), cefoxitin (30 µg), imipenem (10 µg) and meropenem (10 µg) and to non-β-lactam antibiotic ciprofloxacin (5 µg) was also determined. *E. coli* ATCC 25922 was used for quality control.

ESBL and carbapenemase producer resistance phenotype detection

Isolates with different colony morphologies that grew in MacConkey agar with cefotaxime or ceftazidime, were screened for ESBL production by the double disc synergy test and confirmed by clavulanic acid addition to cefotaxime and/or ceftazidime discs, following the CLSI criteria [15]. Isolates that grew in MacConkey agar with meropenem were searched

for carbapenemase production by biochemical Blue-carba test [16].

Genotype of resistance to antibiotics predominant in *E. coli*

DNA was extracted from two *E. coli* isolates from each student. Molecular characterization of predominant resistance to β-lactams and non-β-lactams in *E. coli* isolates from intestinal microbiota in this specific population was done. We searched for bla_{TEM}, bla_{OXA-1}, bla_{SHV}, *tet(A), tet(B)*, *sul1*, *sul2*, *sul3, qnrB, parC, gyrA, aac(3)-IV* and *aac(3)-II* (Supplementary Material S2).

Detection of ESBL and carbapenemase genes

Molecular characterization of ESBL producing *E. coli* was done for bla_{TEM}, bla_{OXA-1}, bla_{SHV} and bla_{CTX-M} groups 1, 2, 8, 9 and 25 (Supplementary Material S2). In isolates that amplified bla_{CTX-M} group 1, $bla_{CTX-M-15}$ was detected with specific primers (Supplementary Material S2). The most prevalent and relevant carbapenemases bla_{IMP}, bla_{VIM}, bla_{KPC}, bla_{NDM} and bla_{OXA-48} were searched in isolates showing reduction of susceptibility to carbapenems (Supplementary Material S2).

Students colonized with ESBL producers were followed-up after 5 months.

Typing, virulence genotyping and conjugation experiments of ESBL-producing *E. coli* isolates

ESBL-producing *E. coli* were searched for phylogenetic groups A, B1, B2 and D using PCR with specific primers for *trpA*, *chuA*, *yjaA* and *TspE4.C2* determinants, and for O25b-ST131 clonal group (Supplementary Material S2). Additionally, virulence genes and pathogenicity associated island (PAI) were also tested by PCR (Supplementary Material S2). Transfer of antibiotic resistance and virulence genes was determined in ESBL-producing *E. coli* isolates by conjugation, using *E. coli* K802N as the recipient strain. Transconjugants were selected in MacConkey agar with cefotaxime (1 µg ml^{-1}) and nalidixic acid (500 µg ml^{-1}). Transconjugants were verified by PCR to confirm plasmid acquisition.

RESULTS

Description of volunteers

Students from all years of university (range 18–25 years, median age 21.5), different gender, different food habits and *Erasmus* students (5/16.7%). None of the volunteer students took antibiotics in the previous 6 months of providing faecal samples. The epidemiological data collected are shown in Tables 1 and 2.

In this study, faecal samples from healthy young adults were analysed to verify the panorama of antibiotic resistance in this population and to try to understand the dynamics of that resistance over time. Veterinary medicine students were specifically analysed, and contact with animals of all students was assessed through the questionnaire, in an attempt to find a relationship between contact with animals and human intestinal colonization, because of the number of animals colonized with antibiotic resistant bacteria, namely with ESBL

Table 2. Description of the changes in the university students that were followed up, after 3 months of the first detection

Volunteer no.	Food changes	Contact with animals changes	International travel	Antibiotic consumption	Gastrointestinal changes	Contact with human patients
1	No	No	No	No	No	No
2	No	No	No	No	No	Gastrointestinal disease
3	No	No	No	No	No	No
4	No	No	No	No	No	No
6	No	No	No	Ciprofloxacin (5 days)	No	Cancer patient
12	No	No	No	Doxycycline (2 months, continued after follow up)	No	No
15	No	No	France and Germany (bottled water consumption)	No	No	No
25	No	No	Germany and Austria (bottled and public water consumption)	No	No	No
28	Vegetarian	Increase (canids, cattle, horses)	No	No	No	No
29	–	–	–	–	–	–

Data were extracted from 9 out of 10 questionnaires.

producers, bacteria resistant to tetracycline and to quinolones [17]. This is due to the use of antibiotics in veterinary practices, with the concern of the possible transfer of antibiotic resistant bacteria between humans and animals. Food habits were also analysed through the questionnaire, specifically meat consumption, as an attempt to evaluate if this is a risk factor for antibiotic resistant bacteria intestinal colonization. The analysis of *Erasmus* students aimed to verify if there are differences in colonization between different countries. Additionally, international travel was assessed through the questionnaire, in order to evaluate if travel to other countries can influence intestinal colonization. At the third detection no volunteer had any alteration relatively to the second detection period, except volunteer number 12 who continued taking doxycycline.

