Molecular Characteristics of Extended-Spectrum Cephalosporin-Resistant Enterobacteriaceae from Humans in the Community

Angela H. A. M. van Hoek, Leo Schouls, Marga G. van Santen, Alice Florijn, Sabine C. de Greeff, Engeline van Duijkeren*

Centre for Infectious Disease Control (Cib), National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands

* engeline.van.duijkeren@rivm.nl

Abstract

Objective
To investigate the molecular characteristics of extended-spectrum cephalosporin (ESC)-resistant Enterobacteriaceae collected during a cross-sectional study examining the prevalence and risk factors for faecal carriage of extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae in humans living in areas with high or low broiler density.

Methods
ESC-resistant Enterobacteriaceae were identified by combination disc-diffusion test. ESBL/AmpC/carbapenemase genes were analysed using PCR and sequencing. For E. coli, phylogenetic groups and MLST were determined. Plasmids were characterized by transformation and PCR-based replicon typing. Subtyping of plasmids was done by plasmid multilocus sequence typing.

Results
175 ESC-resistant Enterobacteriaceae were cultured from 165/1,033 individuals. The isolates were Escherichia coli (n=65), Citrobacter freundii (n=52), Enterobacter cloacae (n=38), Morganella morganii (n=5), Enterobacter aerogenes (n=4), Klebsiella pneumoniae (n=3), Hafnia alvei (n=2), Shigella spp. (n=2), Citrobacter amalonaticus (n=1), Escherichia hermannii (n=1), Kluyvera cryocrescens (n=1), and Pantoea agglomerans (n=1). The following ESBL genes were recovered in 55 isolates originating from 49 of 1,033 (4.7 %) persons: bla_CTX-M-1 (n=17), blaCTX-M-15 (n=16), blaCTX-M-14 (n=9), blaCTX-M-2 (n=3), blaCTX-M-3 (n=2), blaCTX-M-24 (n=2), blaCTX-M-27 (n=1), blaCTX-M-32 (n=1), blashv-12 (n=2), blashv-65 (n=1) and blatem-52 (n=1). Plasmidic AmpC (pAmpC) genes were discovered in 6 out of 1,033 (0.6 %) persons. One person carried two different E. coli isolates, one with blaCTX-M-1 and the other with blaCMY-2 and therefore the prevalence of persons carrying Enterobacteriaceae...
harboring ESBL and/or pAmpC genes was 5.2%. In eight *E. coli* isolates the AmpC phenotype was caused by mutations in the AmpC promoter region. No carbapenemase genes were identified. A large variety of *E. coli* genotypes was found, ST131 and ST10 being most common.

**Conclusions**
ESBL/pAmpC genes resembled those from patients in Dutch hospitals, indicating that healthy humans form a reservoir for transmission of these determinants to vulnerable people. The role of poultry in the transmission to humans in the community remains to be elucidated.

**Introduction**
Extended-spectrum-β-lactamase/AmpC producing Enterobacteriaceae have been found among humans worldwide. Most large-scale studies in humans, however, report data of patients or travelers and/or focus on ESBL-producing bacteria and/or certain bacterial species only (e.g. *Escherichia coli* or *Klebsiella pneumoniae*) [1–4]. Consequently, data on the prevalence of fecal carriage of ESBL/AmpC/carbapenemase producing Enterobacteriaceae in healthy humans in the community are scarce. The major mechanism of resistance to extended spectrum cephalosporins (ESC) in the family Enterobacteriaceae is the production of an extended-spectrum β-lactamase (ESBL) or an AmpC β-lactamase [5]. ESBLs are often plasmid mediated, while the production of AmpC β-lactamases can result either from (over)expression of the chromosomal *ampC* gene or by the acquisition of a plasmid-mediated *ampC* determinant [5]. Initially ESBL/AmpC-producing organisms were associated with hospitals and institutional care in humans, but they are now increasingly found in the community and in food-producing animals [6]. A connection between ESBL/AmpC-producing bacteria in food animals and humans has been suggested [1, 7–9]. ESBL/AmpC-producing Enterobacteriaceae have frequently been reported in broilers and therefore they have been considered as a reservoir for ESBL/AmpC-encoding resistance genes [7, 10]. Transmission from broilers to humans through the food chain has been proposed [11–13], but could also occur through direct contact or through the environment [7]. In 2011, a cross-sectional study was performed to determine the prevalence of, and identify risk factors for, carriage of ESBL-producing Enterobacteriaceae in people living in municipalities with either high or low broiler densities [14]. The prevalence of carriage of ESBL-producing bacteria was 5.1% and this percentage was lower in municipalities with high broiler densities (3.6%) compared to municipalities with low broiler densities (6.7%) [14]. The aim of the present study was to analyse the isolates from this cross-sectional study, including isolates with an AmpC phenotype, with respect to molecular characteristics and compare them to published data on isolates from patients, broilers, and persons in contact with broilers.

