Colonization dynamics of ampicillin-resistant \textit{Escherichia coli} in the infantile colonic microbiota

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Objectives: To compare the colonization dynamics of ampicillin-resistant and ampicillin-susceptible \textit{Escherichia coli} strains in the infantile intestinal microbiota.

Methods: We followed 128 infants over the first year of life with regular quantitative faecal cultures and recordings of antibiotic treatment. \textit{E. coli} strains were quantified, and their resistance pattern and carriage of \(\beta\)-lactamase genes (TEM, SHV and OXA), phylogenetic group (A, B1, B2 or D), virulence gene profile (\(fimA\), \(papC\), \(staD/E\), \(kfiC\) neuB, \(hlyA\) and \(iutA\)) and time of persistence in the microbiota were determined.

Results: Twelve percent (\(n = 32\)) of the \textit{E. coli} strains were resistant to ampicillin, as they carried the \(bla\)\textsubscript{TEM} (84%) or \(bla\)\textsubscript{SHV} genes. Ampicillin-resistant strains belonged mostly to phylogenetic group D and carried \(pap\) genes (\(P = 0.023\)) significantly more often than ampicillin-susceptible strains due to a strong association between carriage of \(pap\) and \(bla\)\textsubscript{SHV}. In 31 of 32 cases, colonization by ampicillin-resistant strains occurred in infants not previously treated with \(\beta\)-lactam antibiotics. Ampicillin-resistant strains were equally capable as susceptible ones of persisting in the intestinal microbiota and did not have lower faecal population counts. Genes encoding \(\beta\)-lactamases were in most cases retained during the entire colonization period.

Conclusions: The results suggest that ampicillin-resistant \textit{E. coli} strains are not hampered in their colonizing capacity, and \(\beta\)-lactamase genes, therefore, may only slowly be eliminated from the commensal \textit{E. coli} strain pool.

Keywords: \textit{E. coli}, \(\beta\)-lactamases, virulence factors, persistence, phylogeny

Introduction

\textit{Escherichia coli} belong to the normal microbiota of the intestines of humans and animals,\(^1\) but may also cause urinary tract infection and neonatal sepsis.\(^2\)–\(^4\) Strains causing such extraintestinal infections carry an array of virulence genes, including \(P\) fimbriae, aerobactin, capsules, haemolysin and aerobactin.\(^5\) Virulent strains mostly belong to the phylogenetic B2 and D sublineages, whereas strains of A and B1 sublineages generally have low extraintestinal pathogenicity.\(^6\),\(^7\)

The capacity to persist in the normal microbiota varies strikingly among \textit{E. coli} strains. Some strains, termed resident, may colonize an individual for months or years.\(^8\)–\(^10\) Others, so-called transient strains, persist in the microbiota only for a short period and may not, in a strict sense, colonize. Interestingly, the same traits that confer extraintestinal pathogenicity are partly identical to those conferring the capacity to persist in the commensal microbiota. Thus, colonic resident strains carry the genes for \(P\) fimbriae,\(^11\)–\(^13\) haemolysin and aerobactin\(^12\),\(^14\),\(^15\) more often than do transient strains. They also have O and K antigens of types similar to those in uropathogenic \textit{E. coli} strains\(^13\),\(^14\) and belong to the B2 sublineage.\(^16\),\(^17\)

Microbial resistance to antibiotics is a growing problem. The intestinal microbiota may provide an important reservoir for antibiotic-resistant bacteria, but the influence of antibiotic resistance gene carriage in such complex bacterial communities has received little attention. Due to the metabolic cost of reproducing antimicrobial resistance elements, susceptible strains would in theory be at advantage in direct competition between strains. In the absence of selective pressure from antibiotics, colonization dynamics of resistant and susceptible strains should favour elimination of resistance genes or make resistant strains less capable of persisting in the complex microbiota of the gut, where competition for nutrients is fierce. However, as we
previously reported, tetracycline-resistant *E. coli* strains persist equally well as susceptible strains in the intestinal microbiota of infants who have never been exposed to this antibiotic. The persistence of resistant strains may be attributed to a genetic linkage between resistance and traits beneficial for persistence, or adaptive mutations may compensate for the cost of carriage resistance elements.