Commensal intestinal microbiota of university students

Through the plating in CHROMagar orientation (ChrOmagar), we classified the commensal intestinal microbiota relatively to the presence of only *E. coli*, predominance of *E. coli* or predominance of KESC group (*Klebsiella* spp., *Enterobacter* spp., *Serratia* spp., *Citrobacter* spp.) and/or *Enterococcus* spp. in the plated dilution. In 30% (9/30), only *E. coli* was detected, in 40% (12/30) the predominance of *E. coli* and in 30% (9/30) predominance of KESC group and/or *Enterococcus* spp. There was a great intestinal microbiota diversity and *E. coli* was the main bacteria found in most university volunteers [21/30 (70%)]. In relation to the colony counting, intestinal colonization ranged over three orders of magnitude from 4.0×10^5 to 3.3×10^8.

Sixty isolates of commensal *E. coli*, two of each student were randomly selected, for characterization of resistance to nine antibiotics. Amoxicillin was the antibiotic that demonstrated less efficacy, with 31.7% of resistance (19/60 isolates). In contrast, resistance to amoxicillin with clavulanic acid, to cefotaxime and to meropenem was not detected. Within non-β-lactam antibiotics, tetracycline was the antibiotic with less efficacy, with 15% of resistance (9/60 isolates). Chloramphenicol, trimethoprim-sulfamethoxazole and ciprofloxacin showed, respectively, 6.7% (4/60 isolates), 5.0% (3/60 isolates) and 3.3% (2/60 isolates) of resistance. Resistance to gentamicin was not found. In total of *E. coli* isolates, 38.3% (23/60 isolates) showed resistance to at least one of the tested antibiotics. Considering the 30 students, 43.3% (13/30 students) were colonized with *E. coli* resistant to one or more antibiotics (Table 3). Additionally, other Gram-negative bacilli resistant to antibiotics, were detected. From the students analysed, nine bacteria with different resistance profiles and non-ESBL producers were isolated, namely *E. coli* [2/30 (6.7%)] *Enterobacter aerogenes* [1/30 (3.3%)], *Enterobacter cloacae* [3/30, (10.0%)], *Pantoea* spp. [1/30,(3.3%)] and *Pseudomonas* spp. [2/30, (6.7%)].

From 19 *E. coli* resistant to amoxicillin, 18 amplified bla_{TEM} and did not amplify bla_{OXA} or bla_{SHV}. In a total of nine isolates resistant to tetracycline, six amplified *tet(A)*, one amplified *tet(B)* and one amplified both *tet(A)* and *tet(B)*. Considering the three *E. coli* resistant to trimethoprim-sulfamethoxazole, two amplified both *sul(1)* and *sul(2)* and one amplified *sul(3)*. All *E. coli* isolates resistant (n=2) and intermediate (n=1) to ciprofloxacin amplified both *parC* and *gyrA* (Table 3).

ESBL-producing *Enterobacteriaceae*

Three distinct ESBL-producing *E. coli* were detected, one isolate in volunteer number 2 and two isolates in volunteer number 24, with a proportion of colonization of 6.7%. Amplification for CTX-M group 1 (n=1), but not for CTX-M-15, and for CTX-M group 9 (n=2) was obtained. Student number 2 was no longer colonized after 5 months of the first detection, but student number 24 remained colonized with an ESBL-producing *E. coli*. The isolate recovered after 5 months from student number 24 amplified for CTX-M group 9, as the previous isolates from this student, and presented the same antibiotic resistance profile and virulence factors as an isolate previously detected, however the phylogenetic group differed (Table 4). All isolates presented virulence factors but none of them amplified for O25b-ST131 clonal group. Additionally, one isolate presented capacity to transfer resistance genes and one isolate presented capacity to transfer both resistance and virulence genes.

Follow up – temporal variability and dynamics associated with phenotypes

Temporal variability of bacteria that colonize the human gut, both in a qualitative and a quantitative level, were verified. From the ten students that were followed up, we verified a change in half, relative to the predominant colonizer group (*E.coli* or KESC group and/or *Enterococcus* spp.). In five students that initially were predominantly colonized by *E. coli* or by KESC group and/or *Enterococcus* spp., the respectively predominant colonization changed after 3 or 4 months. Relative to the quantitative profile, just in one of the ten students (10%) we did not find a change in the order of magnitude. Additionally, in four of the ten students (40%) there was a change in at least two orders of magnitude. In 50% of students there was a change in one order of magnitude.

We also verified that after 3 months, the two *E. coli* randomly selected were different from the *E. coli* selected in the first evaluation in six of the ten students (60%), and in those cases, alteration in the resistance profile to at least one antibiotic in one or both *E. coli* was detected. In the third evaluation, after 1 month of the second evaluation, four students (40%) presented different *E. coli* randomly selected. Six students presented *E. coli* with exactly the same phenotypic and molecular resistance profile (Table 5).