**Materials and Methods**
A cross-sectional study was conducted between August and December 2011. A random sample of adults (>18 years), stratified according to age and gender was taken from eight municipalities across 4 provinces of the Netherlands: North-Brabant, Gelderland, Overijssel and Frisia. In each province the municipality with the highest respectively lowest number of broiler farms per km² was selected. This information was obtained from the Dutch Product Board for
Poultry and Eggs. In total, 3,949 individuals were contacted by post and were asked to return a rectal swab and a questionnaire on demographics, contact with animals, lifestyle, medical history, eating habits and travel. For each respondent, distance to the nearest broiler farm was obtained using geographic data. Exclusion criteria were living or working on a commercial broiler farm [14]. The study was approved by the Medical Ethics Committee of University Medical Centre Utrecht, The Netherlands (protocol number 11–277). All participants provided written informed consent. Rectal swabs were obtained from 1,033 persons and were analysed to determine the presence of ESBL/AmpC/carbapenemase-producing Enterobacteriaceae. Bacteria were isolated by selective enrichment (Luria-Bertani broth (MP Biomedicals, Amsterdam, the Netherlands) supplemented with 1mg/L cefotaxime (Sigma-Aldrich, Zwijndrecht, the Netherlands), and cultured on selective plates (MacConkey agar no. 3, Oxoid, Badhoevedorp, the Netherlands) supplemented with 1 mg/L cefotaxime). Isolates (1–6 per person, depending on the numbers of different phenotypes) were tested phenotypically for ESBL/AmpC-production by a combination disc-diffusion test using cefotaxime and ceftazidime discs, with and without clavulanic acid (Becton Dickinson B.V., Breda, the Netherlands), according to CLSI guidelines [15]. A cefoxitin disc (Becton Dickinson B.V., Breda, the Netherlands) was used to detect AmpC phenotypes [7]. Genotypes of the ESBL/AmpC-positive isolates were determined by PCR and gene sequencing. For isolates with an ESBL phenotype, primers detecting CTX-M-group 1, CTX-M-group 2, CTX-M-group 9, CTX-M-group 8/25, OXA-1 like, SHV and TEM were used. In case of isolates displaying an AmpC phenotype, primers specific for ACC, ACT, BIL, CMY, DHA, FOX, LAT, MIR and MOX were used. In addition, all 71 isolates with an ESBL-phenotype were investigated using primers for the detection of carbapenemase genes of the KPC, NDM, OXA-48, and VIM families. For *E. coli* isolates with an AmpC phenotype, but negative in PCR for β-lactamase genes, chromosomal ampC promoter mutations were detected by PCR and sequencing analysis (Table 1).

DNA was extracted by Chelex-100 chelating resin (Bio-Rad Laboratories B.V., Veenendaal, the Netherlands). Published primer sets were used to screen for the group of ESBL/AmpC gene [22]. Complete ESBL/AmpC gene sequences were obtained by PCR using the primers as indicated in Table 1. Resulting amplicons were treated with ExoSAP-IT (Isogen Life Science, De Meern, the Netherlands) according to manufacturers’ instructions. Aliquots of the purified PCR products were used in sequence reactions on an AB 3730 genetic analyser using the Big Dye Terminator technology (Applied Biosystems, Bleiswijk, the Netherlands). Each sequence was compared with known β-lactamase gene sequences (www.lahey.org/Studies) by multiple-sequence alignment using the BLAST, BioNumerics and Seaview programmes.

Phylogenetic groups were determined for *E. coli* according to Doumith et al. [23]. Strains were sub-grouped according to Escobar-Páramo et al. [24]. For isolates identified as non-*E. coli* the bacterial species was identified by BBL (Becton Dickinson B.V., Breda, the Netherlands) and MALDI TOF MS on a Bruker Microflex LT instrument (Bruker Daltonics GmbH, Bremen, Germany).

Multilocus sequence typing (MLST) of *E. coli* was performed according to Wirth et al. [25]. Plasmids were characterised on a selection of isolates representing different ESBL/AmpC-genes and phylogenetic groups. Plasmids were first isolated using QIAfilter Plasmid Midi Kit (QIAGEN Benelux B.V., Venlo, the Netherlands). Next, the isolated plasmids were transformed into ElectroMAX DH10B cells (Invitrogen, Bleiswijk, the Netherlands) by electroporation [26]. The resulting transformants were cultured on selective plates (LB agar (MP Biomedicals, Amsterdam, the Netherlands) supplemented with 1 μg/ml cefotaxime) to isolate recipients carrying an ESBL/AmpC plasmids. PCR-based replicon typing (PBRT) was conducted to classify the plasmid inside the transformant using the PBRT kit (Diatheva, Fano,
Italy), according to Carattoli et al. [27]. IncF, IncI1 and IncN plasmids were further characterized by plasmid MLST (pMLST) [28, 29, 30].

Results
Out of 1,033 persons investigated, 165 (15.9%) carried ESC-resistant Enterobacteriaceae. Ten persons were positive for two types of ESC-resistant Enterobacteriaceae, yielding a total of 175 isolates with an ESBL/AmpC resistance phenotype.

Species identification
Species identification of the 175 isolates showed that they were *Escherichia coli* (n = 65), *Citrobacter freundii* (n = 52), *Enterobacter cloacae* (n = 38), *Morganella morganii* (n = 5).