The most widely prescribed antibiotics among children are the β-lactam agents, in particular, penicillin V and amoxicillin, which act by interfering with cell wall synthesis. Penicillin V has good activity against Gram-positive bacteria and Gram-negative cocci, whereas amoxicillin/amoxicillin is also active against certain Gram-negative bacteria, including *E. coli*. However, since ampicillin was put on the market, a significant population of *E. coli* has acquired β-lactamases, i.e. enzymes that hydrolyse the β-lactam ring and thus inactivate ampicillin. A range of β-lactamases are produced by Gram-negative bacteria, the most common β-lactamases being TEM-1, TEM-2 and SHV-1, which confer resistance to ampicillin/amoxicillin and early generation cephalosporins. Expression of β-lactamase is regulated by promoter genes with varying efficiency. For *bla*TEM genes, both weak (P3) and strong (P4 and *Pau/Pb*) promoters have been identified, the difference deriving from single nucleotide mutations. Overproduction of β-lactamases due to a strong promoter decreases sensitivity to the β-lactamase inhibitor, clavulanic acid.

The present study describes the colonization dynamics of ampicillin-resistant and -susceptible *E. coli* strains in the gut microbiota of 128 Swedish infants followed over their first year of life. *E. coli* were isolated and quantified in regular stool samples and characterized regarding antibiotic resistance, carriage of virulence genes, phylogenetic group identity and time of persistence in the intestinal microbiota. As the antibiotic consumption of each child was registered, the influence of antibiotic treatment on the establishment of β-lactam-resistant strains could be investigated.

**Materials and methods**

**E. coli strains**

A collection of 272 commensal intestinal *E. coli* strains was studied. They were derived from 128 infants born in 1998–2001 in Göteborg, Sweden. The infants participated in ALLERGYFLORA, a prospective birth-cohort study designed to investigate the relationship between intestinal colonization pattern in infancy and later allergy development. The children’s intake of antibiotics was registered in HotStarTaq Master Mix (Qiagen GmbH, Hilden, Germany). A multiplex PCR was used for this purpose, as described in detail elsewhere.

Each strain was assigned to one of the phylogenetic groups A, B1, B2 or D using PCR as described previously. Data regarding virulence gene carriage and phylogenetic origin of *E. coli* strain strains have previously been published for the cohort.

**Susceptibility testing**

All *E. coli* strains (a total of 272 strains identified among 950 isolates deriving from 128 infants) were tested for antibiotic resistance using the agar disc diffusion method as outlined by the Swedish Reference Group for Antibiotics. If the infant received antibiotics during the first year of life, all isolates of each strain from this infant were tested (30 infants, 55 strains and 125 isolates). Furthermore, if the first isolate of an *E. coli* strain colonizing an infant was resistant to ampicillin, all isolates of this and all other strains appearing in that infant were tested (20 infants, 46 strains and 109 isolates). In the remaining cases (78 infants, 171 strains and 716 isolates), only the first isolate of each strain was tested. The following 14 antibiotic-containing discs (AB Biodisk, Solna, Sweden) were applied to Iso-Sensitest agar: ampicillin, cefoxitin, cefuroxime, mecillinam, cefadroxil, cefazidime, chloramphenicol, gentamicin, tobramycin, nitrofurantoin, nalidixic acid, norfloxicin, tetracycline and trimethoprim. Strains with clearance zones of <11 mm around the ampicillin disc were further analysed by Etest to determine the MIC value of ampicillin, as outlined by the SRGA.

Ampicillin-resistant strains (MIC > 8 mg/L) were further tested for susceptibility to amoxicillin in combination with clavulanic acid by the disc diffusion method. *E. coli* ATCC 25922 (CCUG 17620) was used as a fully susceptible control strain.

