Dynamics of antimicrobial resistance in intestinal *Escherichia coli* from children in community settings in South Asia and sub-Saharan Africa

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The dynamics of antimicrobial resistance (AMR) in developing countries are poorly understood, especially in community settings, due to a sparsity of data on AMR prevalence and genetics. We used a combination of phenotyping, genomics and antimicrobial usage data to investigate patterns of AMR amongst atypical enteropathogenic *Escherichia coli* (aEPEC) strains isolated from children younger than five years old in seven developing countries (four in sub-Saharan Africa and three in South Asia) over a three-year period. We detected high rates of AMR, with 65% of isolates displaying resistance to three or more drug classes. Whole-genome sequencing revealed a diversity of known genetic mechanisms for AMR that accounted for >95% of phenotypic resistance, with comparable rates amongst aEPEC strains associated with diarrhoea or asymptomatic carriage. Genetic determinants of AMR were associated with the geographic location of isolates, not *E. coli* lineage, and AMR genes were frequently co-located, potentially enabling the acquisition of multi-drug resistance in a single step. Comparison of AMR with antimicrobial usage showed that the prevalence of resistance to fluoroquinolones and third-generation cephalosporins was correlated with usage, which was higher in South Asia than in Africa. This study provides much-needed insights into the frequency and mechanisms of AMR in intestinal *E. coli* in children living in community settings in developing countries.

Certain pathotypes of *Escherichia coli* are important causes of diarrhoea in children, especially in the developing countries of sub-Saharan Africa and South Asia. Intestinal *E. coli* is also an important source and reservoir of genes that encode antimicrobial resistance (AMR). One pathotype of intestinal *E. coli*, known as atypical enteropathogenic *E. coli* (aEPEC), is defined by the presence of the locus of enterocyte effacement pathogenicity island, and the absence of Shiga toxins (denoting enterohaemorrhagic *E. coli*) and type IV bundle-forming pili (indicating typical EPEC). Atypical enteropathogenic *E. coli* causes a variety of disease symptoms ranging from sporadic and persistent diarrhoea to asymptomatic carriage. We recently identified distinct lineages of aEPEC, including ten common clonal groups.

AMR has been reported in *E. coli* from various animal species, the environment and in hospitalized patients globally. Many strains exhibit multi-drug resistance (MDR; resistance to one or more agents in at least three different antimicrobial categories). Strains that are resistant to fluoroquinolones and/or produce extended-spectrum β-lactamases (ESBL) or carbapenemases are of particular concern. Although several recent studies of pathogenic *E. coli* from countries in sub-Saharan Africa and South Asia have reported increases in ESBLs, as well as increasing resistance to gentamicin and ciprofloxacin, these data are mostly derived from *E. coli* responsible for extra-intestinal infections in hospital settings. Thus, there remain major gaps in knowledge of the global prevalence of AMR in human intestinal *E. coli*, particularly in developing nations where the burden of infectious diseases is highest and AMR may result in infections that are unresponsive to treatment.

Enhancing our knowledge of AMR amongst gut-dwelling *E. coli* is important for two reasons: (1) *E. coli* is a leading cause of extra-intestinal infections and strains colonising the gastrointestinal tract of patients are the major reservoir of these infections; and (2) most AMR in *E. coli* is encoded on mobile genetic elements that are transferable between bacteria, thus enabling the rapid dissemination and maintenance of resistance genes between bacteria of different species. Although antimicrobials are not recommended to treat dysentery and prolonged diarrhoea, of which aEPEC is a major cause.

Here we present AMR data for 185 aEPEC isolates collected during the Global Enteric Multicenter Study (GEMS). Using phenotypic susceptibility data and whole-genome sequence analysis, we determined the prevalence, mechanisms of resistance and potential drivers of variation in AMR profiles. These isolates, collected from healthy children living in a community setting and children with diarrhoea at seven sites in sub-Saharan Africa and South Asia, provided a unique opportunity to investigate the prevalence of AMR in intestinal bacteria that were not selected on the basis of AMR profile.

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gentamicin. Fluoroquinolone resistance was relatively infrequent, although this antibiotic is not used to treat diarrhoea. Resistance to other aminoglycosides tested was rare (3%), with five isolates from India resistant to tobramycin, four of which were also resistant to other aminoglycosides tested. Resistance to amikacin (an aminoglycoside) or meropenem (a carbapenem) was common (43%), with 121 (65%) isolates resistant to both norfloxacin and ciprofloxacin. Resistance to chloramphenicol (11%) and azithromycin (7%) was also infrequent. Among the β-lactam antibiotics, ampicillin resistance was common (65%), but resistance to ceftriaxone (3%), ceftazidime (3%) and cefepime (2%) was rare (Fig. 1a,b).

