Antibiotic resistance patterns and beta-lactamase identification in *Escherichia coli* isolated from young children in rural Limpopo Province, South Africa: The MAL-ED cohort

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Background. Antibiotic resistance is a growing problem worldwide. Mechanisms of resistance vary, and some can confer resistance to multiple classes of antibiotics.
Objective. To characterise the antibiotic resistance profiles of *Escherichia coli* isolates obtained from stool samples of young rural children exposed or unexposed to antibiotics.
Methodology. The samples were collected from children aged 4 - 12 months who were participants in the Etiology, Risk Factors, and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development (MAL-ED) project at the South Africa research site. We isolated 87 *E. coli* samples (clones) from 65 individual participants, all of which were subjected to disc diffusion assay to determine resistance. We characterised the minimum inhibitory concentration of antibiotics in a subset of strains as well as the mechanism by which these strains were resistant to beta-lactam antibiotics.
Results. Our results revealed high resistance rates to co-trimoxazole (54.0%), penicillin (47.1%) and tetracycline (44.8%) in our isolates, and indicated that the beta-lactamase *TEM-1* is a prevalent source of beta-lactam resistance. We also identified two isolates with the extended-spectrum beta-lactamase CTX-M-14.
Conclusions. This study identified antibiotic-resistant *E. coli* in children with and without prior exposure to antibiotics, with some isolates showing resistance to multiple classes of antibiotics. Clinicians should bear in mind that transmission of extended-spectrum beta-lactamase-resistant *E. coli* exists at the community level, and that children as young as 2 years may be harbouring these resistant phenotypes.

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The problem of antibiotic resistance is not a new one. Multiple drug resistance in *Escherichia coli* was first observed in the 1950s. Transfer of resistance between species had also been observed by this time.[1] There has been evidence for the transfer of resistance genes between members of the human microbiota, as well as from livestock-associated bacteria to human-associated bacteria.[2] The presence of antimicrobial resistance genes even in non-pathogenic isolates therefore represents a problem, as these genes can easily be transferred to a pathogen.

Children acquire bacteria from their mother during birth,[3,4] and their gut microbiomes then undergo maturation during the first 3 years of life.[5] The early colonisation and development of this dynamic environment may predispose individuals to differences in disease incidence and outcomes. Considering that intestinal infectious diseases are the leading cause of death in children aged <14 years in Limpopo Province, South Africa (SA),[6] and that resistance genes can be geographically distinct, identification and monitoring of resistance mechanisms is important in order to foster appropriate treatment regimens. We therefore decided to focus on community isolates from children as opposed to clinical isolates, which are often the source of strains in studies focused on resistant organisms.

The Etiology, Risk Factors, and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development (MAL-ED) project was designed to look for correlations between factors present during childhood in developing regions, focusing on the relationship between enteric pathogen presence and growth and development outcomes. Each participant in MAL-ED had stool samples taken on at least a monthly basis from birth until age 2 years, and information on health events such as the incidence of diarrhoea and exposure to antibiotics was collected. In addition, developmental milestone data for each participant were recorded, such as height, weight and cognitive ability. This rich data set allowed us to consider which antibiotics were most relevant to the community, as we had details of exposure for all the study participants (supplementary Table 1: Appendix 1). Penicillin-class antibiotics are the most frequently administered, so resistance to this class would be most detrimental to health outcomes; we therefore determined that beta-lactamase genes would be an appropriate area of focus.

Objective

To present data on *E. coli* strains isolated from stool samples collected as part of the MAL-ED study. The children from whom the strains

were isolated ranged in age from 4 to 12 months and had varying histories in terms of antibiotic exposure and diarrhoeal events. The antibiotic susceptibilities of the isolates were characterised, along with their minimum inhibitory concentrations (MICs) and identification of some of the beta-lactamase genes responsible. For beta-lactam-resistant isolates, we tested for variants of several narrow-spectrum beta-lactamases (*TEM*, *SHV* and *OXA*), as well as some variants of the *CTX-M* extended-spectrum beta-lactamase (ESBL) type.

Methods

Ethical considerations

The study protocol was approved by the Research Ethics Committee of the University of Venda, SA (ref. no. SMNS/09/MBY/004). Permission was obtained from the Department of Health, Limpopo Province (ref. no. 4/2/2), SA. Signed informed consent was obtained from the parents or legal guardians of all study subjects prior to enrolment and sample collection.