**Molecular detection of TEM, SHV and OXA β-lactamase genes**

Ampicillin-resistant *E. coli* strains were assessed for carriage of the ampicillin resistance genes *bla*TEM, *bla*SHV and *bla*OXA using PCR. In brief, one bacterial colony was picked from an agar culture and suspended in HotStarTaq Master Mix (Qiagen GmbH, Hilden, Germany). First, a PCR was performed for the amplification of the entire *bla*TEM gene using primers and conditions described previously. The following primers for *bla*TEM were used: TEM-F (5’-TTCTTTAGAGCAGCAAGGGC-3’) and TEM-R (5’-ACGCCTCA GTGGAACGAAC-3’). The size of the entire *bla*TEM amplicons was 1150 bp. A duplex PCR was performed for the amplification of the *bla*SHV and *bla*OXA genes using primers and conditions described previously. The following primers were used for *bla*SHV, primers SHV-F (5’-AGGATTTGACTGCTTTTGG-3’) and SHV-R (5’-ATTTTGCTGATTTCGCTCG-3’); and for *bla*OXA,
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Resistance to ampicillin was analysed in relation to phylogenetic group (A, B1, B2 or D). Strains belonging to phylogenetic group D were more often phenotypically ampicillin-resistant (26% versus 9%, \( P = 0.01 \)) and more often carried the bla\(_{TEM-1}\) genes (23% versus 8%, \( P = 0.002 \)) compared with strains of the other phylogenetic groups. Strains belonging to phylogenetic group B1 were significantly less often ampicillin-resistant (0% versus 13%, \( P = 0.03 \)) than those belonging to the other phylogenetic groups. Strains belonging to phylogenetic group A or B2 did not differ significantly in ampicillin resistance, compared with the other phylogenetic groups (group A, 13% versus 18%, \( P = 0.37 \); group B2, 10% versus 14%, \( P = 0.45 \)).

Four strains carrying bla\(_{SHV}\) genes belonged to phylogenetic group B2, whereas only one belonged to group D. The SHV type of \( \beta \)-lactamase has previously been associated with phylogenetic group B2 E. coli.57

Resistance to ampicillin and carriage of virulence genes

We examined whether ampicillin-resistant and -susceptible strains differed in their carriage of genes encoding a range of virulence factors. The results are shown in Figure 1. Strains with phenotypic resistance to ampicillin carried the P-fimbrial gene papC (47% versus 27%, \( P = 0.023 \)) significantly more often than ampicillin-susceptible strains. This association was due to an accumulation of virulence genes among bla\(_{SHV}\)-positive strains. All strains with the bla\(_{SHV}\) genotype carried the same combination of virulence genes, namely fimA, papC, sfaD/E and hlyA, the last three genes being significantly more common among bla\(_{SHV}\)-positive strains than among ampicillin-susceptible strains (\( P = 0.0015 \) for papC and sfaD/E and \( P = 0.0006 \) for hlyA) or among bla\(_{TEM}\)-positive strains (\( P = 0.015 \) for papC, \( P = 0.0013 \) for sfaD/E and \( P = 0.0006 \) for hlyA). Virulence genes papC, sfaD/E and hlyA are linked to the capacity for long-term persistence.11–13,15 bla\(_{TEM}\) genes more often had the aerobactin gene iutA than strains susceptible to ampicillin (46% versus 26%, \( P = 0.039 \), Figure 1). Aerobactin is a virulence factor that is also associated with enhanced capacity to persist in the gut microbiota.15

Figure 1. Virulence gene carriage among ampicillin-resistant (\( n = 32 \)) and ampicillin-susceptible (\( n = 240 \)) E. coli strains from the microbiota of 128 Swedish infants. Ampicillin-resistant strains were further divided into bla\(_{TEM}\)-positive (\( n = 27 \)) and bla\(_{SHV}\)-positive strains (\( n = 5 \). *** \( P < 0.05 \), **P < 0.01 , ***P < 0.001. }\)