**Results**

**Antimicrobial susceptibility profiles.** Susceptibility testing of 185 aEPEC isolates (Supplementary Table 1) to 16 antimicrobials (Supplementary Table 2) revealed resistance to 14 of the drugs investigated (Fig. 1a; Supplementary Table 3), with 121 MDR isolates (65%; Fig. 1c). No resistance was detected to the 'last-line' antimicrobials was common, with 121 (65%) isolates resistant to ampicillin, 124 (67%) to trimethoprim, 122 (66%) to trimethoprim/sulphamethoxazole and 104 (56%) to tetracycline (Fig. 1a). Approximately half (n = 96, 52%) of the isolates were resistant to three or more of these drugs. Streptomycin resistance was common (43%), although this antibiotic is not used to treat diarrhoea. Resistance to other aminoglycosides tested was rare (3%), with five isolates from India resistant to tobramycin, four of which were also resistant to gentamicin. Fluoroquinolone resistance was relatively infrequent, with 31 isolates (17%) resistant to norfloxacin and 8 (4%) resistant to both norfloxacin and ciprofloxacin. Resistance to chloramphenicol (11%) and azithromycin (7%) was also infrequent. Among the β-lactam antibiotics, ampicillin resistance was common (65%), but resistance to ceftriaxone (3%), ceftazidime (3%) and cefepime (2%) was rare (Fig. 1a,b).

**Genetic determinants of AMR.** The genomes of the 185 aEPEC isolates were screened for known genetic determinants of AMR, including horizontally acquired genes and point mutations in chromosomal genes associated with resistance to fluoroquinolones and nitrofurantoin (Fig. 1b; Supplementary Fig. 1 and Supplementary Table 4). More than forty different acquired AMR genes were detected, along with four point mutations (two in gyrA, one in parC (both gyrA and parC are associated with quinolone resistance) and one in nfsA (associated with resistance to nitrofurantoin)). Extensive diversity of AMR genotypes was observed, with 104 distinct combinations of AMR determinants across the 185 isolates (Fig. 1b,...
Supplementary Fig. 1). Nevertheless, four acquired AMR genes were detected in more than half the isolates. These were alleles of bla<sub>TEM</sub> (ampicillin), strA and strB (streptomycin), and sul2 (sulphonamides). Alleles of dihydrofolate reductase (dfr) genes encoding trimethoprim resistance were detected in 132 (71%) isolates. The most common of these were dfrA1 (12%), dfrA5 (23%), dfrA7 (20%) and dfrA8 (16%).

Investigation of mobile genetic elements associated with transfer of AMR genes. As MDR was common in the bacteria studied (Fig. 1), we hypothesized that this was due to the co-transfer of groups of AMR genes via mobile elements. The pairwise co-occurrence matrix of AMR genes was sparse (Supplementary Fig. 2), with only a few clusters of genes frequently detected together in the same genome. The mean co-occurrence value across all gene pairs was 6.4 strains. Figure 2a shows AMR gene co-occurrence networks, constructed using different thresholds for co-occurrence across the aEPEC collection. The most common gene network comprised sul2, strA and strB, which co-occurred in 112 genomes (61%); the combination of sul2, strA and strB occurred with bla<sub>TEM</sub>-194 in 46 (25%) and with bla<sub>TEM</sub>-191 in 20 (11%); and Supplementary Fig. 2 and Fig. 2a) genomes. Using a minimum threshold of co-occurrence in ≥20 strains (mean plus s. d. of all co-occurrence values), we detected a large network of genes comprising sul2, strA, strB and bla<sub>TEM</sub>-194, as well as sul1, bla<sub>TEM</sub>-194, dfrA14, dfrA8, dfrA7, tet(A) and tet(B).

Resolving the genetic context of AMR genes is generally not possible using short-read sequence data, because repeated sequences (such as insertion sequences) and variable plasmid copy numbers cause uncertainty in the de novo assembly graphs<sup>23,24</sup>. We therefore sought only to make broad classifications about the potential mobile elements associated with the AMR gene networks present in the aEPEC genomes, through comparison with elements that have previously been found to mobilize these combinations of genes.

The genes sul2, strA and strB frequently move together on small (approximately 6,000 base pairs (kbps)) plasmids related to pCERC2<sup>29</sup> (Fig. 2b). The combination of the pCERC2 plasmid backbone and the sul2, strA and strB sequences was present in 42 isolates, 40 of which (95%) also carried dfrA gene sequences, including dfrA1, dfrA14 and dfrA8. In some genomes, the plasmid sequences could be resolved completely, showing that the dfr gene was located on the plasmid. For example, GEMS strain 400897 carried dfrA1 gene adjacent to strB on a pCERC2-like plasmid, while GEMS strain 402635 carried dfrA14 inserted within strA as in pCERC1<sup>19</sup> (Fig. 2b).