**Table 1. Primers used to completely sequence the ESBL/AmpC resistance genes.**

| Gene family       | Primer (5'-3')                           | Purpose                  | Reference |
|-------------------|------------------------------------------|--------------------------|-----------|
| ACC group         | gcatgcggattggtggtgc                      | PCR & Sequencing         | This study|
|                   | cagcggcttgagctgagg                       | Sequencing               |           |
|                   | ccccaatgctggctgcc                        | Sequencing               |           |
|                   | aaggcggtcgtgtaacc                       | PCR & Sequencing         |           |
| ACT & MIR group   | cacagctcataccacacacc                     | PCR & Sequencing         | This study|
|                   | ctataagtaaaacccctttacc                  | Sequencing               |           |
|                   | ctgtaatctggcctcttccg                    | Sequencing               |           |
|                   | tttttgtagccggcgaatg                      | PCR & Sequencing         |           |
| AmpC promoter region | aatgcgtttttctcaggtctg                   | PCR & Sequencing         | [16]      |
|                   | ggccagcagaatgtggaac                     | PCR & Sequencing         |           |
| CMY-2 group       | atgtgagaaaaactctttatgtgc                 | PCR & Sequencing         | [17]      |
|                   | cttcagcatggctcgggtg                      | Sequencing               | This study|
|                   | agttcagcatctccgagcc                     | Sequencing               |           |
|                   | gcttttcacagatgtgccag                     | PCR & Sequencing         | [18]      |
| CTX-M-1 group     | gtgtgagagcagctctaa                      | PCR & Sequencing         | This study|
|                   | cggaagagaaaccgaa                        | PCR & Sequencing         |           |
|                   | Cttgggtggcattgtat                       | Sequencing               |           |
|                   | Cttggtaaagcatgtgggt                     | Sequencing               |           |
|                   | Cccaggtgaggtgtat                        | Sequencing               |           |
|                   | Gcacaactctctaaccaca                     | Sequencing               |           |
| CTX-M-2 group     | Atgtgactcagagcatcgg                    | PCR & Sequencing         | [19]      |
|                   | Ttattgcacagaaaccggtg                    | PCR & Sequencing         |           |
| CTX-M-9 group     | Tggtgacaaagagagttggaac                  | PCR & Sequencing         | [20]      |
|                   | Tcagaccccctgtgctgt                      | PCR & Sequencing         |           |
| DHA group         | Gtgaatctgagcagctctgc                    | PCR & Sequencing         | This study|
|                   | Tcagatggtgtgtggtg                       | Sequencing               |           |
|                   | Taaacgtctcctacgcctgc                    | Sequencing               |           |
|                   | Aataatcttaataatctcgcgtccc              | PCR & Sequencing         |           |
|                   | Tccagggggtctgctgc                      | PCR & Sequencing         |           |
| SHV               | Ttatccccctgtgagccacc                    | PCR & Sequencing         | [17]      |
|                   | Gatttctgtacctcgctgg                     | PCR & Sequencing         |           |
| TEM               | Gcgcgaaccccctatttgt                    | PCR & Sequencing         | [21]      |
|                   | Accaatgtaatcttagtg                     | PCR & Sequencing         |           |

doi:10.1371/journal.pone.0129085.t001
Enterobacter aerogenes (n = 4), Klebsiella pneumoniae (n = 3), Hafnia alvei (n = 2), Shigella spp. (n = 2), Citrobacter amalonaticus (n = 1), Escherichia hermannii (n = 1), Kluyvera cryocrescens (n = 1), and Pantoea agglomerans (n = 1).

ESBL/AmpC phenotype and genes of all isolates

Of these 175 isolates, 119 (68.0%) showed an AmpC-phenotype and were recovered from 116 persons. For most isolates, however, no AmpC gene was found. If an AmpC gene was detected that is specific for the species concerned (e.g. blaCMY-2 in C. freundii, blaACC in H. alvei, bladHA in M. morganii, and blaACT/MIR-1 in Enterobacter species) it was considered as chromosomal and these isolates were excluded from further analysis. Six isolates carried plasmidic AmpC (pAmpC) genes: 4 E. coli isolates, 1 P. agglomerans isolate and 1 C. freundii isolate. The prevalence of pAmpC-producing Enterobacteriaceae was 5.0% (6/119) of the isolates with an AmpC phenotype. The prevalence of persons carrying a pAmpC-positive isolate was 0.6% (6/1,033).

The remaining 56 (32.0%) ESC-resistant isolates displayed an ESBL-phenotype. In 55 (98.2%) of these 56 isolates an ESBL-gene was found. Isolates carrying an ESBL-gene were E. coli (n = 51), E. hermannii (n = 1), Klebsiella pneumonia (n = 2) and Citrobacter freundii (n = 1) and were recovered from 49 persons yielding an ESBL prevalence of 4.7% (49/1,033). The most frequently identified ones were blaCTX-M-1 (n = 17), blaCTX-M-15 (n = 16) and blaCTX-M-14 (n = 9). For one E. coli isolate no ESBL gene was found, but the isolate carried blaOXA-1 and blaTEM-1b.

Three persons carried two isolates with different ESBL-genes: two of them (P33, P39) carried two different E. coli genotypes with blacTX-M-14 and blacTX-M-15, respectively, and one individual (P1) carried a C. freundii with blacTX-M-15 and an E. coli with blacTX-M-1. Two persons (P17, P44) carried two E. coli with different MLSTs with the same ESBL-gene. One individual (P14) carried two E. coli isolates with blacTX-M-1, but one of these isolate also contained blatEM-1b. One person (P3) carried an E. coli with blacTX-M-1 and an E. coli carrying blacMY-2. The prevalence of persons carrying an ESBL/pAmpC positive isolate was 5.2 (54/1,033).

No genes encoding for the production of carbapenemases were found.

Characteristics of the E. coli isolates

AmpC genes were found in only four of the 13 phenotypically AmpC E. coli isolates (blacMY-2 (n = 3) and bladHA-1 (n = 1)). However, eight of the isolates belonging to MLST ST88, ST95, ST131 (n = 2), ST345, ST453 (n = 2) and ST500 carried mutations in the promoter region of ampc (−42T−18A−1T+58T+81G (n = 4); −32A−28A (n = 2); −32A−28A+17T+30A (n = 1) and −18A−14INS(G)−1T+58T+81G (n = 1). Most of the 65 E. coli isolates displaying an ESBL-phenotype (n = 52). The predominant ESBL-genes in E. coli were blacTX-M-1 (n = 17), blacTX-M-15 (n = 13) and blacTX-M-14 (n = 9). Other ESBL-genes found were blacTX-M-2 (n = 3), blacTX-M-3 (n = 2), blacTX-M-24 (n = 2), blasHV-12 (n = 2), blacTX-M-27 (n = 1), blacTX-M-32 (n = 1), blatEM-52 (n = 1). Other β-lactamase genes found were blatEM-1b (n = 20), blatEM-84 (n = 1), and blaoxa-1 (n = 6) (Table 2).