Strain isolation and growth

E. coli strains were isolated from stool samples collected as part of the MAL-ED study, and in addition *E. coli* ATCC 25922 (Microbiologics, USA) was maintained as a control strain. Initial isolation of lactose fermenters (pink colonies) was performed on MacConkey agar (Neogen, USA), followed by screening on EMB agar (Neogen, USA), on which *E. coli* produce a characteristic green sheen. In total, 87 strains were isolated from 65 study participants. Cultures were maintained in nutrient broth (Oxoid, UK) or on nutrient agar (Neogen, USA) at 37°C.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed using the disc diffusion assay.[7] Briefly, colonies were resuspended in sterile saline to a McFarland standard of 0.5, and were uniformly spread on Mueller-Hinton agar (Mast Diagnostics, UK) using sterile cotton swabs. Antibiotic discs (Mast Diagnostics, UK) were dispensed using the Discmaster 3 Dispenser (Mast Diagnostics, UK), and plates were incubated overnight at 37°C. Zones of inhibition were then measured against a black, non-reflecting background. *E. coli* ATCC 25922 was utilised as a control strain to ensure that zones of inhibition were within an appropriate range, according to Clinical and Laboratory Standards Institute (CLSI) standards.[7] The CLSI zone diameter interpretive criteria for Enterobacteriaceae were used for interpretation of all antibiotic inhibitory zones except those of the macrolides. For azithromycin, a zone ≤13 mm was considered to indicate resistance, a zone of 14 - 17 mm was considered the intermediate range, and a zone ≥18 mm was considered to indicate sensitivity.

Detection and identification of beta-lactamases

Multiplex polymerase chain reactions (PCRs) for detection of beta-lactamase genes were designed by Dallenne *et al*.[8] The first multiplex PCR amplified bla_{TEM}/bla_{SHV}/bla_{OXA-1}-like genes using the following primers: MultiTSO-T_for CATTTCCGTGTCGCCCTTATTC (0.4 µM)/MultiTSO-T_rev CGTTCATCCATAGTTGCCTGAC (0.4 µM) – product size 800 bp, MultiTSO-S_for AGCCGCTTGAGCAAATTAAAC (0.4 µM)/MultiTSO-S_rev ATCCCGCAGATAAATCACCAC (0.4 µM) – product size 713 bp, and MultiTSO-O_for GGCACCAGATTCAACTTTCAAG (0.4 µM)/MultiTSO-O_rev GACCCCAAGTTTCCTGTAAGTG (0.4 µM) – product size 564 bp. The second amplified bla_{CTX-M} phylogenetic groups 1, 2 and 9 using the following primers: MultiCTXMGp1_for TTAGGAARTGTGCCGCTGYA (0.4 µM)/MultiCTXMGp1-2_rev CGATATCGTTGGTGGTRCCAT (0.2 µM) – product size 688 bp, MultiCTXMGp2_for CGTTAACGGCACGATGAC (0.2 µM)/MultiCTXMGp1-2_rev CGATATCGTTGGTGGTRCCAT (0.2 µM) – product size 404 bp, and MultiCTXMGp9_for TCAAGCCTGCCGATCTGGT (0.4 µM)/MultiCTXMGp9_rev TGATTCTCGCCGCTGAAG (0.4 µM) – product size 561 bp. PCRs were performed in duplicate, one replicate using DNA isolated with the GeneJET Plasmid Miniprep Kit (Thermo Scientific, USA) and one using colonies resuspended in 100 µL of water and subjected to heating at 95°C for 10 minutes.

PCRs were performed using DreamTaq Green Master Mix (Thermo Scientific, USA) in 50 µL reactions, using the primer concentrations specified above. The amplification reaction was performed as in Dallenne *et al*.:[8] 94°C for 10 minutes; [94°C for 40 seconds, 60°C for 40 seconds and 72°C for 1 minute] × 30 cycles; 72°C for 7 minutes. Amplified beta-lactamase genes were visualised under ultraviolet light following separation on a 2% agarose gel containing ethidium bromide, along with a GeneRuler 100 bp Plus ladder (Thermo Scientific, USA). Products were purified using the GeneJET PCR Purification Kit (Thermo Scientific, USA) and sent for sequencing (Inqaba Biotec, SA).

Sequencing results were analysed using Geneious 7 (http://www.geneious.com), and the data were searched using the NCBI Nucleotide BLAST Megablast tool (http://blast.ncbi.nlm.nih.gov/), optimised for highly similar sequences.

Minimum inhibitory concentrations

MICs were determined using MIC Test Strips (Liofilchem, Italy). Antimicrobial susceptibility testing was performed according to the manufacturer's guidelines. Briefly, colonies of each strain were suspended in sterile saline solution to achieve a 0.5 McFarland standard turbidity level, and were uniformly spread on Mueller-Hinton agar (Mast Diagnostics, UK) using sterile cotton swabs. Once the agar surface was completely dry, an MIC test strip was applied to each plate with sterile forceps and the plates were incubated at 37°C for 18 - 24 hours. The MIC was read where inhibition of growth intersected the strip.