The sul2, strA and strB genes also occur together with bla<sub>TEM</sub>-194 genes (predominately bla<sub>TEM</sub>-194) in transposon Tn6029, which is commonly found in <em>E. coli</em> in a range of distinct plasmid backbones<sup>27,28,30</sup> (Fig. 2c). Tn6029 is mobilized by the flanking copies of IS26. A third copy of IS26 is located between bla<sub>TEM</sub> and the other AMR genes, resulting in separation of the transposon into two separate contigs in short-read assemblies (Fig. 2c). We detected the presence of both Tn6029 contigs in 33 genomes, which are therefore likely to carry the complete transposon. As the flanking IS26 sequence is present in many different locations, we could not determine the insertion site of Tn6029 within the draft genomes. However, Tn6029 is frequently located within Tn1696, which includes a class 1 integron that carries variable AMR genes (often including dfr genes) within the cassette, and sul1 downstream of the cassette. For an example of a composite transposon structure from <em>Salmonella</em> plasmid pSRC264, see Fig. 2c. In total, we identified class 1 integron sequences in 38 aEPEC genomes, 26 of which included both Tn6029 contigs. An example assembly graph, showing how these contigs are connected to one another in a manner consistent with previously sequenced composite transposons, is shown in Fig. 2c. Overall, we identified five different integron gene cassettes, the most prevalent of which carried dfrA7 (n = 30 genomes, including 25 with Tn6029), whereas the others carried dfrA1 (n = 4), dfrA1 and adaA1 (n = 1 genome, which also carried Tn6029), dfrA17 and ada5 (n = 2), and dfrA5 (n = 1).

We found two common genes that encode tetracycline resistance efflux pumps. The most prevalent was tet(A), which was found in 63 (34%) aEPEC genomes. Although tet(A) is associated with the Tn1721 transposon, the full transposon was detected in only three genomes. The tet(B) gene was detected in 50 (27%) genomes, five of which also carried tet(A). The tet(B) gene can be mobilized by Tn10, which is flanked by IS10 genes, but the complete transposon was present in only two genomes. The linkage of tet(A) or tet(B) to other AMR-related elements was not resolvable from the draft genome assemblies, however both were found in association with other common AMR genes (Fig. 2a).

Although it is not possible to resolve plasmid sequences from draft short-read assemblies or determine linkage between specific plasmid replicons and AMR genes<sup>40</sup>, our screening for markers of plasmid replicons revealed several that are often associated with large AMR plasmids (Supplementary Fig. 1d). The most prevalent amongst the aEPEC collection were FII (n = 131, 71%) and FIBA (n = 104, 56%). Notably, F plasmids are also associated with the carriage of genes for virulence determinants, such as adhesins of enteropathogenic <em>E. coli</em>, but our data did not permit the determination of which plasmids were associated with AMR genes versus virulence genes. An IncC plasmid replicon (also known as IncA/C) was detected in four genomes that carried bla<sub>TEM</sub>-194, but not sul2, strA or strB. It was not possible, however, to determine whether the bla<sub>TEM</sub>-194 gene was located on this plasmid.

Prediction of AMR phenotypes from genotypes. In vitro resistance was largely explained by the presence of known genetic determinants of AMR (Fig. 1, Table 1). For most drugs, the detection of resistance genes was both sensitive (>95%) and specific (>90%) in predicting AMR phenotype. The frequency of very major errors (failure to detect phenotypic resistance) exceeded the minimum acceptable threshold of 1.5% for five drugs (Table 1). These were: ampicillin (4.9%), streptomycin (2.2%), trimethoprim (2.2%), trimethoprim/sulphamethoxazole (2.2%) and tetracycline (2.2%). Major errors (predicting resistance when none is present) were also detected for these and several other antimicrobials (Table 1). The highest major error rates were observed for streptomycin (26.5%), ampicillin (7.0%), trimethoprim (5.9%), trimethoprim/sulphamethoxazole (5.4%) and tetracycline (4.3%). The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for each drug are shown in Supplementary Table 5. Sensitivity and NPV were greater than 90% for all drugs tested with the exception of azithromycin (85% sensitivity) and ampicillin (85% NPV). Specificity and PPV were more variable, reflecting the error rates summarized above (Table 1).

Potential sources of variation in AMR profiles. Given the diversity of AMR profiles in our aEPEC strains, we determined whether the distribution of AMR genes was associated with disease status, phylogenetic lineage or the geographic location from which each strain was isolated. First, we compared the frequency of AMR phenotypes and genotypes among aEPEC isolated from diarrhoea cases and asymptomatic controls. Only data from confirmed cases (n = 94) and controls (n = 88) were used for this analysis. For each drug class, neither AMR phenotype nor AMR predicted from genotype, were statistically different between cases and controls (Supplementary Table 6). The frequencies of individual AMR genes were also similar in isolates obtained from cases and from controls (Fig. 3a). As AMR determinants were equally distributed among cases and controls, all isolates were pooled for further analysis of lineage and region.

Discriminant analysis of principal components<sup>43</sup> on the binary matrix of AMR genetic determinants (Supplementary Table 4; Fig. 3b,c) revealed that the first 20 principal components accounted...
for >93% of variation in AMR profiles and were retained for discriminant analysis by phylogenetic lineage (clonal groups as defined previously; Supplementary Fig. 1) or by geographic origin (East Africa, West Africa and Asia; Fig. 3 and Supplementary Fig. 3).