Considering the selection of Gram-negative isolates that grew in MacConkey agar with antibiotic, three *Pseudomonas* spp. (two in the second detection and one in the third detection) were detected from volunteer number 25. One isolate of the second detection and one isolate of the third

Table 3. Predominant colonizing bacteria and antibiotic resistance profile in two intestinal commensal *E. coli* randomly selected, in 30 university students of University of Porto (UP)

Student ID	Faculty	Sex/ Nationality	Colonies counting	Predominant bacteria colonizing	Antibiotic resistance profile in intestinal commensal *E. coli*			
					Phenotypic		Molecular	
					β-lactams	Non-β-lactams	β-lactams	Non-β-lactams
1	FFUP	F/Pt	$4{,}0\times10^{6}$	KESC group	None	None	–	–
2	FFUP	F/Pt	$3{,}3\times10^{8}$	KESC group	AML	TE, SXT, C	bla_{TEM}	*tetA, sul3*
3	FFUP	F/Pt	$1{,}5\times10^{7}$	KESC group	None	TE	–	*tetA*
4	FFUP	F/Pt	$2{,}3\times10^{7}$	*E. coli*	None	None	–	–
5	FFUP	F/Sp	$1{,}1\times10^{7}$	*E. coli*	AML	TE, C	bla_{TEM}	*tetA*
6	FFUP	F/Pt	$4{,}9\times10^{7}$	*E. coli*	AML	None	bla_{TEM}	–
7	FFUP	F/Pt	$1{,}7\times10^{8}$	*E. coli*	None	None	–	–
8	FFUP	M/Pt	$4{,}7\times10^{5}$	KESC group	None	None	–	–
9	FFUP	M/Pt	$4{,}6\times10^{6}$	*E. coli*	None	None	–	–
10	FFUP	F/Bz	$1{,}8\times10^{8}$	*E. coli*	None	None	–	–
11	FFUP	F/Sp	$5{,}9\times10^{7}$	*E. coli*	AML*	None	None	–
12	FFUP	F/Pt	$7{,}9\times10^{7}$	*E. coli*	AML	None	bla_{TEM}	–
13	FFUP	F/Geor	$3{,}1\times10^{7}$	KESC group	AML	TE, C, CIP*, CN*	bla_{TEM}	*parC, gyrA*
14	FFUP	F/Pt	$1{,}7\times10^{8}$	*E. coli*	AML	TE, SXT, C	bla_{TEM}	*tetA, tetB, sul1, sul2*
15	FFUP	F/Pt	$3{,}3\times10^{7}$	*E. coli*	AML	None	bla_{TEM}	–
16	FFUP	M/Bz	$1{,}3\times10^{8}$	*E. coli*	AML	None	bla_{TEM}	–
17	FFUP	F/Pt	$1{,}5\times10^{6}$	KESC group	AML	CIP	bla_{TEM}	*parC, gyrA*
18	FFUP	F/Pt	$4{,}3\times10^{7}$	*E. coli*	AML	None	bla_{TEM}	–
19	FFUP	F/Pt	$4{,}0\times10^{5}$	KESC group	None	None	–	–
20	FFUP	F/Pt	$1{,}5\times10^{7}$	*E. coli*	None	None	–	–
21	FFUP	F/Pt	$4{,}6\times10^{7}$	KESC group	AML	None	bla_{TEM}	–
22	FFUP	F/Pt	$6{,}0\times10^{6}$	*E. coli*	None	None	–	–
23	FFUP	F/Pt	$9{,}6\times10^{7}$	*E. coli*	None	None	–	–
24	FFUP	F/Pt	$1{,}3\times10^{8}$	*E. coli*	AML*, AMC*	None	None	–
25	ICBAS	M/Pt	$1{,}4\times10^{6}$	*E. coli*	None	None	–	–
26	ICBAS	M/Pt	$4{,}3\times10^{6}$	KESC group	None	None	–	–
27	ICBAS	F/Pt	$1{,}5\times10^{8}$	*E. coli*	None	None	–	–
28	ICBAS	M/Pt	$4{,}8\times10^{5}$	*E. coli*	AML*	None	None	–
29	ICBAS	M/Pt	$5{,}2\times10^{6}$	*E. coli*	None	None	–	–
30	ICBAS	F/Pt	$4{,}3\times10^{6}$	*E. coli*	AML, AMC*	None	bla_{TEM}	–

*Isolates with intermediate resistance.
FFUP, Faculty of Pharmacy of University of Porto; ICBAS, Institute of Biomedical Sciences Abel Salazar; F, Female; M, Male; Pt, Portuguese; Sp, Spanish; Bz, Brazilian; Geor, Georgian; AML, amoxicillin; TE, tetracycline; SXT, trimethoprim-sulfamethoxazole; CIP, ciprofloxacin; CN, gentamicin; C, chloramphenicol.

Table 4. Characteristics of bla_{CTX-M} harbouring *E. coli* isolates recovered from the two university students of FFUP

Student ID	Sex/ age*	Isolate ID	Isolation date	Resistance profile to β-lactam and non-β-lactam antibiotics†	ESBL genes	Other β-lactamases	Resistance to non-β-lactams genes	Virulence genes‡	Phylogenetic group
2	F/ 21	2A	October 2015	AML, PRL, CXM§, CTX, ATM§, CAZ§, TET	CTX-M group 1	TEM, SHV	*tet(A)*	*fimH*, *iutA*, traT, cvaC	B1
24	F/ 22	24A	March 2016	AML, PRL, CXM, CTX, CPT	CTX-M group 9	–	–	*fimH*, *fyuA*, *traT*	A
		24B‖	March 2016	AML, PRL, CXM, CTX, CPT, TET	CTX-M group 9	TEM	*tet(A)*	*fimH*, *iutA*, *fyuA*, *traT*	B1
		24C‖, ¶	October 2016	AML, PRL, CXM, CTX, CPT, TET	CTX-M group 9	TEM	*tet(A)*	*fimH*, *iutA*, *fyuA*	D

*F, Female.
†AML, amoxicillin; PRL, piperacillin; CXM, cefuroxime; CTX, cefotaxime; ATM, aztreonam; CAZ, ceftazidime; CPT, ceftaroline; TET, tetracycline.
‡*fimH* - type one fimbriae; *iutA* - ferric aerobactin receptor; *fyuA* - yersiniabactin receptor; *traT* - serum survival associated; *cvaC* - colicin V; underlined - antibiotic resistance genes and virulence genes transferred in conjugation assay; (-) not detected.
§Isolates with intermediate resistance.
‖Colonies with mucoid aspect.
¶Isolate recovered after 5 months of the first detection (October 2016).