The predominant E. coli phylogenetic groups found were B1 (n = 17) and A1 (n = 16), followed by D2 (n = 11), B2 (n = 8), D1 (n = 6), A0 (n = 5), and B2 (n = 2). The most prevalent E. coli MLST types were ST10 (n = 6), ST131 (n = 6), followed by ST58 (n = 5), and ST38 (n = 4) but a great diversity of different genotypes was found. Plasmid family incI1 was most commonly identified, followed by incF. pMLST revealed that within incI1 subtype ST58 was found most often. All incN plasmids had subtype ST1. For the incF plasmid family a variety of subtypes were found (Table 2).
Table 2. Characteristics of the ESBL/pAmpC-producing isolates.

| Person | Bacterial species | Isolate | Phylotype | MLST | ESBL/AmpC gene | Other β-lactamase gene | Plasmid | pMLST or FAB formula |
|--------|------------------|---------|-----------|------|----------------|------------------------|---------|---------------------|
| P1     | C. freundii      | 0754_1  |           |      | CTX-M-15       | nd                     |         |                     |
|        | E. coli          | 0754_6  | A1        | ST10 | CTX-M-3        | nd                     |         |                     |
| P2     | C. freundii      | 2090_2  |           |      | DHA-1          | nd                     |         |                     |
| P3     | E. coli          | 0331_3  | B2        | ST131| CMY-2          | incl1                  | ST12 (CC-12) |                     |
|        | E. coli          | 0331_4  | D1        | ST69 | CTX-M-1        | incl1                  | ST36 (CC-5) |                     |
| P4     | E. coli          | 3745_1  | A0        | ST93 | CMY-2          | inclA/C                |         |                     |
| P5     | E. coli          | 1517_1  | B2        | ST219| CMY-2          | incl1                  | ST12 (CC-12) |                     |
| P6     | E. coli          | 3325_1  | A1        | ST10 | CTX-M-1        | TEM-1b                 |         | ST58 (CC-58)        |
| P7     | E. coli          | 3554_3  | A1        | ST10 | CTX-M-1        | TEM-1b                 | incl1   | ST58 (CC-58)        |
| P8     | E. coli          | 3745_1  | A0        | ST93 | CMY-2          | inclA/C                |         |                     |
| P9     | E. coli          | 0610_4  | A1        | ST10 | CTX-M-1        | TEM-1b                 | nd      |                     |
| P10    | E. coli          | 2079_1  | B1        | ST58 | CTX-M-1        | TEM-1b                 | NTP     |                     |
| P11    | E. coli          | 2115_1  | B1        | ST58 | CTX-M-1        | TEM-1b                 | incl1   | ST7 (CC-7)          |
| P12    | E. coli          | 2362_1  | A1        | ST10 | CTX-M-1        | TEM-1b                 | nd      |                     |
| P13    | E. coli          | 2555_5  | B1        | ST58 | CTX-M-1        | TEM-1b                 | incl1   | ST58 (CC-58)        |
| P14    | E. coli          | 2643_1  | B1        | ST5037| CTX-M-1       | incN                   | ST1     |                     |
| P15    | E. coli          | 2668_1  | A1        | ST88 | CTX-M-1        | TEM-1b                 | incl1   | ST3 (CC-3)          |
| P16    | E. coli          | 2760_1  | B1        | ST2536| CTX-M-1       | incl2                  |         |                     |
| P17    | E. coli          | 2865_3  | D2        | ST657| CTX-M-1        | incN                   | ST1     |                     |
| P18    | E. coli          | 2865_5  | A1        | ST744| CTX-M-1        | TEM-1b                 | nd      |                     |
| P19    | E. coli          | 2870_1  | D1        | ST59 | CTX-M-1        | incl1                  | ST58 (CC-58) |                     |
| P20    | E. coli          | 2870_1  | B1        | ST5037| CTX-M-1       | incN                   | ST1     |                     |
| P21    | E. coli          | 2316_3  | A0        | ST1178| CTX-M-3       | nd                     |         |                     |
| P22    | E. coli          | 0002_1  | A1        | ST10 | CTX-M-14       | NTP                    |         |                     |
| P23    | E. coli          | 0164_2  | B1        | ST58 | CTX-M-14       | incl1                  | ST80    |                     |
| P24    | E. coli          | 0413_1  | D1        | ST69 | CTX-M-14       | inclF                  | F2:A--B1|                     |
| P25    | E. coli          | 0482_3  | D2        | ST38 | CTX-M-14       | TEM-1b                 | nd      |                     |
| P26    | E. coli          | 2968_2  | B2        | ST1982| CTX-M-14      | incB/O                 |         |                     |
| P27    | E. coli          | 3055_1  | A1        | ST5039| CTX-M-14      | incl1                  | ST80    |                     |
| P28    | E. coli          | 3055_1  | A1        | ST5039| CTX-M-14      | incl1                  | ST80    |                     |
| P29    | E. coli          | 3055_1  | A1        | ST5039| CTX-M-14      | incl1                  | ST80    |                     |
| P30    | E. coli          | 3055_1  | A1        | ST5039| CTX-M-14      | incl1                  | ST80    |                     |
| P31    | E. coli          | 3055_1  | A1        | ST5039| CTX-M-14      | incl1                  | ST80    |                     |
| P32    | E. coli          | 3055_1  | A1        | ST5039| CTX-M-14      | incl1                  | ST80    |                     |
| P33    | E. coli          | 3055_1  | A1        | ST5039| CTX-M-14      | incl1                  | ST80    |                     |
| P34    | E. coli          | 3055_1  | A1        | ST5039| CTX-M-14      | incl1                  | ST80    |                     |
| P35    | E. coli          | 3055_1  | A1        | ST5039| CTX-M-14      | incl1                  | ST80    |                     |
| P36    | E. coli          | 3055_1  | A1        | ST5039| CTX-M-14      | incl1                  | ST80    |                     |
| P37    | E. coli          | 3055_1  | A1        | ST5039| CTX-M-14      | incl1                  | ST80    |                     |
| P38    | E. coli          | 3055_1  | A1        | ST5039| CTX-M-14      | incl1                  | ST80    |                     |
| P39    | E. coli          | 3055_1  | A1        | ST5039| CTX-M-14      | incl1                  | ST80    |                     |
| P40    | E. coli          | 3055_1  | A1        | ST5039| CTX-M-14      | incl1                  | ST80    |                     |
| P41    | E. coli          | 3055_1  | A1        | ST5039| CTX-M-14      | incl1                  | ST80    |                     |