Variation in AMR was not associated with clonal group, apart from CG378 which was characterized by the absence of the most common AMR genes: \( \text{bla}_{\text{TEM}} \) variants, \( \text{sul2} \), \( \text{tet(A)} \) and \( \text{tet(B)} \), and the presence of the uncommon \( \text{catA} \) gene, detected in 7 of 9 CG378 isolates compared to 15 of 176 non-CG378 (\( P < 10^{-4} \), Fisher’s exact test, two-tailed). Variation in AMR determinants was associated with the region of origin (Fig. 3b and Supplementary Fig. 3b). Discriminant function 1 (DF1) separated Asian from African isolates and was associated with \( \text{gyrA} \) single nucleotide polymorphisms; DF2 separated East from West African isolates and was associated with \( \text{dfrA8} \), \( \text{dfrA5} \), \( \text{tet(A)} \) and \( \text{sul2} \). Figure 3d shows the distribution of the \( \text{dfrA} \) alleles across the GEMS sites. For example,
Table 1 | Comparison of phenotypic and genotypic AMR profiles of 185 aEPEC isolates

|                      | Resistant phenotype | Resistant genotype | Very major error | Susceptible phenotype | Susceptible genotype | Major errorb |
|----------------------|---------------------|--------------------|------------------|-----------------------|----------------------|--------------|
| **β-lactams**        |                     |                    |                  |                       |                      |              |
| Ampicillin           | 121                 | 112                | 9 (4.9%)         | 64                    | 51                   | 13 (7.0%)    |
| Cefepime             | 3                   | 3                  | 0                | 182                   | 178                  | 4 (2.2%)     |
| Ceftriaxone          | 6                   | 6                  | 0                | 179                   | 174                  | 5 (2.7%)     |
| Ceftazidime          | 6                   | 6                  | 0                | 179                   | 174                  | 5 (2.7%)     |
| Meropenem            | 0                   | 0                  | 0                | 185                   | 185                  | 0            |
| **Aminoglycosides**  |                     |                    |                  |                       |                      |              |
| Streptomycin         | 80                  | 76                 | 4 (2.2%)         | 105                   | 56                   | 49 (26.5%)   |
| Gentamicin           | 5                   | 5                  | 0                | 180                   | 180                  | 0            |
| Tobramycin           | 5                   | 5                  | 0                | 180                   | 180                  | 0            |
| Amikacin             | 0                   | 0                  | 0                | 185                   | 185                  | 0            |
| **Folate pathway inhibitors** | | | | | | |
| Trimethoprim         | 124                 | 120                | 4 (2.2%)         | 61                    | 50                   | 11 (5.9%)    |
| Trimethoprim/sulphamethoxazolec | 122               | 118                | 4 (2.2%)         | 63                    | 53                   | 10 (5.4%)    |
| **Nitrofurantoin**   |                     |                    |                  |                       |                      |              |
| Nitrofurantoin       | 0                   | 0                  | 0                | 185                   | 182                  | 3 (1.6%)     |
| **Chloramphenicol**  |                     |                    |                  |                       |                      |              |
| Chloramphenicol      | 21                  | 19                 | 2 (1.1%)         | 164                   | 160                  | 4 (2.2%)     |
| **Macrolides**       |                     |                    |                  |                       |                      |              |
| Azithromycin         | 13                  | 11                 | 2 (1.1%)         | 172                   | 166                  | 6 (3.2%)     |
| **Tetracyclines**    |                     |                    |                  |                       |                      |              |
| Tetracycline         | 104                 | 100                | 4 (2.2%)         | 81                    | 73                   | 8 (4.3%)     |
| **Fluoroquinolones** |                     |                    |                  |                       |                      |              |
| Ciprofloxacin        | 8                   | 8                  | 0                | 177                   | 173                  | 4 (2.2%)     |
| Norfloxacin          | 31                  | 30                 | 1 (0.5%)         | 154                   | 149                  | 5 (2.7%)     |

aVery major errors (resistant isolate genotyped as susceptible) at frequencies >15% are shown in bold. bMajor errors (susceptible isolate genotyped as resistant) at frequencies >3% are shown in bold.
cWhen two genes are required for resistance, both were required for genotypic resistance: strA and strB for streptomycin, and a dfr gene plus a sul gene for resistance to trimethoprim/sulphamethoxazole.
dfrA1 predominated at Asian sites and dfrA8 was most common at West African sites, whereas dfrA14 and dhfr7 were common in Mozambique and Kenya, respectively. Further, tet(A) was more common at West and East African sites than in Asia. These genetic differences were reflected in AMR phenotypes, as resistance to ciprofloxacin (n = 8, 12%) and third-generation cephalosporins (ceftazidime and ceftriaxone, both n = 6; 9%) was identified only in strains from Asia, while resistance to tetracycline was more common in African (East Africa, n = 44, 60%; West Africa, n = 33, 72%) than in Asian isolates, n = 27, 41%; P < 0.05, Fisher’s exact test, two-tailed; Fig. 4a).