Results

Antibiotic susceptibility of isolates

Each *E. coli* clone was tested for susceptibility to 13 antibiotics or combination therapies using the disc diffusion method.[7] Results of testing are shown in Table 1. The highest incidence of resistance was to the trimethoprim-sulfamethoxazole combination antibiotic (co-trimoxazole), with 54.0% of isolates showing resistance. The second highest incidence of resistance was towards penicillin-class antibiotics, with 47.1% of isolates showing resistance to ampicillin and amoxicillin. Much of this resistance appeared to be reversed by the inclusion of a beta-lactamase inhibitor, clavulanic acid. We did not observe resistance to several antibiotics including imipenem, ciprofloxacin, and both aminoglycosides tested. Although not typically included in Gram-negative susceptibility studies owing to their inefficient penetration of the cell wall,[9] a macrolide antibiotic was tested, as *E. coli* has been shown to be a potential reservoir for macrolide resistance genes.[10] The azithromycin results are reported in Table 1, and indicate that ~20% of isolates show some enhanced resistance to this class.

Isolates showing no resistance were most abundant (n=29), whereas the second-largest subset of isolates (n=24) showed resistance to three antibiotics, indicating that resistance to more than one antibiotic is more common than resistance to one (n=14 isolates) or two (n=7). Additional information detailing which resistances were observed in which clones can be found in supplementary Table 2 (Appendix 2).

Table 1. Results of disc diffusion tests for antimicrobial resistance

Class of antibiotic	Antibiotic (μg*)	Resistant, *n* (%)†	Intermediate, *n* (%)†	Sensitive, *n* (%)†
Penicillins	Ampicillin (10) or amoxicillin (10)‡	41 (47.1)	0	46 (52.9)
	Amoxicillin (20) + clavulanic acid (10)	1 (1.2)	1 (1.2)	85 (97.7)
Cephalosporins	Cefotaxime (30)	2 (2.3)	0	85 (97.7)
Carbapenems	Imipenem (10)	0	0	87 (100)
Quinolones	Nalidixic acid (30)	5 (5.7)	4 (4.6)	78 (89.7)
Fluoroquinolones	Ciprofloxacin (5)	0	0	87 (100)
Folate pathway inhibitor/ sulfonamides	Trimethoprim (1.25) + sulfamethoxazole (23.75)	47 (54.0)	0	40 (46.0)
Phenicols	Chloramphenicol (30)	9 (10.3)	0	78 (89.7)
Tetracyclines	Tetracycline (30)	39 (44.8)	0	48 (55.2)
Aminoglycosides	Gentamicin (10)	0	0	87 (100)
	Amikacin (30)	0	0	87 (100)
Macrolides	Azithromycin (15)	10 (11.5)	7 (8.1)	70 (80.5)

*The mass of the antibiotic, or the mass of each component for mixtures.
†Number of clones that exhibit a given phenotype, and percentage of all clones showing this phenotype.
‡Although results of ampicillin testing can be used to predict results for amoxicillin,[7] both were included. The results for the two were identical.

Table 2. Beta-lactamases present in penicillin-resistant strains

Beta-lactamase gene*	Positive clones, *n* (%)	Gene identity
TEM (includes *TEM-1*, *TEM-2*)	39 (95.1)	18/39 tested, all *TEM-1*
CTX-M group 9 (includes *CTX-M-9* and *CTX-M-14*)	2 (4.9)	2/2 tested, both *CTX-M-14*

*Other beta-lactamases tested but not identified in these samples include variants of SHV, OXA, and CTX-M groups 1 and 2.

Presence and identity of beta-lactamase genes

All strains resistant to penicillins were subjected to multiplex PCR amplification to determine which beta-lactamases were present. The multiplex PCRs, originally designed by Dallenne *et al.*,[8] can identify the presence of certain *TEM*, *SHV*, *OXA* and *CTX-M* variants. TEM, SHV and OXA are narrow-spectrum beta-lactamases, whereas CTX-M is an ESBL. The 41 strains tested included one isolate with resistance to the combination treatment of amoxicillin + clavulanic acid. Results of multiplex PCR are shown in Table 2. A subset of the PCR products were sequenced and analysed by BLAST, which revealed that both of the CTX-M group 9 beta-lactamases present were in fact CTX-M-14. As for the samples positive for TEM, products from 18 clones were sent for sequencing, which revealed that all 18 were TEM-1. This result is not surprising considering that this is the most frequently observed resistance gene in enterobacteria.[11] The sequences of the amplified regions can be found in supplementary Table 3 (Appendix 3).

Minimum inhibitory concentrations

MIC ranges were 0.016 - 0.094 μg/mL for ciprofloxacin, 0.125 - 4.0 μg/mL for imipenem, 0.38 - 2 μg/mL for gentamicin, 1.5 - 3 μg/mL for amikacin, 1.0 - 24.0 μg/mL for amoxicillin + clavulanic acid, 1.5 - 32 μg/mL for azithromycin, 0.047 - 0.125 μg/mL for cefotaxime, 4.0 - 48 μg/mL for chloramphenicol and 0.125 - 24.0 μg/mL for nalidixic acid (Table 3). Some MIC values outside the test range were observed for a subset of strains tested when exposed to chloramphenicol and nalidixic acid.