(Continued)
The E. hermannii isolate carried blaCTX-M-15 and blaOXA-1. Of the K. pneumoniae isolates, two had an ESBL phenotype (Table 2); one harboured blaSHV-65, while the other contained blaCTX-M-15 and blaSHV-11, both genes had synonymous mutations. The third K. pneumoniae isolate displayed an AmpC phenotype, but no gene could be characterized. The P. agglomerans isolate carried blaCMY-48. The C. freundii isolate with the ESBL-phenotype contained blaCTX-M-15.

Analysis of risk factors

After analysis of the questionnaires we found no clear evidence that certain genes were more often found in specific exposure categories. However, blaCTX-M-15 genes were relatively more often found than other genes among persons owning or in contact with a horse compared to persons not frequently exposed to a horse (p = 0.04) (Table 3).

Discussion

In the present study the prevalence of persons carrying Enterobacteriaceae harboring ESBL and/or pAmpC genes was 5.2%. Response analysis with respect to age, sex, and province and broiler density showed that a representative sample of Dutch adults was obtained [14]. Medical histories of 1,025/1,033 persons were available and 7.9% reported admission to a hospital and 6.4% urinary tract infection in the 6 month prior to inclusion in the present study and neither of these two factors was identified as a risk factor for being ESBL-positive [14]. Therefore, this study population represents a predominantly healthy general population. Most studies on ESBL/AmpC producing bacteria include either hospitalized patients or persons visiting a general practitioner.
To date only limited data are available on the prevalence of pAmpC-producing bacteria in the open population. The prevalence of persons carrying pAmpC genes in the present study (0.6%) was slightly lower than the 1.3% found in a study in community-dwelling individuals in the densely populated region of Amsterdam [31]. The disparity might be caused by the different study population: participants of the current study were living in rural areas, but dissimilarities in the methodology might also be an explanation. The prevalence of ESBL and pAmpC carriers in patients and out-patients of a Spanish University teaching hospital was 5.0% and 0.6%, respectively, which is similar to our findings [32]. The pAmpC gene discovered most frequently was bla\textsubscript{CMY-2} [31, 32] and this corresponds with the findings of the present study. Altogether, the findings indicate that healthy humans form a reservoir for transmission of these determinants to vulnerable people.

Interestingly, only two persons carrying ESBL-positive \textit{K. pneumoniae} were found (0.2% of all persons tested), although the prevalence of ESC-resistant \textit{K. pneumoniae} is increasing in Dutch hospitals. The European Antimicrobial Resistance Surveillance System that collects resistance data from invasive isolates throughout Europe showed that third-generation cephalosporin resistance in The Netherlands has increased from 3.5% in 2005 to 7.5% in 2013 in \textit{K. pneumoniae} [33]. This might be explained by the fact that ESBL-producing \textit{K. pneumoniae} have different transmission dynamics compared to ESBL-producing \textit{E. coli}, the predominant ESBL-positive species in the present study. A recent study showed a higher rate of community acquisition among ESBL-producing \textit{E. coli} compared to ESBL-producing \textit{K. pneumoniae} in patients with bacteremia [34]. In addition, ESBL-producing \textit{E. coli} isolates had more different genotypes and patients infected with ESBL-producing \textit{E. coli} were more likely to come from high prevalence countries compared to ESBL-producing \textit{K. pneumoniae} supporting the notion that ESBL-producing \textit{E. coli} is more likely to be acquired in community settings while \textit{K. pneumoniae} is more often associated with hospital outbreaks and clonal transmission within the hospital [34].

| Table 3. Distribution of ESBL/pAmpC-genes over the different risk categories. |
|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|
| Broiler density               | Number of persons carrying an isolate with bla\textsubscript{CTX-M-1} | Number of persons carrying an isolate with bla\textsubscript{CTX-M-15} | Number of persons carrying an isolate with other gene | Total number of positive persons |
| Low                          | 11 (32.4%)                  | 9 (26.5%)                   | 14 (41.2%)                  | 34                             |
| High                         | 4 (20.0%)                   | 7 (35.0%)                   | 9 (45.0%)                   | 20                             |
| Owning/contact with a pet     | No                          | 4 (19.0%)                   | 7 (33.3%)                   | 10 (47.6%)                    | 21                             |
|                             | Yes                         | 11 (33.3%)                  | 9 (27.3%)                   | 13 (39.4%)                    | 33                             |
| Owning/contact with a horse   | No                          | 10 (22.2%)                  | 13 (28.9%)                  | 22 (48.9%)                    | 45                             |
|                             | Yes                         | 5 (55.6%)                   | 3 (33.3%)                   | 1 (11.1%)                     | 9                              |
| Urinary tract infection       | No                          | 12 (24.5%)                  | 16 (32.7%)                  | 21 (42.9%)                    | 49                             |
|                             | Yes                         | 3 (60.0%)                   | 0 (0.0%)                    | 2 (40.0%)                     | 5                              |
| Hospital admission            | No                          | 6 (25.0%)                   | 5 (20.8%)                   | 13 (54.2%)                    | 24                             |
|                             | Yes                         | 9 (30.0%)                   | 11 (36.7%)                  | 10 (33.3%)                    | 30                             |
| Eating meat                  | No                          | 0 (0.0%)                    | 0 (0.0%)                    | 0 (0.0%)                      | 0                              |
|                             | Yes                         | 15 (27.8%)                  | 16 (29.6%)                  | 23 (42.6%)                    | 54                             |
| Travelling abroad             | No                          | 8 (40.0%)                   | 4 (20.0%)                   | 8 (40.0%)                     | 20                             |
|                             | Yes                         | 7 (20.6%)                   | 12 (35.3%)                  | 15 (44.1%)                    | 34                             |