Results and discussion

Prevalence and mechanism of ampicillin resistance among commensal E. coli strains

Of the E. coli strains isolated from the commensal infantile microbiota, 12% (32/272) were phenotypically resistant to ampicillin. They all had MICs exceeding 256 mg/L. They were all susceptible to the cephalosporins tested: cefadroxil, cefotixin, cefuroxime and cefazidime. The prevalence of ampicillin resistance was very moderate compared with the 59% ampicillin resistance found among commensal infantile E. coli strains15 and TEM-positive PCR products were purified, analysed (3730 DNA Sequencer, Applied Biosystems) and aligned (Sequencher 4.2.2 software, Gene Codes Corporation, MI, USA). The nucleotide sequences obtained were compared with previously described sequences obtained from the GenBank database.

Statistics

Frequencies were compared using Fisher’s exact test. The time of persistence in the bowel flora of susceptible and resistant strains was compared using the Mann–Whitney test. Population counts of susceptible and resistant strains colonizing the same individual were compared using the Wilcoxon signed rank test.

Primers OXA-F (5’-ATATCTCTACTTGGCATCTCC-3’) and OXA-R (5’-AAACCCATTGACGATCC-3’). The sizes of the bla\(_{SHV}\) and bla\(_{OXA}\) amplicons were 392 and 619 bp, respectively. The strains carrying known bla genes were used as positive controls (CCUG 30600 for bla\(_{TEM}\), CCUG 45421 for bla\(_{SHV}\) and CCUG 52541 for bla\(_{OXA}\) all from the Culture Collection of the University of Gothenburg, CCUG, Sweden).

The bla\(_{TEM}\)-positive PCR products were purified with magnetic beads and sequenced using the Big Dye Terminator Cycle Sequencing Kit v. 3.1 (Applied Biosystems). Three forward primers (TEM-F, TEM-M1 and TEM-M2) and two reverse primers (TEM-R and TEM-end-R3) were used to obtain sequences covering both strands of the entire bla\(_{TEM}\) gene.31,33 Sequence-PCR products were purified, analysed (3730 DNA Sequencer, Applied Biosystems) and aligned (Sequencher 4.2.2 software, Gene Codes Corporation, MI, USA). The nucleotide sequences obtained were compared with previously described sequences obtained from the GenBank database.

Ampicillin-resistant strains were analysed with respect to carriage of bla\(_{TEM}\), bla\(_{SHV}\) and bla\(_{OXA}\) genes by PCR. All ampicillin-resistant strains carried at least one gene belonging to these groups. The bla\(_{TEM-1}\) genes were most common, found in 84% (27/32) of the resistant strains, one of which also carried the bla\(_{OXA}\) genes. The rest (\( n = 5 \)) had bla\(_{SHV}\) genes. Strains carrying bla\(_{TEM-1}\) were more often resistant to other antibiotics (63%) than ampicillin-susceptible strains (12%, \( P = 0.0017 \)) or bla\(_{SHV}\)-positive strains (0%, \( P = 0.01 \)).

The promoter region was sequenced in all 27 bla\(_{TEM}\) amplicons. The weak \( P3 \) promoter was found in 24 and the strong Pa/Pb promoter in 3 of these sequences. The occurrence of the stronger promoter coincided with a decreased susceptibility to amoxicillin/clavulanic acid in these three strains, as previously reported.36

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<th>papC</th>
<th>sfaD/E</th>
<th>hlyA</th>
<th>iutA</th>
<th>neuB</th>
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<td>40</td>
<td>20</td>
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Figure 1. Virulence gene carriage among ampicillin-resistant (\( n = 32 \)) and ampicillin-susceptible (\( n = 240 \)) E. coli strains from the microbiota of 128 Swedish infants. Ampicillin-resistant strains were further divided into bla\(_{TEM}\)-positive (\( n = 27 \)) and bla\(_{SHV}\)-positive strains (\( n = 5 \). *** \( P < 0.05 \), **P < 0.01 , ***P < 0.001. }\)
Impact of antibiotic treatment on *E. coli* colonization dynamics