Table 5. Follow up results in ten participants. First detection, after 3 months of the first detection (second detection) and after 1 month of the second detection (third detection)

Student ID	First detection		Second detection		Third detection	
	c.f.u. per gram of faeces*	*E. coli* antibiotic resistance profile†	c.f.u. per gram of faeces*	*E. coli* antibiotic resistance profile†	c.f.u. per gram of faeces*	*E. coli* antibiotic resistance profile†
1	4.0×10^6	Without resistance	9.2×10^6	Without resistance	2.4×10^8	Without resistance
4	2.3×10^7	Without resistance	1.1×10^8	Without resistance	5.3×10^6	Without resistance
25	1.4×10^6	Without resistance	2.6×10^5	Without resistance	1.5×10^5	Without resistance
29	5.2×10^6	Without resistance	6.1×10^7	Without resistance	5.2×10^6	Without resistance
3	1.5×10^7	TE | *tetA*	1.7×10^7	Without resistance	1.3×10^6	Without resistance
6	4.9×10^7	AML | bla_{TEM}	1.6×10^7	Without resistance	1.7×10^6	Without resistance
15	3.3×10^7	AML | bla_{TEM} AML	7.1×10^7	Without resistance	7.9×10^7	AML‡ AML‡
28	4.8×10^5	AML‡	1.1×10^7	AML‡ Without resistance	8.3×10^6	Without resistance
2	3.3×10^8	AML, TE, SXT, C | bla_{TEM}, *tetA*, *sul3* AML, TE | bla_{TEM}, *tetA*	9.7×10^5	AML, TE, SXT| bla_{TEM}, *tetA*, *sul1* Without resistance	1.0×10^8	AML, TE, SXT | bla_{TEM}, *tetA*, *sul1*, *sul2*
12	7.9×10^7	AML | bla_{TEM}	1.5×10^8	AML, TE, CIP, CN SXT | bla_{TEM}, *tetB*, *parC*, *gyrA*, *aac(3)-II*, *sul1*, *sul2*, *sul3* TE | *tetB*	1.0×10^8	AML, TE, CIP, CN SXT | bla_{TEM}, *tetB*, *parC*, *gyrA*, *aac(3)-II*, *sul1*, *sul3*

*Only Gram-negative bacteria were counted.
†From two *E. coli* isolates randomly selected from each student. When only one profile is presented, this means that both *E. coli* isolates are the same, phenotypic and molecular.
‡Isolates with intermediate resistance.
AML, amoxicillin; CTX, cefotaxime; TE, tetracycline; SXT, trimethoprim-sulfamethoxazole; CIP, ciprofloxacin; CN, gentamicin; C, chloramphenicol

detection presented native resistance profile, however one *Pseudomonas* spp. resistant to meropenem was detected. Neither in the blue-carba test nor in the PCR for the prevalent carbapenemases a positive result was obtained.

DISCUSSION

Qualitative and quantitative analysis of commensal intestinal microbiota

In most of the university students, microbiota presented *E. coli* as the main colonizer. This result is as expected as *E. coli* is the predominant facultative aerobic organism in the gut [18]. The abundance of Gram-negative bacteria, varied between 4.0×10^5 to 3.3×10^8 c.f.u. per gram of faeces. This result is consistent with previous reports, namely *E. coli* and other Gram-negative bacteria incidence between 2.0×10^4 and 1.7×10^8 [19]. In fact, in the gut there are a lot of Gram-negative bacteria, which may present resistance and virulence genes. Based on these values we verify that even the less abundant strains represent a significant reservoir.

Prevalent antibiotic resistant phenotype in *E. coli* in intestinal microbiota

E. coli has consolidated resistances that have appeared over the years, such as the TEM-1 β-lactamase or more recently the ESBL, carbapenemases or the plasmid-mediated quinolone resistance. In healthy humans the incidence of resistance in commensal *E. coli* has been increasing, although the knowledge in this area is still scarce [19].

In this study two colonies of *E. coli* were randomly isolated from each student in an attempt to evaluate the phenotypic resistance profile of this species in the studied population. Of the isolates resistant to amoxicillin, bla_{TEM} was the only antibiotic resistance gene that was amplified. The pair of primers used amplify both TEM-1 and TEM-2 β-lactamases, but due to high prevalence of TEM-1 all over the world, even in healthy populations, it was probably the enzyme that confers resistance in these isolates [20, 21]. The combination of amoxicillin with clavulanic acid showed only two intermediate resistant isolates (3.3%). Once this combination is widely used in clinical practice, these data suggest that, for now, it seems to be a good therapeutic option. No resistant isolate to cefotaxime and meropenem was found, which demonstrates that these antibiotics are still effective and an alternative to be used in treatment. In the 30 students analysed only one intermediate resistance to gentamicin was found. Regarding resistance to trimethoprim-sulfamethoxazole there was no trend or predominance in the amplified genes. Considering resistance to ciprofloxacin, all intermediate resistant isolates amplified both *gyrA* and *parC*, confirming that these two genes are responsible for conferring much of the resistance to this antibiotic in *E. coli*, being described as the principal mechanism of resistance to quinolones in this species [22].