Strains showing resistance phenotypes to beta-lactam antibiotics were subjected to multiplex PCR to determine the presence of specific beta-lactamase genes. A subset of these products was sequenced, allowing identification of the specific beta-lactamase gene using BLAST.

Discussion and conclusion

When deciding which antibiotics to test in the disc diffusion assays, we considered the most frequently used antibiotics in the SA site MAL-ED participant group (supplementary Table 1). This provided useful information on the local clinical usage of various classes of antibiotics, with the penicillin class being most common, followed by sulfonamides, macrolides and others. With penicillin-class antibiotics being most commonly employed in treatment of illness, resistance to this class would have the most negative impact, so we chose to focus on resistance mechanisms to this class. Penicillin resistance was in fact the second most prevalent in our study, after co-trimoxazole resistance, which is a worrying trend considering the common use of penicillin in the management of bacterial infections.

Multidrug resistance is resistance to multiple classes of antibiotics. Of the 87 isolates tested, 2 showed resistance to five antibiotics (ampicillin/amoxicillin, co-trimoxazole, chloramphenicol, tetracycline and nalidixic acid) and 11 showed resistance to four antibiotics. Interestingly, the two most multidrug-resistant isolates were from individuals with no reported exposure to antibiotics. The two strains that harbour the *CTX-M-14* resistance gene both show resistance to three antibiotics: cefotaxime, ampicillin/amoxicillin and tetracycline. Although antibiotic exposure increases the selective pressure for organisms to develop and maintain antibiotic-resistant elements, the presence of resistance genes apparently does not necessarily correlate with prior exposure to antimicrobial agents.

Table 3. MIC (µg/mL) of different antibiotics against *E. coli* clones

Clone no.	CIP	IMI	CN	AK	AUG	AZM	CTX	C	NA
91	0.023	0.5	0.75	2	6	256	0.047	8	3
92	0.023	0.19	0.75	2	6	2	0.094	4	4
96	0.023	0.125	0.75	2	3	1.5	0.064	6	3
98	0.094	0.38	0.5	2	6	8	0.047	8	4
102	0.023	0.25	0.75	2	3	256	0.094	6	6
111	0.023	0.25	0.5	2	6	24	0.094	8	4
112	0.016	1	1	3	6	32	0.094	256	2
115	0.019	4	0.75	3	6	8	0.064	6	256
116.1	0.023	0.19	0.75	1.5	3	1.5	0.064	6	4
116.2	0.023	0.19	0.75	2	6	1.5	0.094	4	3
120	0.016	0.25	1.5	1.5	8	256	0.125	8	4
121	0.023	1	0.75	2	1	4	0.047	8	4
127	0.023	4	0.75	3	12	32	0.094	8	6
130	0.023	3	1	2	4	3	0.047	6	4
131	0.094	3	0.75	2	24	8	0.125	8	6
132	0.023	3	0.75	3	3	16	0.064	8	4
133	0.023	0.25	0.75	2	8	8	0.064	8	6
138	0.023	0.38	0.75	2	3	4	0.064	6	6
140	0.023	4	0.75	2	3	4	0.094	8	4
141	0.023	0.25	0.75	1.5	8	6	0.094	6	4
145	0.023	0.25	0.75	3	6	4	0.047	8	6
151	0.023	0.19	0.75	3	6	8	0.064	8	6
158.1	0.032	0.5	2	3	4	4	0.094	24	16
158.2	0.047	4	0.75	3	6	16	0.094	48	24
159	0.023	1.5	0.75	2	4	8	0.064	12	3
163	0.012	0.25	1.5	3	6	8	0.064	8	3
167	0.023	1	1.5	2	12	256	0.125	12	6
172	0.023	0.38	0.75	3	4	6	0.064	12	6
173	0.016	4	1.5	3	16	256	0.125	12	12
181	0.016	0.38	0.75	1.5	8	6	0.125	6	6

CIP = ciprofloxacin; IMI = imipenem; CN = gentamicin; AK = amikacin; AUG = amoxicillin + clavulanic acid; AZM = azithromycin; CTX = cefotaxime; C = chloramphenicol; NA= nalidixic acid.

To characterise the range of MICs for our isolates, a subset was tested using MIC test strips. Results for amikacin, amoxicillin + clavulanic acid, chloramphenicol, ciprofloxacin, cefotaxime, gentamicin and nalidixic acid were all consistent with the disc diffusion assay results. For imipenem, ~16% of the MIC results indicated resistance where the disc diffusion assay had not indicated this, as did ~7% for azithromycin. This indicates that the proportion of resistant isolates may actually be higher than we reported. There was also one azithromycin test that showed resistance in the disc diffusion and sensitivity in the MIC testing.