We examined whether colonization by ampicillin-resistant strains occurred in relation to antibiotic treatment. In all, 30 infants were treated with antibiotics at least once during the first year of life. Fourteen of these received amoxicillin or cephalosporin antibiotics that could select for β-lactamase-producing *E. coli*. Figure 2 shows the *E. coli* strain colonization pattern during the first year of life in relation to antibiotic treatment in these 14 infants. In only one case could a temporal relation between β-lactam treatment and colonization by β-lactamase-producing *E. coli* be detected: infant no. 12 was colonized by an ampicillin-resistant strain at 12 months of age after having received amoxicillin shortly after 6 months of age. However, 12% (11/92) of *E. coli* strains picked up by non-treated infants between 6 and 12 months of age were also ampicillin-resistant, indicating that the acquisition of a resistant strain in 1/14 treated infants could occur by chance without relation to treatment. In all, 31 of 32 ampicillin-resistant strains were found in infants who had not previously been treated with β-lactam antibiotics that could select for such resistance. Thus, it appeared as if β-lactam-resistant *E. coli* are part of the common pool of circulating strains and may establish, unpaved by their resistance, in the commensal microbiota of non-treated hosts. In one infant (no. 5), an *E. coli* strain that was initially susceptible to ampicillin acquired resistance after treatment with ampicillin due to transfer of a resistant plasmid from another *E. coli* strain that co-inhabited the gut flora of that infant (Figure 2). This event has been described in detail elsewhere. 38

**Figure 2.** *E. coli* colonization pattern of the 14 infants who were treated with amoxicillin or a cephalosporin at least once during their first year of life. Each *E. coli* strain colonizing an infant is represented by a symbol. Filled symbols indicate strains resistant to ampicillin (filled circles) or ampicillin and trimethoprim (filled upside-down triangles). Open symbols indicate strains susceptible to ampicillin; different symbols indicating individual strains colonizing an infant. A horizontal line connecting the strain symbols indicates persistence of a strain in the intestinal microbiota. A vertical line marks treatment with antibiotics: a solid line representing amoxicillin/ampicillin or a cephalosporin, and a dashed line representing treatment with other antibiotics. The antibiotics given are specified on the right-hand side of the diagram as follows: AMP, ampicillin; AMX, amoxicillin; PEN, penicillins (PCV or PCG); TMP, trimethoprim; CTB, cefetobuten; NIT, nitrofurantoin; CAZ, ceftazidime; TOB, tobramycin; ERY, erythromycin.

Faecal population levels of ampicillin-resistant *E. coli* strains

The stool population counts of each strain were determined on each culture occasion, which permitted us to compare the population levels of ampicillin-resistant and -susceptible strains. Because the population levels of facultative bacteria, including *E. coli*, decrease with time as the infantile gut microbiota become increasingly complex, 27 the population levels should be compared in infants of the same age. Figure 3(a) shows the

**Figure 3.** (a) Faecal population counts (log 10 values) of all ampicillin-resistant (filled circles) and ampicillin-susceptible (open circles) *E. coli* strains at each time point in infants not receiving any antibiotics during their first year of age. The numbers within parentheses show the number of ampicillin-resistant strains at each sampling occasion. (b) Faecal population counts of ampicillin-resistant and ampicillin-susceptible strains colonizing simultaneously in eight infants. In one infant, one ampicillin-resistant strain and three ampicillin-susceptible strains were present simultaneously; the population counts of the susceptible strains were averaged before comparison with those of the resistant strain.
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faecal population levels of ampicillin-resistant and ampicillin-susceptible strains at different time points in infants who never received antibiotics during their first year of life. At no time point were the faecal population counts of ampicillin-resistant strains lower than those of ampicillin-susceptible strains in this cross-sectional analysis.