Comparing the values of prevalence of resistance to antibiotics obtained with the values described in the literature, we can verify some similarities with the results of this work. In a study conducted in 2010, where *E. coli* isolates were analysed from healthy humans, the antibiotics ampicillin, tetracycline and trimethoprim-sulfamethoxazole were the ones that showed lowest efficacy. No strains were found with resistance to cefotaxime, as demonstrated by our results [19]. In another study, which included 110 humans apparently healthy, the higher levels of antibiotic resistance were found with ampicillin (77.3%) and tetracycline (66.5%) [23]. However, this last study was carried out in Asia, where prevalence of antibiotic resistance is higher in comparison to Europe, and included individuals of all age groups, including the elderly, who are colonized by antibiotic resistant bacteria in more extent than the younger population. In Mexico, Spain and Turkey, in a study that covered all age groups, ampicillin resistance values above 50% were described, but in Germany these values were lower [24].

When analysing *E. coli* from intestinal microbiota, there was no prevalence of antibiotic resistance in students of veterinary medicine nor in *Erasmus* students. Relating to the parameters evaluated in the questionnaire, namely contact with animals, food habits and international travel, it was also not possible to establish any clear relation. Finally, it was found that in the majority of the volunteers the two *E. coli* randomly isolated presented the same phenotypic profile.

ESBL-producing *Enterobacteriaceae*, virulence factors and gene transfer

E. coli, both a human intestinal commensal and an important pathogen, is the species most often responsible for community-acquired infections, and in fact all ESBL producers detected in this study, were *E. coli* isolates [21]. CTX-M-producing faecal carriage in healthy humans have been rising in recent years, and currently supplanted TEM and SHV enzymes worldwide [21, 25]. CTX-M group 1 and group 9 increase are in accordance with previous reports in healthy humans, however the non-amplification for CTX-M-15 and clonal group O25b-ST131, both highly disseminated in healthcare facilities in Portugal, suggests that neither this CTX-M variant nor this clonal group are associated with young healthy community settings [26–29]. Additionally, only co-resistance to tetracycline was detected, differing from ESBL clinical isolates that in most of the cases present co-resistance to multiple antibiotics. In fact, in *E. coli*, tetracycline is an antibiotic with high levels of resistance described [19, 23, 26, 30]. The presence of the virulence factors described in the healthy intestinal microbiota alerts for the colonization of healthy humans by bacteria that can be responsible for extra-intestinal infections, namely urinary tract infections (UTIs), and for the hypothesis of transfer of resistance and virulence genes to other bacteria. It should be noted that the university students colonized with ESBL-producing *E. coli* reported taking many antibiotics in childhood, due to recurrent UTIs (student number 2) and to tonsillitis (student number 25). However, no other association with other parameters analysed by the questionnaire could be established. Relative to the follow up after 5 months, results demonstrate that the human intestinal colonization with ESBL producers are dynamic and apparently transitory, as described in the literature [31, 32].

Other antibiotic resistant Gram-negative isolates

One volunteer was colonized with *Enterobacter aerogenes* and three volunteers were colonized with *Enterobacter cloacae*, commensal bacteria of the intestinal tract and intrinsically resistant to amoxicillin, amoxicillin with clavulanic acid, first-generation cephalosporins and cefoxitin [33]. However, the isolated bacteria presented additional resistance to β-lactam antibiotics. These bacteria are part of ESKAPE group (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.), described as main contributors to human infections and that effectively escape to antibiotics [34]. They present the ability to develop antibiotic resistance mechanisms and currently *Enterobacter cloacae* has been associated with ESBL and carbapenemases [33]. However, none of these isolates were ESBL or carbapenemase producers. There were also three students colonized with *Pseudomonas aeruginosa*, and although it is not a typicalenteric bacteria, an intestinal colonization incidence of 1.47% in the community, was observed [35].

The fact that antibiotic resistant bacteria was found colonizing healthy humans, is important because these antibiotics are widely used in clinical practice, namely amoxicillin with clavulanic acid, alerting to the limitations of therapeutic options.

Follow up – variability and temporal dynamics associated with phenotypes

Selected volunteers had not taken antibiotics for at least 6 months in the eventuality that if any of them started taking antibiotics during the follow up, the changes should be observed. At the quantitative level there was temporal dynamic, however the changes were not very significant in none of the students. These results demonstrate that there are variability and dynamic in the composition and amount of bacteria in the gut, and that this variability is constant, since there were no differences between the analysis performed in the 3 month interval and the analysis performed with 1 month interval.