Some participants in the MAL-ED study went on to develop severe acute malnutrition. Treatment for this condition often involves administration of antibiotics, as recommended by the World Health Organization (WHO); however, there is no strong evidence that this is the best course of action.[12] Considering our evidence that even without antibiotic exposure, children at risk of malnutrition often harbour resistance genes, it seems that the introduction of additional selective pressure could actually contribute to ill health rather than recovery. A 2014 study[13] found that the diversity of antibiotic resistance genes in the human gut microbiota appears to increase with age, although they did not look at individuals <3 years of age. This would indicate that even without antibiotic administration, the burden of resistance would increase over time.

The diversity of beta-lactamases is high, and many novel variants are reported year after year. In the early 1990s there were fewer than 150 known beta-lactamases, and by 2009, over 890 unique beta-lactamase sequences had been identified.[14] New enzymes often emerge in isolated areas and go on to expand their host range and also their geographical range. *TEM-1*, which we observed in a majority of penicillin-resistant isolates, is the most commonly found secondary beta-lactamase in ampicillin-resistant *E. coli*,[11] with much greater prevalence than *TEM-2*, *SHV* and *OXA-1* (the other narrow-spectrum beta-lactamases that we tested for).[15] ESBLs are beta-lactamases with enhanced activity against cephalosporins, early examples of which were similar to TEM and SHV. The CTX-M-type ESBL was first observed in the late 1980s, and is not TEM- or SHV-derived.[16]

Although previous studies in SA found examples of multiple ESBLs being produced by clinical isolates,[17-20] the first report of CTX-M-type ESBLs was not until 2003, where CTX-M-2 and CTX-M-3 were found in *Klebsiella pneumoniae*.[21] Since then, other CTX-M types have been found in SA, including CTX-M-14, CTX-M-15 and CTX-M-37.[22-25] The WHO reports that in the African Region there are insufficient data concerning antibiotic resistance,[26] so additional reports such as this are important in this regard. The data presented are limited to the MAL-ED SA Dzimauli community study site of Limpopo Province.[27] However, our identification of the *CTX-M-14* ESBL in community *E. coli* isolates, even in young children who have not received antibiotics, adds to the greater picture of the antibiotic resistance landscape in SA and in the African Region.

The prescribing patterns of antibiotics, either through excessive use or through sub-therapeutic doses, in addition to the use of antibiotics in animal production, are factors known to contribute to the development and spread of antibiotic resistance at community level. Our finding has clinical relevance. Firstly, it adds to the body of evidence on the spread of antibiotic resistance in rural communities, and secondly, it supports the increasing need for a reduction in the frequency of empirically prescribing antibiotics, a common practice

in communities without diagnostic laboratory support. Discouraging empirical prescription has been proposed in the SA national approach to 'antibiotic stewardship'.[28] In conclusion, clinicians and public health practitioners should bear in mind that transmission of ESBL-resistant *E. coli* exists at the community level and that children as young as 2 years, even without prior exposure to antibiotics, may be harbouring these resistant phenotypes, an awareness that should guide prescription practices.

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Appendix 1

Supplementary Table 1. Antibiotic exposure of SA site MAL-ED participants and antibiotics used in the study*

Class	Days of exposure	Representative antibiotic(s) tested
Penicillins	3 083	Ampicillin, amoxicillin, amoxicillin + clavulanic acid
Sulfonamides	395	Trimethoprim + sulfamethoxazole
Macrolides	289	Azithromycin, erythromycin
Metronidazole	140	-
Other/unknown	107	Imipenem, chloramphenicol, gentamicin, amikacin
Tetracyclines	27	Tetracycline
Cephalosporins	6	Cefotaxime
Fluoroquinolones	1	Ciprofloxacin, nalidixic acid

*This table lists the total days of exposure to different classes of antibiotics for all SA site participants in the MAL-ED study. This provided information on the most frequently used antibiotics in the community, which informed our selection of antibiotics.

Appendix 2

Supplementary Table 2. Demographic and microbial characteristics of the individual clones studied