Discussion

AMR and usage data reported here were collected at seven study sites in Asia and Africa, using the same protocols thus enabling comparisons between the sites17–34. No national AMR surveillance data are available from these countries; and AMR data on E. coli in these countries pertain mostly to isolates causing extra-intestinal infections, and to a limited number of drugs (mainly third-generation cephalosporins and fluoroquinolones)14,19,21,22,35. Furthermore, publicly available background data on antimicrobial usage at the seven study sites are limited. For example, the IMS Health MIDAS database includes usage data for India, Pakistan and Bangladesh reported in aggregate without detailed methods of data collection or interpretation; and no data for Mozambique, The Gambia, Kenya and Mali.

Nearly half of all isolates were resistant to penicillins, trimethoprim and tetracyclines. The rates of resistance to these drugs were generally lower amongst Asian isolates (21–62%) than in Asia, while resistance to tetracycline was more common at West African sites (Fig. 4). We therefore investigated the associations between usage and resistance for the two drugs that showed substantial usage (>10%) at three or more study sites: ciprofloxacin and trimethoprim. Across the seven sites, ciprofloxacin usage was significantly associated with the prevalence of substitutions in the quinolone resistance-determining regions (QRDRs), gyrA and parC (Coefficient of determination (R²) = 0.87, P = 0.002; Fig. 5). By contrast, trimethoprim usage was not associated with the prevalence of horizontally acquired dfr genes that confer resistance to the drug (R² = 0.04, P > 0.5).
Africa isolates (59–84%); whereas resistance to newer drugs such as ceftriaxone, fluoroquinolones and azithromycin were detected at the Asian sites (Fig. 4a). These patterns were broadly consistent with the antimicrobial usage data at the corresponding study sites, which showed that ciprofloxacin and azithromycin were commonly used to treat diarrhoea in Asia, whereas trimethoprim-sulphamethoxazole was the mainstay of treatment in Africa (Fig. 4b,c). The high frequencies of ciprofloxacin resistance at the Asian sites are similar to rates previously reported among clinical cases of intestinal *E. coli* (including multiple pathotypes) in these countries\(^{17,18,21,22,36}\). We detected ESBL-producing isolates at the Asian, but not the African, study sites. Much higher levels have been reported from extra-intestinal *E. coli* infections (including bacteremia) in hospitals in Asia and Africa\(^{16,19,35}\), we speculate that this may...
reflect selection due to use of third-generation cephalosporin at higher rates in hospitals than in the community, and/or the dissemination of the ESBL-producing extra-intestinal E. coli lineages, such as ST131.

In agreement with our data relating to AMR phenotypes, we found that the genetic determinants of resistance were similar in bacteria isolated from diarrhoeal cases and asymptomatic controls (Fig. 3a, Supplementary Table 6) and were not associated with the clonal lineage of the strain, but associated with the geographic region where the bacteria were isolated (Fig. 3b,c; Supplementary Fig. 3). These findings are consistent with the hypothesis that differences in the frequencies of AMR-encoding genes in different regions reflect selection due to differences in antimicrobial exposure (Fig. 4b,c).

In strong support of this explanation, mutations in the QRDRs of gyrA and parC were significantly associated with the frequency of ciprofloxacin use across the seven study sites (Fig. 5). The same pattern of point mutations in the QRDRs of chromosomal genes has been observed in other Enterobacteriaceae associated with South Asia, including Salmonella enterica serovar Typhi and Shigella sonnei.

The situation was more complex for horizontally transferred AMR genes associated with resistance to older drugs. Although individual dfr alleles were distributed differently across sites (Fig. 3d) and contributed to orthogonal components of the regional discriminant function (Fig. 3b), the overall prevalence of dfr genes was relatively high (50–90%) at each site and not significantly associated with use of trimethoprim for diarrhoea (Figs. 3d and 4b,c). Similarly, genes encoding resistance to ampicillin, streptomycin and tetracycline were common at sites where these drugs were seldom or never used for the treatment of diarrhoea. We also found evidence of several common elements mediating AMR to these older drugs, including small plasmids and class I integrons (Fig. 2). This could be due to: (1) a lack of fitness cost associated with these resistances, resulting in maintenance of the genes in the E. coli population after drug usage declines; (2) co-selection for resistance to multiple drugs whose associated genes are present on the same mobile elements; and/or (3) selection due to drug exposure unrelated to the treatment of diarrhoea. We could not distinguish between hypotheses using our data, although exposure to antimicrobials from other sources is quite likely. For example, although we found that trimethoprim, alone or combined with sulfamethoxazole, was used less frequently in India than alternative agents for the treatment of diarrhoea, other studies have reported frequent use of trimethoprim in hospitals, community settings.

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**Fig. 4 | AMR phenotypes by region and antimicrobial use at study sites.**

**a**, AMR phenotypes of GEMS isolates, stratified by region of isolation. **b,c**, Percentage of antimicrobials prescribed to patients with watery diarrhoea (b) or dysentery (c) at each of the seven study sites.
and agriculture in India; there is also evidence of its presence in the environment, particularly surface waters\(^1\). Future studies would benefit from additional data on clinical and agricultural antimicrobial exposures, and assays for selected antimicrobials agents in urine and the environment.