The variability was also verified in the antibiotic resistance profile, but in this case, alterations were verified between the analysis performed with a 3 month interval and with a 1 month interval. It should be noted that, at the antibiotic resistance level, it was not only in the phenotype that we detected changes, but also at molecular level. For example, in volunteer number 2, the gene *sul3* was detected in the first evaluation, but in the next two evaluations both genes *sul1* and *sul2* were detected. This shows that, in this case, a variability in antibiotic resistance genes exists, although at phenotypic level this variability was not detected, with different genes conferring the same antibiotic resistance in the same host. Analysing these parameters shows that in addition to inter-individual there is intra-individual variability.

Of note is the situation of volunteer number 12. Two months before the second evaluation this student started a treatment with doxycycline and continued till the third evaluation. In the first evaluation only resistance to tetracycline was detected, but in the second and third evaluation MDR bacteria were detected. In addition, it should be noted that in the second evaluation we isolated *E. coli* with resistance only to tetracycline, the only antibiotic tested that belongs to the same class of doxycycline, and *E. coli* resistant to amoxicillin, tetracycline, ciprofloxacin, gentamicin and trimethoprim-sulfamethoxazole. However, in the third evaluation the two *E. coli* isolates already presented an antibiotic resistance profile equal to MDR *E. coli* detected in the second evaluation. The use of antibiotics induces profound changes at the individual microbiome structure [36]. Both *in vivo* and *in vitro* studies demonstrate that antibiotic treatment selects and favours resistant strains, leading to changes in the amount and composition of bacteria due to selective pressure exerted by the antibiotic [36]. Genes conferring resistance to other antibiotics than the one used, are also favoured, as happened in this case, due to co-selection of several antibiotic resistance genes in the same genetic platform originating selection of multiple genes when only one is the target of selection [37]. In fact, it is reported that even the administration of antibiotics during a short period of time may lead to the stabilization of populations of antibiotic resistant bacteria in the human gut, which may persist for years [36]. However, the microbiome demonstrates to be variable between people and antibiotics also contribute to that [38].

Volunteer number 6 and volunteer number 50, who were colonized with *Enterobacter aerogenes* and *Enterobacter cloacae* respectively, were no longer colonized with these bacteria in the second and third evaluations. They probably corresponded to transient strains, demonstrating once again the dynamics of microbiota.

Regarding volunteer number 50, in the second evaluation a carbapenem-resistant *Pseudomonas* spp. was detected. By analysing the questionnaire, the only change relatively to the previous evaluation was a trip to Germany and Austria in the week prior to the evaluation. Circulation of this type of bacteria in Germany was described, which raises the possibility of origin of the student's colonizing bacteria [39, 40]. In *P. aeruginosa* the resistance to carbapenems may be caused by the acquisition of carbapenemases, however the presence of intrinsic mechanisms responsible for antibiotic resistance is more common, such as hyperexpression of efflux pump systems, overexpression of cephalosporinases or loss of expression of the outer membrane protein OprD [41]. Of note is the fact that, in the third evaluation, this isolate has not been detected.

Despite the great variability in the composition of microbiome in healthy humans, temporal intra-individual variability is considerable and the level of variability of the individual experiences over time can be a determinant factor in disease and differential treatment success.

CONCLUSION

This is the first study that analyses the antibiotic resistance present in the colonizing intestinal flora of young adults from the university of Porto in *E. coli*. In fact, our study demonstrates that *E. coli* is the gut main colonizer in young

healthy adults and that this species presents multidrug antibiotic resistance in this reservoir, as ESBL. The fact that ESBL-producing *E. coli* have been found, with important virulence factors for the establishment of urinary tract infections and with the ability to transfer genes, demonstrates that healthy young adults can represent reservoirs of this type of bacteria and be responsible for the transfer of these genes. Additionally, we alert for limitations in therapeutic options, but also demonstrate that amoxicillin with clavulanic acid and gentamicin still effective and cefotaxime and meropenem, with none antibiotic resistance found, are a viable alternative.

It is important to study the bacteria that colonize the gut, in order to understand its diversity, composition and dynamics. It has been found that these bacteria vary over the time, although this knowledge is still limited. Our study demonstrates that the microbiome is dynamic, both in qualitative and quantitative ways. In addition, it has been found that the use of antibiotics causes changes in the type of bacteria that colonize the gastrointestinal tract.

The increase in the proportion of individuals colonized with antibiotic resistant bacteria in the community, increases the risk of other individuals becoming colonized, as a consequence of transmission between humans or through the environment, thereby increasing the antibiotic resistance and facilitating the acquisition of antibiotic resistance mechanisms by susceptible bacteria. Monitoring antibiotic resistance can help to identify factors that might contribute to the spread of antibiotic resistant bacteria and be a tool for the therapeutic decision to be instituted over time. In addition, it is necessary to characterize commensal bacteria to understand how commensals can become a dangerous pathogen.

Funding information
This work received no specific grant from funding agency

Acknowledgements
We are grateful to the students of FFUP and ICBAS who agreed to participate in this study. We thank Professor Luísa Peixe for supplying O25b-ST131 control strain and phylogenetic groups controls.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Voluntaries participating in this work gave their informed consent in the questionnaire This work received exemption from the ethical committee of Faculty of Pharmacy of University of Porto.