Sample information			Antibiotic resistance									Beta-lactamase presence			Prior antibiotic exposure						
Clone	PID	Age	A/AP	ATH	AUG	C	CTX	NA	T	TS	#RES	PCR 1	PCR 2	Gene	PEN	SUL	MAC	TET	MET	OTH	#EXP
1	306	10	S	S	S	S	S	S	S	S	0	*	*	*	0	0	0	0	0	0	0
5.1	302	8	S	S	S	S	S	S	R	R	2	*	*	*	4	0	0	0	0	0	1
5.2	302	8	R	S	S	S	R	S	R	S	3	-	Group 9	*CTX-M-14*	4	0	0	0	0	0	1
8	299	6	S	S	S	S	S	S	R	S	1	*	*	*	0	0	0	0	0	0	0
11.1	307	9	S	S	S	S	S	S	S	S	0	*	*	*	25	7	0	0	0	0	2
11.2	307	9	R	S	S	R	S	S	S	R	3	*TEM*	-	*TEM-1*	25	7	0	0	0	0	2
12	308	8	R	R	S	S	S	S	R	R	4	*TEM*	-	*TEM-1*	0	11	0	0	0	0	1
14	238	9	R	R	S	S	S	S	R	R	4	*TEM*	-	*TEM-1*	0	0	0	0	0	0	0
15	309	10	S	S	S	S	S	S	S	S	0	*	*	*	0	0	0	0	0	0	0
16	310	8	S	S	S	S	S	S	S	S	0	*	*	*	0	0	0	0	0	0	0
17	310	9	R	S	R	S	S	R	S	R	4	*TEM*	-	*TEM-1*	0	0	0	0	0	0	0
18	310	10	S	S	S	S	S	I	S	S	0	*	*	*	0	0	0	0	0	0	0
21	316	8	S	S	S	S	S	S	R	S	1	*	*	*	0	0	0	0	0	0	0
22	315	11	R	S	S	S	S	S	R	R	3	*TEM*	-	*TEM-1*	0	0	24	0	0	0	1
25	314	8	R	S	S	S	R	S	R	S	3	-	Group 9	*CTX-M-14*	7	0	1	0	0	2	4
29	313	11	R	S	S	R	S	S	S	R	3	*TEM*	-	*TEM-1*	30	0	0	0	0	0	1
30	312	10	R	S	S	S	S	S	R	R	3	*TEM*	-	*TEM-1*	10	5	0	0	0	0	2
31	257	12	R	S	S	R	S	R	R	R	5	*TEM*	-	*TEM-1*	0	0	0	0	0	0	0
32	258	9	S	S	S	S	S	S	R	R	2	*	*	*	0	0	0	0	0	0	0
34.1	260	11	R	S	S	S	S	S	R	R	3	*TEM*	-	*	0	0	0	0	0	0	0
34.2	260	11	S	S	S	S	S	S	S	S	0	*	*	*	0	0	0	0	0	0	0
35	261	10	S	S	S	S	S	S	S	S	0	*	*	*	0	0	0	0	0	0	0
37	262	11	S	S	S	S	S	S	S	S	0	*	*	*	0	0	0	0	0	0	0
39	264	12	R	S	S	R	S	S	S	R	3	*TEM*	-	*TEM-1*	2	0	0	0	0	0	1
42.1	272	12	R	S	S	S	S	S	S	R	2	*TEM*	-	*	0	0	0	0	0	0	0
42.2	272	12	R	S	S	S	S	S	R	R	3	*TEM*	-	*	0	0	0	0	0	0	0
46	270	10	R	S	S	S	S	S	R	R	3	*TEM*	-	*TEM-1*	0	0	0	0	0	0	0

Continued ...

Supplementary Table 2. (continued) Demographic and microbial characteristics of the individual clones studied