doi:10.1371/journal.pone.0129085.t003
One third of all ESC-resistant isolates in the present study carried an ESBL-gene and in all but one isolate with an ESBL-phenotype an ESBL-gene was found. $bla_{CTX-M-1}$ was found most frequently and exclusively in $E. coli$, followed by $bla_{CTX-M-15}$ and $bla_{CTX-M-14}$. In a study investigating ESBL-producing Enterobacteriaceae in Dutch community patients with gastrointestinal complaints, the most prevalent ESBL gene was $bla_{CTX-M-15}$, comprising 47% of all ESBL-genes and 85% of the genes of the CTX-M-group 1 [3]. In another study, $bla_{CTX-M-15}$ was also most prevalent (39%), followed by $bla_{CTX-M-1}$ (15%), among clinical Enterobacteriaceae obtained from Dutch patients [35]. Another Dutch study, however, found $bla_{CTX-M-1}$ most often in faecal samples from persons admitted to the hospital, whereas $bla_{CTX-M-14}$ was predominant in isolates from blood cultures, followed by $bla_{CTX-M-1}$ [1].

In this study, $E. coli$ ST131, ST10, ST58 and ST38 were found most often. This is in accordance with the findings of Reuland et al. [3]: the predominant $E. coli$ ST’s in their study were ST38, ST131, ST648 and ST10. $E. coli$ ST131 has emerged as a global epidemic, multidrug-resistant clade. $E. coli$ ST131 may cause extraintestinal infections, especially of the urinary tract, and its ESBL production is most often due to the presence of $bla_{CTX-M-15}$ [36]. In an international study investigating 240 ESBL-producing $E. coli$ with ST131 from nine countries, 193 (80%) contained $bla_{CTX-M-15}$ [36]. In a Dutch study investigating clinical isolates most of the ST131 $E. coli$ contained $bla_{CTX-M-15}$, and presence of this gene was associated with higher levels of resistance [35]. In the present study only one person carrying $E. coli$ ST131 containing $bla_{CTX-M-15}$ was found indicating that this ST131 clade does not seem to be endemic in humans in the community in the Netherlands.

Most $E. coli$ isolates belonged to phylogroups A1 and B1. These phylogroups are less often recovered from extraintestinal body sites. Isolates belonging to phylogroups B2 and D, however, were also found. Virulent strains causing extraintestinal infections belong mainly to groups B2 and D [24, 37]. This indicates that humans in the community carry $E. coli$ isolates that have the potential to cause disease.

The prevalence of ESBL carriage was higher in areas with low broiler densities than in areas with high broiler densities and therefore living in areas with high broiler density was not identified as a risk factor [14]. The prevalence of ESBL/pAmpC carriage among people on broiler farms (19.1%) was higher than in the present study and an increased risk of carriage was shown among individuals having a high degree of contact with live broilers [7]. The most prevalent ESBL/AmpC genes in isolates from humans on broiler farms as well as broilers were $bla_{CMY-2}$, $bla_{CTX-M-1}$ and $bla_{SHV-12}$, followed by $bla_{TEM-52}$, while $bla_{CTX-M-15}$ was not found [7, 10]. In contrast, in the present study, $bla_{CTX-M-1}$, $bla_{CTX-M-15}$ and $bla_{CTX-M-14}$ were among the most prevalent ESBL-genes identified and $E. coli$ isolates carrying $bla_{CMY-2}$, $bla_{SHV-12}$ and $bla_{TEM-52}$ were only found sporadically although 94.5% of the study participants reported eating...
chicken meat [14]. It has been postulated that humans acquire ESBL-producing bacteria by eating chicken meat, because Dutch chicken meat has been shown to be contaminated with *E. coli* strains containing ESBL-genes similar to those found in patients [1, 12]. The same genes are, however, present in many different potential reservoirs, including cattle, companion animals, horses and pigs, and therefore conclusions regarding their origin cannot be drawn [6, 38].

Genes encoding for the production of carbapenemases were not detected, signifying that the prevalence of carbapenemase producing Enterobacteriaceae in the community is low.

**Conclusions**

ESBL/pAmpC genes found in healthy humans in the community are similar to those in Dutch patients indicating that humans in the community could be a reservoir for these resistant determinants. While contact with broilers has previously been identified as a risk factor, the role of poultry in transmission to humans through the environment or the food chain remains to be elucidated.

**Author Contributions**

Conceived and designed the experiments: ED SG LS AH. Performed the experiments: AH MS AF. Analyzed the data: AH ED LS SG. Wrote the paper: AH LS SG ED.