We were also unable to determine the precise location of most AMR genes and associated mobile elements from our short-read sequence data. Further experiments such as conjugation or long-read sequencing\(^\text{34,35,40}\) could resolve this in future. It is notable, however, that the pCERC-like plasmids (which often carried resistance to streptomycin, sulphonamides and trimethoprim) are common in \(E.\ coli\), possibly because their small size (~6 kb) imposes a low fitness cost\(^\text{29,40}\). It is also notable that these and many of the other AMR genes we detected are also associated with composite transposons that can integrate into the bacterial chromosome, where they are maintained at lower fitness cost than large resistance encoding plasmids\(^\text{27,31,38}\).

Many of the mobile elements we detected have been reported widely in \(E.\ coli\) and other Enterobacteriaceae from human intestinal and extra-intestinal samples and animal samples\(^\text{29,31,48-50}\). Atypical enteropathogenic \(E.\ coli\) has multiple reservoirs and our collection included extensive phylogenetic diversity within the \(E.\ coli\) collection. Atypical enteropathogenic \(E.\ coli\) has multiple reservoirs and our study showed that presence of known acquired resistance genes in each genome\(^\text{\text{-}}\). The \(E.\ coli\) they are core genes that normally do not confer antibiotic resistance. The results were transformed into a binary table in R to indicate presence/absence of acquired resistance gene alleles (Supplementary Table 4).

Resistance to older drugs was also common at these sites, such that only Asian isolates were resistant to seven or eight categories of antimicrobials, indicating that changing patterns of antimicrobial use leads to an accumulation of resistance determinants rather than their replacement.

**Methods**

\(aEPEC\) isolates and corresponding whole-genome sequences. A total of 185 confirmed \(aEPEC\) isolates from children aged 0–5 years at GEMS sites located in The Gambia, Mali, Kenya, Mozambique, Bangladesh, India and Pakistan\(^\text{1,6,26,34}\) were included in the analysis\(^\text{\text{-}}\). Their collection, selection for sequencing, whole-genome sequencing and phylogenetic analysis have been described previously\(^\text{\text{-}}\). Briefly, the isolates were mostly from fecal samples in which \(aEPEC\) alone (or with \(Giardia \) lamblia) was the only pathogen detected, where a pure culture could be obtained. All such isolates from diarrhoeal cases were sequenced \((n=943);\) controls matched for age, sex and study site were also included \((n=88)\). Three isolates were from children whose case/control status was uncertain. Fecal samples were collected at the study sites before antimicrobial treatment, although previous exposure to antimicrobials from other sources cannot be ruled out. Control children were also not receiving any antimicrobial treatment.

Whole-genome sequences were generated for all 185 \(aEPEC\) isolates at the Wellcome Trust Sanger Institute using the Illumina HiSeq platform (100bp paired-end reads) and assembled using Velvet, as described previously\(^\text{\text{-}}\). Details of the individual isolates, accession numbers for the corresponding genome sequence reads and assemblies (deposited collectively under BioProject ERP001141), and associated metadata are provided in Supplementary Table 1.

**Phenotypic characterization of AMR profiles.** Antimicrobial susceptibility testing to 16 antimicrobials was performed using the VITEK2 (bioMérieux) system or an agar-dilution method. A summary of the drugs, testing methods, and the minimum inhibitory concentration (MIC) breakpoints used to determine susceptible, intermediate or resistant status for each drug is shown in Supplementary Table 2. The controls used were three reference \(S.\ enterica\) isolates with known resistance profiles that were kindly provided by the Microbiological Diagnostic Unit Public Health Laboratory. These strains had the following profiles: (1) susceptible to all drugs tested; (2) resistant to ampicillin, streptomycin, tetracycline, chloramphenicol, sulphadiazine, trimethoprim, kanamycin, spectinomycin and gentamicin; and (3) resistant to streptomycin,\(^\text{\text{-}}\) tetracycline, kanamycin, nalidixic acid and ciprofloxacin.

For VITEK2 assays, pure isolates were streaked on MacConkey agar plates and incubated at 37 \(^\circ\)C overnight. Isolates were then subcultured onto horse blood agar (HBA) plates for fresh culture and incubated overnight at 37 \(^\circ\)C. One to three colonies were selected from each HBA plate and suspended in saline to an absorbancy of ~0.5 McFarlane Units before being subjected to VITEK2 analysis. The raw MIC data from the VITEK2 assays are shown in Supplementary Table 7.

Susceptibility to streptomycin, chloramphenicol, azithromycin and tetracycline were determined using an agar-dilution method. Bacterial suspensions were prepared as described above. To each of 32 stainless steel wells, 450 \(\mu\)l nutrient broth containing 0.05% agar was added, followed by 50 \(\mu\)l bacterial suspension. Each Mueller Hinton agar antimicrobial-containing plate for susceptibility testing and two control Mueller Hinton and MacConkey agar plates were inoculated using a 32-pin replicator. Each pin delivered 2 \(\mu\)l to the plate such that the final number of colony-forming units in each sample was ~10\(^8\). Plates were incubated overnight at 37\(^\circ\)C and inspected the next day. Growth on an antimicrobial-containing Mueller Hinton plate was recorded as phenotypically resistant to the drug, whereas no growth was recorded as susceptible.