Sample information			Antibiotic resistance									Beta-lactamase presence			Prior antibiotic exposure						
Clone	PID	Age	A/AP	ATH	AUG	C	CTX	NA	T	TS	#RES	PCR 1	PCR 2	Gene	PEN	SUL	MAC	TET	MET	OTH	#EXP
47	268	10	R	R	S	S	S	S	R	R	4	*TEM*	-	*TEM-1*	3	0	0	0	0	0	1
48	269	11	S	S	S	S	S	S	S	S	0	*	*	*	0	0	0	0	0	0	0
49	267	12	S	S	S	S	S	R	S	S	1	*	*	*	0	0	0	0	0	0	0
55	274	12	S	S	S	S	S	S	S	R	1	*	*	*	0	0	0	0	0	0	0
56	275	11	R	S	S	S	S	S	R	R	3	*TEM*	-	*	0	0	0	0	0	0	0
58	276	9	S	S	S	S	S	S	R	S	1	*	*	*	2	0	0	0	0	0	1
60	237	12	S	S	S	S	S	S	R	S	1	*	*	*	0	0	0	0	0	0	0
61	309	8	R	S	S	R	S	R	R	R	5	*TEM*	-	*TEM-1*	0	0	0	0	0	0	0
63	239	9	R	S	S	R	S	S	R	R	4	*TEM*	-	*TEM-1*	5	0	0	0	0	0	1
66	244	11	S	I	S	S	S	S	S	S	0	*	*	*	4	0	0	0	4	0	2
67	245	11	R	R	S	S	S	S	S	R	3	*TEM*	-	*TEM-1*	10	0	0	0	0	0	1
68	244	12	S	S	S	S	S	S	S	S	0	*	*	*	4	0	0	0	4	0	2
70	247	10	R	S	S	S	S	S	R	R	3	*TEM*	-	*	0	0	0	0	0	0	0
71	208	11	S	S	S	S	S	S	S	S	0	*	*	*	0	0	0	0	0	0	0
72	202	12	S	S	S	S	S	S	S	S	0	*	*	*	0	0	0	0	0	0	0
73	197	12	R	S	S	R	S	I	R	R	4	*TEM*	-	*TEM-1*	0	0	0	0	0	0	0
75	255	12	S	S	S	S	S	S	S	S	0	*	*	*	6	0	0	0	0	0	1
77	250	12	S	I	I	S	S	S	S	S	0	*	*	*	2	0	0	0	2	0	2
78	255	10	R	S	S	S	S	S	R	R	3	*TEM*	-	*TEM-1*	0	0	0	0	0	0	0
80	248	12	S	S	S	S	S	S	S	R	1	*	*	*	4	0	0	0	0	0	1
85	217	11	S	S	S	S	S	S	R	R	2	*	*	*	1	0	0	0	0	0	1
89	227	11	R	S	S	S	S	S	R	R	3	*TEM*	-	*TEM-1*	3	0	0	0	1	0	2
90	234	12	S	S	S	S	S	S	S	S	0	*	*	*	0	0	0	0	0	0	0
91	283	9	R	R	S	S	S	S	S	R	3	*TEM*	-	*	0	0	0	0	0	0	0
92	282	10	R	S	S	S	S	S	S	R	2	*TEM*	-	*	2	0	0	0	0	0	1
96	280	12	S	S	S	S	S	S	S	S	0	*	*	*	13	0	0	0	0	0	1
98	280	12	R	R	S	S	S	S	S	R	3	*TEM*	-	*	20	0	0	0	0	0	1
102	293	10	S	S	S	S	S	S	S	R	1	*	*	*	3	0	0	0	0	0	1
103	291	8	R	R	S	S	S	S	R	R	4	*TEM*	-	*	0	0	0	0	0	0	0
104	288	11	S	S	S	S	S	S	S	S	0	*	*	*	0	0	0	0	0	0	0
105	288	10	S	I	S	S	S	S	S	S	0	*	*	*	0	0	0	0	0	0	0

Continued ...

Supplementary Table 2. (continued) Demographic and microbial characteristics of the individual clones studied

Sample information			Antibiotic resistance									Beta-lactamase presence			Prior antibiotic exposure						
Clone	PID	Age	A/AP	ATH	AUG	C	CTX	NA	T	TS	#RES	PCR 1	PCR 2	Gene	PEN	SUL	MAC	TET	MET	OTH	#EXP
109	283	10	R	S	S	S	S	S	R	R	3	*TEM*	-	*	0	0	0	0	0	0	0
110	342	8	S	S	S	S	S	S	S	S	0	*	*	*	16	0	0	0	0	0	1
111	342	7	S	S	S	S	S	S	S	S	0	*	*	*	10	0	0	0	0	0	1
112	341	8	R	I	S	R	S	S	S	R	3	*TEM*	-	*TEM-1*	15	0	0	0	3	0	2
115	341	6	R	S	S	S	S	R	R	R	4	*TEM*	-	*	15	0	0	0	0	0	1
116.1	295	8	S	S	S	S	S	S	S	S	0	*	*	*	0	0	0	0	0	0	0
116.2	295	8	S	S	S	S	S	S	S	R	1	*	*	*	0	0	0	0	0	0	0
117	294	10	R	S	S	S	S	S	R	R	3	*TEM*	-	*	0	0	0	0	0	0	0
120	354	8	R	R	S	S	S	S	R	R	4	*TEM*	-	*	0	0	0	0	0	0	0
121	354	4	S	S	S	S	S	S	S	S	0	*	*	*	0	0	0	0	0	0	0
127	348	8	R	R	S	S	S	S	R	R	4	*TEM*	-	*	3	0	0	0	0	0	1
130	342	9	S	S	S	S	S	S	S	R	1	*	*	*	18	0	0	0	0	0	1
131	363	5	S	S	S	S	S	S	S	S	0	*	*	*	4	0	0	0	0	0	1
132	361	8	S	S	S	S	S	S	S	S	0	*	*	*	11	0	0	0	0	0	1
133	361	6	R	S	S	S	S	S	R	S	2	*TEM*	-	*	11	0	0	0	0	0	1
138	358	10	S	S	S	S	S	S	S	S	0	*	*	*	0	0	0	0	0	0	0
140	202	4	S	I	S	S	S	S	S	R	1	*	*	*	0	0	0	0	0	0	0
141	294	8	R	S	S	S	S	S	R	R	3	*TEM*	-	*	0	0	0	0	0	0	0
145	157	7	R	S	S	S	S	S	S	S	1	*TEM*	-	*	0	0	0	0	0	3	1
151	317	11	S	S	S	S	S	S	S	S	0	*	*	*	3	0	0	0	0	0	1
158.1	322	8	S	S	S	S	S	I	R	S	1	*	*	*	13	0	0	0	0	0	1
158.2	322	8	R	S	S	R	S	I	R	S	3	*TEM*	-	*	13	0	0	0	0	0	1
159	279	10	S	S	S	S	S	S	S	S	0	*	*	*	26	0	0	8	0	0	2
163	226	12	S	S	S	S	S	S	S	R	1	*	*	*	0	0	0	0	0	0	0
167	333	8	R	R	S	S	S	S	R	R	4	*TEM*	-	*	0	3	0	0	0	2	2
172	334	9	S	I	S	S	S	S	S	S	0	*	*	*	0	0	0	0	0	0	0
173	301	10	S	I	S	S	S	S	R	R	2	*	*	*	2	0	0	0	0	0	1
176	363	4	R	S	S	S	S	S	R	R	3	*TEM*	-	*	4	0	0	0	0	0	1
181	204	12	R	S	S	S	S	S	R	R	3	*TEM*	-	*	20	0	0	0	0	0	1