**References**

1. Overdevest I, Willemsen I, Rijnsburger M, Eustace A, Xu L, Hawkey P, et al. Extended-spectrum β-lactamase genes of *Escherichia coli* in chicken meat and humans, the Netherlands. Emerg Infect Dis. 2011; 17: 1216–1222. doi:10.3201/eid1707.110209 PMID: 21762575

2. Paltansing S, Vlot JA, Kraakman MEM, Mesman R, Bruining ML, Bernards AT, et al. Extended-spectrum β-lactamase-producing *Enterobacteriaceae* among travellers from the Netherlands. Emerg Infect Dis. 2013; 19: 1206–1213. doi:10.3201/eid1908.130257 PMID: 23885972

3. Reuland EA, Overdevest ITMA, al Naiemi N, Kalpoe JS, Rijnsburger MC, Raadsen SA, et al. High prevalence of ESBL-producing *Enterobacteriaceae* carriage in Dutch community patients with gastrointestinal complaints. Clin Microbiol Infect. 2013; 19: 542–549. doi:10.1111/j.1469-0691.2012.03947.x PMID: 22756722

4. Valenza G, Nickel S, Pfeiffer Y, Eller C, Krupa E, Lehner-Reindl V, et al. Extended-spectrum-β-lactamase-producing *Escherichia coli* as intestinal colonizers in the German community. Antimicrob Agents Chemother. 2014; 58: 1228–1230. doi:10.1128/AAC.01993-13 PMID: 24295972

5. Pfeiffer Y, Cullik A, Witte W. Resistance to cephalosporins and carbapenems in Gram-negative bacterial pathogens. Int J Med Microbiol. 2010; 300: 371–379. doi:10.1016/j.ijmm.2010.04.005 PMID: 20537585

6. Ewers C, Bethe A, Semmler T, Guenther S, Wieler LH. Extended-spectrum β-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. Clin Microbiol Infect. 2012; 18: 646–655. doi:10.1111/j.1469-0691.2012.03850.x PMID: 22519857

7. Huijbers PMC, Graat EAM, Haenen APJ, van Santen MG, van Essen-Zandbergen A, Mevius DJ, et al. Extended-spectrum β-lactamase- and AmpC β-lactamase-producing *Escherichia coli* in broilers and people living and/or working on broiler farms: prevalence, risk factors, and molecular characteristics. J Antimicrob Chemother. 2014; 69: 2669–2675. doi:10.1093/jac/dku178 PMID: 24879667

8. Liebana E, Carattoli A, Coque TM, Hasman H, Magiorakos AP, Mevius D, et al. Public health risks of enterobacterial isolates producing extended-spectrum β-lactamases or AmpC β-lactamases in food and food-producing animals: an EU perspective of epidemiology, analytical methods, risk factors, and control options. Clin Infect Dis. 2013; 56: 1030–1037. doi:10.1093/cid/cis1043 PMID: 23243183

9. Smet A, Martel A, Persoons D, Dewulf J, Heyndrickx M, Cloeckaert A, et al. Comparative analysis of extended-spectrum-β-lactamase-carrying plasmids from different members of *Enterobacteriaceae* isolated from poultry, pigs and humans: evidence for a shared β-lactam resistance gene pool? J Antimicrob Chemother. 2009; 63: 1286–1288. doi:10.1093/jac/dkp101 PMID: 19297376

10. Dienikx CM, van der Goot J, Fabri T van Essen-Zandbergen A, Smith H, Mevius D. Extended-spectrum-β-lactamase-and AmpC-β-lactamase-producing *Escherichia coli* in Dutch broilers and broiler farmers. J Antimicrob Chemother. 2013; 68: 60–67. doi:10.1093/jac/dks349 PMID: 22949623
11. Kluytmans JAJW, Overdevest ITMA, Willemsen I, Kluytmans-van den Bergh MF, van der Zwaluw K, Heck M, et al. Extended-spectrum β-lactamase producing Escherichia coli from retail chicken meat and humans: comparison of strains, plasmids, resistance genes, and virulence factors. Clin Infect Dis. 2013; 56: 478–487. doi: 10.1093/cid/cis929 PMID: 23243181

12. Leverstein-van Hall MA, Dierikx CM, Cohen Stuart J, Voets GM, van den Munchhof MP, van Essen-Zandbergen A, et al. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. Clin Microbiol Infect. 2011; 17: 873–880. doi: 10.1111/j.1469-0691.2011.03497.x PMID: 21463397

13. Voets GM, Fluit AC, Scharringa J, Schapendonk C, van den Munchhof T, Leverstein-van Hall MA, et al. Improved multiplex PCR strategy for rapid assignment of multidrug resistant Salmonella enterica serovars. J Antimicrob Chemother. 2013; 68: 2429–2432. doi: 10.1093/jac/dkt402 PMID: 23875310

14. Escobar-Páramo P, Le Menac’h A, Le Gall T, Amorin C, Gouriou S, Picard B, et al. Identification of plasmids by PCR-based replicon typing. J Microbiol Methods. 2010; 83: 244–248. doi: 10.1016/j.mimet.2010.02.013 PMID: 20263884

15. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Twenty-second Informational Supplement M100–S22. CLSI, Wayne, PA, USA, 2012.

16. Caroff N, Espaze E, Bérard I, Richet H, Reynaud A. Mutations in the ampC promoter of Escherichia coli isolates resistant to oxypeptimcephalosporins without extended spectrum β-lactamase production. FEMS Microbiol Lett. 1999; 173: 459–465. PMID: 10227175

17. Dierikx C, van Essen-Zandbergen A, Veldman K, Smith H, Mevius D. Increased detection of extended spectrum β-lactamase producing Salmonella enterica and Escherichia coli isolates from poultry. Vet Microbiol. 2010; 145: 273–278. doi: 10.1016/j.vetmic.2010.03.019 PMID: 20395076

18. Hasman H, Mevius D, Veldman K, Olesen I, Aarestrup FM. β-Lactamases among extended-spectrum β-lactam (ESBL)-resistant Salmonella from poultry, poultry products and human patients in The Netherlands. J Antimicrob Chemother. 2005; 56: 115–121. PMID: 15941775

19. Steward CD, Rasheed JK, Hubert SK, Biddle JW, Raney PM, Anderson GJ, et al. Characterization of clinical isolates of Klebsiella pneumoniae from 19 laboratories using the National Committee for Clinical Laboratory Standards extended-spectrum β-lactamase detection methods. J Clin Microbiol. 2001; 39: 2864–2872. PMID: 11474005