References

1. Ruppé E, Andremont A. Causes, consequences, and perspectives in the variations of intestinal density of colonization of multidrug-resistant enterobacteria. *Front Microbiol* 2013;4:129.
2. Karanika S, Karantanos T, Arvanitis M, Grigoras C, Mylonakis E. Fecal colonization with extended-spectrum beta-lactamase-producing *Enterobacteriaceae* and risk factors among healthy individuals: a systematic review and Metaanalysis. *Clin Infect Dis* 2016;63:310–318.
3. Sarkar S, Hutton ML, Vagenas D, Ruter R, Schüller S *et al*. Intestinal Colonization Traits of Pandemic Multidrug-Resistant *Escherichia coli* ST131. *J Infect Dis* 2018;218:979–990.
4. Rodrigues C, Machado E, Fernandes S, Peixe L, Novais Ângela. An update on faecal carriage of ESBL-producing *Enterobacteriaceae* by Portuguese healthy humans: detection of the H30 subclone of B2-ST131 *Escherichia coli* producing CTX-M-27. *J Antimicrob Chemother* 2016;71:1120–1122.
5. Maharjan A, Bhetwal A, Shakya S, Satyal D, Shah S *et al*. Ugly bugs in healthy guts! Carriage of multidrug-resistant and ESBL-producing commensal *Enterobacteriaceae* in the intestine of healthy Nepalese adults. *Infect Drug Resist* 2018;11:547–554.
6. Chirindze LM, Zimba TF, Sekyere JO, Govinden U, Chenia HY *et al*. Faecal colonization of *E. coli* and *Klebsiella* spp. producing extended-spectrum beta-lactamases and plasmid-mediated AmpC in Mozambican university students. *BMC Infect Dis* 2018;18:244.
7. Decker BK, Lau AF, Dekker JP, Spalding CD, Sinaii N *et al*. Healthcare personnel intestinal colonization with multidrug-resistant organisms. *Clin Microbiol Infect* 2018;24:82.e1–8282.
8. Hertz F, Nielsen K, Frimodt-Møller N. Selection of ESBL-producing *E. coli* in a mouse intestinal colonization model antibiotic resistance protocols. *Methods Mol Biol* 2018;1736:105–115.
9. Ebrahimi F, Mózes J, Monostori J, Gorácz O, Fésűs A *et al*. Comparison of rates of fecal colonization with extended-spectrum beta-lactamase-producing enterobacteria among patients in different wards, outpatients and medical students. *Microbiol Immunol* 2016;60:285–294.
10. Angelin M, Forsell J, Granlund M, Evengård B, Palmgren H *et al*. Risk factors for colonization with extended-spectrum beta-lactamase producing *Enterobacteriaceae* in healthcare students on clinical assignment abroad: A prospective study. *Travel Med Infect Dis* 2015;13:223–229.
11. Woerther P-L, Lepeule R, Burdet C, Decousser J-W, Ruppé Étienne *et al*. Carbapenems and alternative β-lactams for the treatment of infections due to extended-spectrum β-lactamase-producing *Enterobacteriaceae*: what impact on intestinal colonisation resistance? *Int J Antimicrob Agents* 2018;52:762–770.
12. Pires J, Kuenzli E, Hauser C, Tinguely R, Kasraian S *et al*. Intestinal colonisation with extended-spectrum cephalosporin-resistant *Enterobacteriaceae* in different populations in Switzerland: prevalence, risk factors and molecular features. *J Glob Antimicrob Resist* 2018;12:17–19.
13. Pires J, Kuenzli E, Kasraian S, Tinguely R, Furrer H *et al*. Polyclonal intestinal colonization with extended-spectrum cephalosporin-resistant *Enterobacteriaceae* upon traveling to India. *Front Microbiol* 2016;7:1069.
14. Mota R, Pinto M, Palmeira J, Gonçalves D, Ferreira H. Intestinal microbiota as a reservoir of extended-spectrum β-lactamase-producing *Escherichia coli*: An exploratory study in healthy university students. *J Glob Antimicrob Resist* 2018;14:10–11.
15. CLSI. *Performance Standards for Antimicrobial Susceptibility Testing. Twenty-Sixth Informational Supplement. CLSI document M100-S26*. Wayne, PA: Clinical and Laboratory Standards Institute; 2016.
16. Pires J, Novais A, Peixe L. Blue-Carba, an easy biochemical test for detection of diverse carbapenemase producers directly from bacterial cultures. *J Clin Microbiol* 2013;51:4281–4283.
17. Garcia-Migura L, Hendriksen RS, Fraile L, Aarestrup FM. Antimicrobial resistance of zoonotic and commensal bacteria in Europe: the missing link between consumption and resistance in veterinary medicine. *Vet Microbiol* 2014;170:1–9.
18. Tenaillon O, Skurnik D, Picard B, Denamur E. The population genetics of commensal *Escherichia* coli. *Nat Rev Microbiol* 2010;8:207–217.
19. Bailey JK, Pinyon JL, Anantham S, Hall RM. Commensal *Escherichia coli* of healthy humans: a reservoir for antibiotic-resistance determinants. *J Med Microbiol* 2010;59:1331–1339.
20. Dallenne C, Da Costa A, Decré D, Favier C, Arlet G. Development of a set of multiplex PCR assays for the detection of genes encoding important beta-lactamases in *Enterobacteriaceae*. *J Antimicrob Chemother* 2010;65:490–495.
21. Woerther P-L, Burdet C, Chachaty E, Andremont A. Trends in human fecal carriage of extended-spectrum β-lactamases in the

community: toward the globalization of CTX-M. *Clin Microbiol Rev* 2013;26:744–758.