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) MIC breakpoints (version 6) were used where available\(^\text{\text{-}}\). Differences exist between the EUCAST and Clinical and Laboratory Standards Institute (CLSI) guidelines in terms of MIC breakpoints and the drugs to be tested. As tetracycline does not have defined MIC breakpoints under the EUCAST scheme, we used the CLSI MIC breakpoint. Streptomycin and azithromycin do not have established MIC breakpoints under either scheme\(^\text{\text{-}}\). Previous research proposed a breakpoint of 16 \(\mu\)g ml\(^{-1}\) for streptomycin in \(E.\ coli\)\(^\text{\text{-}}\). Little information is available on the MIC distribution of azithromycin for \(E.\ coli\). A breakpoint of 16 \(\mu\)g ml\(^{-1}\) has been proposed for \(S.\ enterica\) based on a study in which the majority of isolates displayed MICs of 4–89 \(\mu\)g ml\(^{-1}\)\(^\text{(ref. \text{18})}\). For the present study, the breakpoint MIC for each of these drugs was set at the conservative value of 16 \(\mu\)g ml\(^{-1}\). The antibiogram susceptibility data for each isolate are shown in Supplementary Table 3.

**Detection of AMR genes.** An SRS2-formatted version of the ARG-ANNOT ARMS database\(^\text{\text{-}}\) was downloaded from https://github.com/katholt/srs2. All sequence read sets were screened against the database using SRS2 to detect the presence of known acquired resistance genes in each genome\(^\text{\text{-}}\). The \(\beta\)-lactamase genes, \(ampC1\), \(ampC2\) and \(ampH\), were excluded from analysis, as in \(E.\ coli\) they are core genes that normally do not confer antibiotic resistance. The results were transformed into a binary table in R to indicate presence/absence of acquired resistance gene alleles (Supplementary Table 4).
Detection of single nucleotide polymorphisms conferring resistance to fluoroquinolones and nitrofurantoin. Chromosomal mutations, known to be associated with resistance to fluoroquinolones in E. coli, were extracted from the genome-wide single nucleotide polymorphism calls obtained previously based on mapping the reads to the E. coli strain 12009 O103:H2 reference genome. These included specific mutations in the quinolone-resistance-determining regions of gyrA, gyrB, parC and parE, and non-synonymous substitutions in nfsA (residues 11–15) that confer resistance to nitrofurantoin.

Statistical analysis of AMR phenotype prediction from genotype. All statistical analyses were performed using the R Stats Package version 3.4.0. The ability of genotypes to predict drug susceptibility phenotypes was assessed by comparing antimicrobial susceptibility phenotypes (S, I and R) with the presence of known AMR-associated genes and mutations. Errors in predicting antimicrobial susceptibility were characterized as very major (calling a resistant isolate susceptible) or major (calling a susceptible isolate resistant). The currently accepted standards for very major error and major error rates are <1.5% and <3%, respectively. Here, very major errors were said to have occurred when an isolate was phenotypically resistant, but no known resistance genes or mutations were detected, while major errors were made when an isolate carried known resistance determinant(s) but was phenotypically susceptible. Statistical analysis to determine specificity, PPV and NPV were calculated in the epiR package representing the relationships between the 185 GEMS aEPEC isolates was extracted.

Visualization of AMR and plasmid genotypes against a core gene tree. The presence/absence in each genome. The results were transformed into a binary table in R to indicate membership. The two principal components contributing the most to discriminant analysis were plotted and labelled with the genetic determinants whose variation contributed the most to those components. The posterior group membership probabilities for each discriminant function were also plotted.

Construction of co-occurrence network. A pairwise co-occurrence matrix of acquired AMR genes was constructed by transforming the binary AMR gene content matrix in R. The co-occurrence relationships were visualised between all pairs of genes using the pheatmap package (v1.0.8) in R (https://CRAN.R-project.org/package=pheatmap) (Supplementary Fig. 2). Networks of co-occurring genes, in which nodes represent genes and edges represent a frequency of co-occurrence exceeding a given threshold (set to ≥20, ≥33, ≥46, ≥100 genomes), were visualized in R using the igraph package (v1.1.2) in R.

Plasmid replicon screening. An SRST2-formatted version of the PlasmidFinder database was downloaded from https://github.com/katholt/srst2 (https://github.com/katholt/srst2) containing 80 known plasmid replicon marker sequences. All sequence read sets were screened against the database using SRST2 to detect the presence of these replicons in each genome. The results were transformed into a binary table in R to indicate presence/absence.

Visualisation of AMR and plasmid genotypes against a core gene tree. A subtree representing the relationships between the 185 GEMS aEPEC isolates was extracted from the full core phylogeny we published previously by pruning all other tips using R packages ape (v5.1.0) and geiger (v2.0.6). The presence of acquired AMR genes, mutations and plasmid replicons was plotted as a heatmap against the core gene tree in which nodes represent genes and edges represent a frequency of co-occurrence exceeding a given threshold (set to ≥20, ≥33, ≥46, ≥100 genomes), were visualized in R using the igraph package (v1.1.2) in R.