PID = child ID; Age = rounded age (age in days/30.44); A/AP = amoxicillin/ampicillin; ATH = azithromycin; AUG = amoxicillin + clavulanic acid; C = chloramphenicol; CTX = cefotaxime; NA = nalidixic acid; T = tetracycline; TS = trimethoprim + sulfamethoxazole.
Note: Ciprofloxacin, imipenem, gentamicin and amikacin were tested but are not listed because all isolates were susceptible to these antibiotics. #RES = total number of classes tested to which resistance was observed; in this case A/AP are counted as one class;
PCR1 and PCR2 = the results of the multiplex PCRs to distinguish *TEM*, *SHV* and *OXA* variants and *CTX-M* variants, respectively; Gene = the specific beta-lactamase identified as a result of sequencing the PCR product. The individual exposures to penicillins (PEN), sulfonamides (SUL), macrolides (MAC), tetracyclines (TET), metronidazole (MET) and other (OTH) are listed next, with the number of days of exposure to each class indicated. #EXP = how many classes of antibiotics the individual was exposed to prior to sample collection.
Note: none of the participants had exposure to cephalosporins or fluoroquinolones.
*PCR was not performed on this sample, indicating that no product was observed.

Appendix 3

Supplementary Table 3. Derived nucleotide sequences of beta-lactamase genes studied*

Strains tested	Group	Sequence of beta-lactamase gene
Clones 5.2 and 25	CTX-M-14	TTGATTCTCGCCGCTGAAGCCAGCACATCGCGGCGGCTCTCTGCGTTCTGTTGCGGCTGGGT AAAATAGGTCACCAGAACCAGCGGCGCACGACCCTGCGGCCAGATCACCGCAATATCATTGG TGGTGCCGTAGTCGCCGCTGCCGGTCTTATCACCCACAGTCCACGACGTCGGTAAGCCGGCC CGAATGCTGGCTGCGCCGGTCGTATTGCCTTTGAGCCACGTCACCAACTGCGCCCGCTGGGT TTCGCCCAGCGCATGACCCAGCGTAAGCTGACGCAACGTCTGCGCCATCGCCCGCGGCGTGG TGGTGTCTCTCGGGTCGCCGGGAATGGCGGTATTCAGCGTAGGTTCAGTGCGATCCAGACGA AACGTCTCATCGCCGATCGCGCGGGCAAAAGCCGTCACGCCTCCCGGGCCACCGAGCTGGG CAATCAATTTGTTCATGGCGGTATTGTCGCTGTACTGCAACGCGGCCGCGCTCAGTTCTGC CAGCGTCATTGTGCCGTTGACGTGTTTTTCGGCAATCGGATTGTAGTTAACCAGATCGGC AGGCTTGAA
Clones 11.2, 12, 14, 17, 22, 29, 30, 31, 39, 46, 47, 61, 63, 67, 73, 78, 89, 112	TEM-1	TGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACG CTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGAT CTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTT TTAAAGTTCTGCTATGTGGTGCGGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACTCGGTCG CCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACG GATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCTGCC AACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGG GATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGA GCGTGACACCACGATGCCTGCAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAAC TACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGA CCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTG AGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCG TAGTTATCTACACGACGGGGAGTCAGGCAACTATGGG

*This table lists the sequences obtained for the beta-lactamase gene regions amplified by PCR. The first column indicates which clones these sequences were amplified from, the second column indicates which beta-lactamase group these genes fall into (as determined by BLAST), and the third column contains the sequence information. No variation in sequence was observed for clones that fell into the same beta-lactamase group.