22. Vila J, Sáez-López E, Johnson JR, Römling U, Dobrindt U *et al*. *Escherichia* coli: an old Friend with new tidings. *FEMS Microbiol Rev* 2016;40:437–463.
23. Cho S-H, Lim Y-S, Park M-S, Kim S-H, Kang Y-H. Prevalence of antibiotic resistance in *Escherichia* coli fecal isolates from healthy persons and patients with diarrhea. *Osong Public Health Res Perspect* 2011;2:41–45.
24. Erb A, Stürmer T, Marre R, Brenner H. Prevalence of antibiotic resistance in *Escherichia* coli: overview of geographical, temporal, and methodological variations. *Eur J Clin Microbiol Infect Dis* 2007;26:83–90.
25. Bevan ER, Jones AM, Hawkey PM. Global epidemiology of CTX-M β-lactamases: temporal and geographical shifts in genotype. *Journal of Antimicrobial Chemotherapy* 2017;72:2145–2155.
26. Geser N, Stephan R, Korczak BM, Beutin L, Hächler H. Molecular identification of extended-spectrum-β-lactamase genes from *Enterobacteriaceae* isolated from healthy human carriers in Switzerland. *Antimicrob Agents Chemother* 2012;56:1609–1612.
27. Vinué L, Sáenz Y, Martínez S, Somalo S, Moreno MA *et al*. Prevalence and diversity of extended-spectrum beta-lactamases in faecal *Escherichia coli* isolates from healthy humans in Spain. *Clin Microbiol Infect* 2009;15:954–957.
28. Hammerum AM, Lester CH, Jakobsen L, Porsbo LJ. Faecal carriage of extended-spectrum β-lactamase-producing and AmpC β-lactamase-producing bacteria among Danish army recruits. *Clin Microbiol Infect* 2011;17:566–568.
29. Gonçalves D, Cecílio P, Ferreira H. Nursing homes and long-term care facilities: Reservoirs of CTX-M-15-producing *Escherichia coli* O25b-ST131 in Portugal. *J Glob Antimicrob Resist* 2016;7:69–71.
30. Massot M, Daubié A-S, Clermont O, Jauréguy F, Couffignal C *et al*. Phylogenetic, virulence and antibiotic resistance characteristics of commensal strain populations of *Escherichia coli* from community subjects in the Paris area in 2010 and evolution over 30 years. *Microbiology* 2016;162:642–650.
31. Barreto Miranda I, Ignatius R, Pfüller R, Friedrich-Jänicke B, Steiner F *et al*. High carriage rate of ESBL-producing *Enterobacteriaceae* at presentation and follow-up among travellers with gastrointestinal complaints returning from India and South-east Asia. *J Travel Med* 2016;23:tav024.
32. Tängdén T, Cars O, Melhus A, Löwdin E. Foreign travel is a major risk factor for colonization with *Escherichia coli* producing CTX-M-type extended-spectrum beta-lactamases: a prospective study with Swedish volunteers. *Antimicrob Agents Chemother* 2010;54:3564–3568.
33. Davin-Regli A, Pagès J-M, Davin A. *Enterobacter aerogenes* and *Enterobacter cloacae*; versatile bacterial pathogens confronting antibiotic treatment. *Front Microbiol* 2015;6:392.
34. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D *et al*. Bad bugs, no drugs: no ESKAPE! an update from the infectious diseases Society of America. *Clin Infect Dis* 2009;48:1–12.
35. Valenza G, Tuschak C, Nickel S, Krupa E, Lehner-Reindl V *et al*. Prevalence, antimicrobial susceptibility, and genetic diversity of *Pseudomonas aeruginosa* as intestinal colonizer in the community. *Infect Dis* 2015;47:654–657.
36. Jernberg C, Löfmark S, Edlund C, Jansson JK. Long-term impacts of antibiotic exposure on the human intestinal microbiota. *Microbiology* 2010;156:3216–3223.
37. Modi SR, Collins JJ, Relman DA. Antibiotics and the gut microbiota. *J Clin Invest* 2014;124:4212–4218.
38. Gerber GK. The dynamic microbiome. *FEBS Lett* 2014;588:4131–4139.
39. Pfennigwerth N, Geis G, Gatermann SG, Kaase M. Description of IMP-31, a novel metallo-β-lactamase found in an ST235 *Pseudomonas aeruginosa* strain in Western Germany. *J Antimicrob Chemother* 2015;70:1973–1980.
40. Rieber H, Frontzek A, Baum H, Pfeifer Y. Emergence of Metallo-β-Lactamases GIM-1 and VIM in multidrug-resistant *Pseudomonas aeruginosa* in North Rhine–Westphalia, Germany. *J Antimicrob Chemother* 2012;64:1043–1045.
41. Castanheira M, Deshpande LM, Costello A, Davies TA, Jones RN. Epidemiology and carbapenem resistance mechanisms of carbapenem-non-susceptible *Pseudomonas aeruginosa* collected during 2009-11 in 14 European and Mediterranean countries. *J Antimicrob Chemother* 2014;69:1804–1814.