20. Psauw A,Fluit AC,Verhoef J,Leverstein-van Hall MA. Enterobacter cloacae outbreak and emergence of quinolone resistance gene in Dutch hospital. Emerg Infect Dis. 2006; 12: 807–812. PMID: 16704842

21. Olesen I, Hasman H, Aarestrup FM. Prevalence of β-lactamases among ampicillin resistant Escherichia coli and Salmonella isolated from food animals in Denmark. Microbial Drug Res. 2004; 10: 334–340. PMID: 15650379

22. Dallenec C, Da Costa A, Decré D, Favier C, Arlet G. Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in Enterobacteriaceae. J Antimicrob Chemother. 2010; 65: 490–495. doi: 10.1093/jac/dkp498 PMID: 20071363

23. Doumith M, Day MJ, Hope R, Wain J, Woodford N. Improved multiplex PCR strategy for rapid assignment of the four major Escherichia coli phylogenetic groups. J Clin Microbiol. 2012; 50: 3108–3110. doi: 10.1128/JCM.01468-12 PMID: 22785193

24. Escobar-Páramo P, Le Menac’h A, Le Gall T, Amorin C, Gouriou S, Picard B, et al. Identification of forces shaping the commensal Escherichia coli genetic structure by comparing animal and human isolates. Environ Microbiol. 2006; 8: 1975–1984. PMID: 17014496

25. Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, et al. Sex and virulence in Escherichia coli: an evolutionary perspective. Mol Microbiol. 2006; 60: 1136–1151. PMID: 16689791

26. Hordijk J, Wagenaar JA, Kant A, van Essen-Zandbergen A, Dierikx C, Veltman K, et al. Cross-sectional study on prevalence and molecular characteristics of plasmid mediated ESBL/AmpC-producing Enterobacteriaceae from humans living in municipalities with high and low broiler density. Clin Microbiol Infect. 2013; 19: E256–E259. doi: 10.1111/j.1469-0691.2013.12150 PMID: 23397953

27. Huijbers PMC, de Kraker M, Graat EAM, van Hoek AH, van Santen MG, de Jong MC, et al. Prevalence of extended-spectrum β-lactamase-producing Enterobacteriaceae in humans living in municipalities with high and low broiler density. Clin Microbiol Infect. 2013; 19: E256–E259. doi: 10.1111/j.1469-0691.2013.12150 PMID: 23397953

28. Garcia-Fernández A, Chiaretto G, Bertini A, Villa L, Fortini D, Ricci A, et al. Multilocus sequence typing of IncF plasmids carrying extended-spectrum β-lactamases in Escherichia coli and Salmonella of human and animal origin. J Antimicrob Chemother. 2008; 61: 1229–1233. doi: 10.1093/jac/dkn131 PMID: 18367460
30. García-Fernández A, Villa L, Moodley A, Hasman H, Miragou V, Guardabassi L, et al. Multilocus sequence typing of IncN plasmids. J Antimicrob Chemother. 2011; 66: 1987–1991. doi:10.1093/jac/dkr225 PMID: 21653604

31. Reuland EA, Halaby T, Hays JP, de Jongh DM, Snetselaar HD, van Keulen M, et al. Plasmid-mediated AmpC: Prevalence in community-acquired isolates in Amsterdam, the Netherlands, and risk factors for carriage. PLoS One http://www.ncbi.nlm.nih.gov/pubmed/?term=Reuland+EA%2C+Halaby+T%2C+Hays+JP%2C+de+Jongh+DM%2C+Snetselaar+HD 2015; 10: e0113033. doi:10.1371/journal.pone.0113033 PMID: 25587716

32. Garrido A, Seral C, Gude MJ, Casado C, González-Domínguez M, Sáenz Y, et al. Characterization of plasmid-mediated β-lactamases in fecal colonizing patients in the hospital and community setting in Spain. Microb Drug Resist. 2014; 20: 301–304. doi:10.1089/mdr.2013.0109 PMID: 24328895

33. EARS-Net. Available: http://www.ecdc.europa.eu/en/activities/surveillance/EARS-Net/Pages/index.aspx. Accessed 29 January 2015.

34. Freeman JT, Rubin J, McAuliffe GN, Peirano G, Roberts SA, Drinković D, et al. Differences in risk-factor profiles between patients with ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae*: a multicentre case-case comparison study. Antimicrob Resist Infect Control. 2014; 3: 27. doi:10.1186/2047-2994-3-27 PMID: 25237477

35. Voets GM, Platteel TN, Fluit AC, Scharringa J, Schapendonk CM, Stuart JC, et al. Population distribution of β-lactamase conferring resistance to third-generation cephalosporins in human clinical Enterobacteriaceae in the Netherlands. PLoS One. 2012; 7: e52102. doi:10.1371/journal.pone.0052102 PMID: 23284986

36. Peirano G, van der Bij AK, Freeman JL, Poirel L, Nordmann P, Costello M, et al. The characteristics of *Escherichia coli* ST131 that produce extended-spectrum β-lactamases: global distribution of the H30-Rx sublineage. Antimicrob Agents Chemother. 2014; 58: 3762–3767. doi: 10.1128/AAC.02428-14 PMID: 24752265

37. Johnson JR, Delavari P, Kuskowski M, Stell AL. Phylogenetic distribution of extraintestinal virulence-associated traits in *Escherichia coli*. J Infect Dis. 2001; 183: 78–88. PMID: 1106538

38. Dierikx CM, van Duijkeren E, Schoormans AHW, van Essen-Zandbergen A, Veldman K, Kant A, et al. Occurrence and characteristics of extended-spectrum β-lactamase and AmpC-producing clinical isolates derived from companion animals and horses. J Antimicrob Chemother. 2012; 67: 1368–1374. doi: 10.1093/jac/dks049 PMID: 22382469