Investigation of mechanisms of AMR gene mobilisation. Common AMR-associated genes that were shown to co-occur, specifically blasoja, sul1, sul2, strB, strR, multiple dfrA alleles, tet(A) and tet(B), were further investigated in the aEPEC genome assemblies to determine whether they were carried on the same mobile elements. The aEPEC genome assemblies generated previously were interrogated with BLAST (v2.3.0), using as queries the AMR genes and the sequences of the plasmids pCERC1 (accession [N012467] and pCERC2 (accession KK291024), and the transposons Tn6029 (accession GQ150541), Tn1722 (accession X61367) and Tn10 (accession AF223162). For example, if the pCERC2 backbone and AMR and plasmid genotypes were also detected in a single contig in the genome assembly, we inferred that these genes were moving together on a pCERC2-related plasmid.

Two representative aEPEC isolates that were identified as harbouring a pCERC2-like plasmid backbone with different dfr gene insertions (strains 402635 and 400879) were selected as representatives for further analysis. These genomes were re-assembled with Unicycler (v0.2.0), annotated using Prokka (v1.12) and compared to the reference sequences for pCERC1 and pCERC2 using BLAST. The comparisons were then explored using Artemis Comparison Tool and plotted with genoplotR (v0.8.7) in R.

Antimicrobial usage and data and correlation with resistance. Data on the use of antimicrobials at each of the seven GEMS sites were collected as part of the original GEMS protocol. These data included details of the antimicrobials prescribed to all cases presenting with watery diarrhoea or dysentery at the study clinics and were documented by a member of the GEMS clinical team. Two of the recorded drugs were excluded from the current analysis: pivmecillinam, because it was not used at any discernible level, and metronidazole, which is active against obligate anaerobic protozoa and bacteria only and therefore does not pertain to E. coli, which is intrinsically resistant to this agent. The frequency of prescriptions for each drug at each site was visualized in R, using the ggplot2 package (v2.2.1).

The frequencies of ciprofloxacin and trimethoprim usage and associated genetic determinants was investigated via linear regression modelling in R using the lme function. For ciprofloxacin, the genetic determinants were either one or more quinolone resistance-associated point mutations in gyrA (point mutations in parC only occurred when gyrA mutations were also present), or the presence of the plasmid-borne genes qepA or qnrS. Genetic evidence of trimethoprim/sulphanemethoxazole resistance required the combination of at least one dfrA gene together with sul1 or sul2. The data were visualized in R using the ggplot2 package.

Data availability. Accession numbers for the short-read data and associated metadata are listed in Supplementary Table 1. The phenotypic resistance data are provided in Supplementary Tables 3 and 7 and the genotypic resistance profiles are shown in Supplementary Table 4.

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**Author contributions**

D.J.I., K.E.H. and R.M.R.-B. contributed to the design of the study and data interpretation. D.J.I. performed the experimental analyses. D.J.I. performed the majority of bioinformatics analyses with input from K.E.H. M.M.L. and K.L.K. oversaw the original GEMS study and provided bacterial isolates and associated metadata. All authors contributed to the writing of the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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- State explicitly what error bars represent (e.g. SD, SE, CI)

Software and code

Policy information about availability of computer code

Data collection: Not applicable

Data analysis

- adegenet package (v2.1.0); pheatmap package (v1.0.8); igraph package (v1.1.2); ape (v5.1); geiger (v2.0.6); genoplotR (v0.8.7); epiR package (v0.9-93); Unicycler (v0.2.0); Prokka (v1.12); Bandage (v0.8.1); MUMmer (v3.23); Artemis Comparison Tool; BLAST (v2.3.30).

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All studies must disclose on these points even when the disclosure is negative.

Sample size

No sample size calculation was performed. As described in the methods section, we used all of the bacterial strains that were available from the original GEMS study.

Data exclusions

As described in the MS, for some analyses we excluded samples where the case/control status of the isolate was unknown.

Replication

Not applicable

Randomization

Not applicable

Blinding

Resistance phenotypes were determined and recorded without knowledge of the genotype. Antibiotic usage data were examined only after all the phenotypes and genotypes related to AMR had been analysed and recorded. All other data were computer-generated and not adjusted.

Reporting for specific materials, systems and methods

Materials & experimental systems

n/a Involved in the study

☒ X Unique biological materials
☒ X Antibodies
☒ X Eukaryotic cell lines
☒ X Palaeontology
☒ X Animals and other organisms
☒ X Human research participants

Methods

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☒ X ChIP-seq
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☒ X MRI-based neuroimaging

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

All unique materials are available from the authors.

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Population characteristics

The bacteria used in this study were of human origin. The way patients and controls were selected and the way samples were
| Population characteristics | collected have been described in Kotloff et al. Lancet 382, 209–222 (2013); Panchalingam et al. Clin Infect Dis 55, S294–S302 (2012), and Ingle et al. Nat Microbiol 1, 15010 (2016). |
|-----------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Recruitment                 | See above                                                                                                                                                                                                  |