To investigate a situation with direct competition between resistant and susceptible E. coli strains, we examined the eight infants who simultaneously harboured an ampicillin-resistant strain and at least one fully susceptible strain in the same stool sample. The population counts were slightly lower for resistant than for susceptible strains (average \(10^7.36\) versus \(10^7.79\)), but the difference was not significant in paired analysis (\(P = 0.10\)) (Figure 3b). These results show that ampicillin-resistant genes have a low fitness cost on E. coli strains and that they are able to compete favourably in the gut flora.

Impact of ampicillin resistance on capacity to persist in the intestinal microbiota

Ampicillin-resistant and -susceptible strains were examined with respect to their capacity to persist in the microbiota. Strains that were isolated at least twice in faecal samples collected at least 3 weeks apart were classified as resident, whereas strains whose colonization period was shorter than a month were classified as transient. Strains appearing only once in samples spaced >1 month in time could neither be grouped as resident nor as transient and were not included in the analysis. Among ampicillin-resistant strains, 82% were resident and 18% transient. Among bla\textsubscript{TEM}-positive strains, 87% (13/15) were resident, and among the five bla\textsubscript{SHV}-positive strains, one strain was resident and one transient, while the three remaining strains could not be classified. Among ampicillin-susceptible strains, 81% were resident.

The average time of persistence was 34 ± 15 (SD) weeks for the 13 resident strains that were resistant to ampicillin and 29 ± 17 weeks for the 82 resident strains that were susceptible (\(P = 0.35\)). Thus, ampicillin-resistant strains were equally able to persist in the infantile microbiota as ampicillin-susceptible strains.

To exclude the possibility that the good colonizing capacity of ampicillin-resistant strains was the result of selection by antibiotic treatment, we excluded all infants ever treated with antibiotics from the analysis. Among strains that colonized infants never treated with antibiotics, 91% (10 of 11) of the ampicillin-resistant and 85% (63 of 74) of the ampicillin-susceptible strains were resident in the microbiota. The average time of persistence was 29 ± 15 weeks for the ampicillin-resistant resident strains and 26 ± 17 weeks for the susceptible strains (\(P = 0.42\)). Thus, even in the absence of any antibiotic, ampicillin-resistant strains persisted equally well as fully susceptible strains in the microbiota.

Stability of ampicillin resistance

We examined the stability of the resistance phenotype in the 13 ampicillin-resistant E. coli strains that persisted in the microbiota of an infant for at least 3 weeks. Four of the 13 infants harbouring such a strain received β-lactam antibiotics. Eleven of the strains kept their resistance genes during the entire colonization period (the average time of persistence being 36 ± 15 weeks).

Resistance to β-lactam antibiotics tended to be a fairly stable trait. However, two strains, both from infants who received no antibiotics, lost their bla\textsubscript{TEM} genes. The strain identity of these isolates as determined by RAPD was confirmed by PFGE and virulence gene profiles. The strains also lost resistance to tetracycline and trimethoprim simultaneously with the loss of bla\textsubscript{TEM}, suggesting the loss of a multiple drug resistance plasmid.\(^1\) As described previously in a paper reporting on the effect of tetracycline resistance genes on colonization dynamics,\(^1\) the loss of the resistance phenotype was associated with increased population levels in one case and decreased population levels in the other.

Conclusions

Our results suggest that carriage of β-lactamase genes does not impact negatively on survival of E. coli in the gut microbiota because: (i) resistant strains are freely established in children not treated with antibiotics; (ii) resistant strains persist equally well in the microbiota as susceptible ones and attain approximately equal population levels; and (iii) resistance genes were, in most cases, retained during long-time colonization in the absence of selective pressure. The good colonizing ability could be due to the carriage, in ampicillin-resistant strains, of virulence factor genes that promote persistence in the colonic milieu. These findings suggest that resistance genes may not be easily eliminated from the commensal strain pool.

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Transparency declarations

None to declare.

References


