

## Molecular relatedness of ESBL/AmpC-producing *Escherichia coli* from humans, animals, food and the environment: a pooled analysis

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**Background:** In recent years, ESBL/AmpC-producing *Escherichia coli* (ESBL/AmpC-EC) have been isolated with increasing frequency from animals, food, environmental sources and humans. With incomplete and scattered evidence, the contribution to the human carriage burden from these reservoirs remains unclear.

**Objectives:** To quantify molecular similarities between different reservoirs as a first step towards risk attribution.

**Methods:** Pooled data on ESBL/AmpC-EC isolates were recovered from 35 studies in the Netherlands comprising >27 000 samples, mostly obtained between 2005 and 2015. Frequency distributions of ESBL/AmpC genes from 5808 isolates and replicons of ESBL/AmpC-carrying plasmids from 812 isolates were compared across 22 reservoirs through proportional similarity indices (PSIs) and principal component analyses (PCAs).

**Results:** Predominant ESBL/AmpC genes were identified in each reservoir. PCAs and PSIs revealed close human-animal ESBL/AmpC gene similarity between human farming communities and their animals (broilers and pigs) (PSIs from 0.8 to 0.9). Isolates from people in the general population had higher similarities to those from human clinical settings, surface and sewage water and wild birds (0.7–0.8), while similarities to livestock or food reservoirs were lower (0.3–0.6). Based on rarefaction curves, people in the general population had more diversity in ESBL/AmpC genes and plasmid replicon types than those in other reservoirs.

**Conclusions:** Our 'One Health' approach provides an integrated evaluation of the molecular relatedness of ESBL/AmpC-EC from numerous sources. The analysis showed distinguishable ESBL/AmpC-EC transmission cycles in different hosts and failed to demonstrate a close epidemiological linkage of ESBL/AmpC genes and plasmid replicon types between livestock farms and people in the general population.

### Introduction

Since 2000, the incidence of ESBL-producing Enterobacteriaceae infections increased globally both in hospitals and in the community.<sup>1–3</sup> In parallel, ESBL/AmpC-producing *Escherichia coli* (ESBL/

AmpC-EC) have increasingly been reported in livestock, in the food chain and in companion animals.<sup>4–9</sup> The public health burden of these animal reservoirs as sources of human infections remains an issue of controversy.<sup>10–17</sup>

Resistance transmission pathways for ESBL/AmpC-EC are complex. Bacterial populations can acquire resistance genes horizontally through mobile genetic elements in a wide variety of intestinal or extra-intestinal environments.<sup>13</sup> There are multiple direct and indirect transmission routes from animal or environmental sources to humans and vice versa.<sup>13</sup> Parallel occurrence of ESBL gene subtypes, plasmids and/or bacterial clones in livestock and humans has been documented and is considered evidence of poultry being an important source of animal-associated ESBL/AmpC-EC infections in humans.<sup>11,13,18</sup> A study based on WGS showed direct transmission of ESBL-producing *E. coli* between pigs and pig farmers, whereas transmission to humans from poultry through the food chain was not considered to be likely.<sup>19</sup> This study also demonstrated widespread prevalence of specific plasmids, which is compatible with a scenario of plasmid-mediated transmission between different reservoirs.<sup>19</sup>

Despite the availability of numerous studies on molecular characteristics, the epidemiology of ESBL/AmpC-EC is not fully understood.<sup>20–31</sup> *E. coli* ESBL types, phylogroups and antimicrobial susceptibilities from a meta-collection of ESBL/AmpC-EC isolates ( $n = 1329$ ) across humans, livestock and companion animal populations have only been compared in a single study.<sup>18</sup>

The purpose of the present study was to investigate molecular relatedness in a pooled analysis of a substantially larger collection of ESBL/AmpC-EC isolates from diverse human and animal populations, food and environmental reservoirs in the Netherlands. The isolate collection was analysed by dimension reduction techniques to quantify the abundance, diversity and similarity of genes, plasmid replicons and strains between the different reservoirs as a first step towards an analysis of attribution.

## Methods

### Selection of studies

Peer-reviewed publications containing collections of ESBL/AmpC-EC in the Netherlands were systematically reviewed using PubMed (Figure S1, available as [Supplementary data](#) at JAC Online). The search strategy consisted of the combination of terms 'ESBL', 'extended-spectrum' or 'β-lactamase', with either terms related to the bacterial species of interest (i.e. '*Escherichia coli*' or '*E. coli*') or terms referring to the country where isolates were collected ('the Netherlands' or 'Dutch'). The last search was done in February 2016. Studies were only included when they contained isolates with at least ESBL/AmpC gene typing information for all gene groups found; this was the primary outcome. Exclusion criteria were applied to studies containing only isolates collected prior to 2000, reports of outbreak investigations, studies that focused on the molecular characterization of only specific plasmids and/or ESBL/AmpC genes, abstracts from scientific conferences and studies written in languages other than English or Dutch. Other reasons for exclusion were the lack of gene typing data for any of the gene groups CTX-M, TEM or SHV and the impossibility of data extraction at the isolate level when reporting of specific genes was aggregated (Figure S1). From the 25 publications selected after full text screening, partners within the ESBLAT (ESBL Attribution) research consortium (<http://www.1health4food.nl/nl/show/ESBL-attributie-3.htm>) provided detailed data from laboratory records ( $n = 20$  studies).<sup>7,8,11,12,21–26,28,29,31–38</sup> Data were exclusively extracted from the publications for five studies.<sup>20,27,30,39,40</sup> Additionally, ESBLAT partners provided finalized data from epidemiological studies within the consortium ( $n = 9$  studies, from which 3 publications became available after the systematic search)<sup>41–43</sup> and from the isolates collected as part of the Dutch Antimicrobial National Surveillance System (MARAN) for years

2012 to 2016 (Figure S1).<sup>44</sup> A total of 35 studies were included for the pooled analysis (Table S1 and Figure S1).

### Data extraction and synthesis

From each of the selected studies the number of ESBL genes from the CTX-M, TEM or SHV families associated with phenotype 2be (ESBL-producing),<sup>45</sup> and the number of AmpC plasmid-mediated genes from the CMY, ACC, ACT, DHA and MIR families were extracted. Additionally, where available, the number of replicon types of plasmids harbouring ESBL/AmpCs identified by PCR-based replicon typing (PBRT) and/or number of *E. coli* genotypes identified by MLST were extracted (Table S1 and Figure S2). Multi-F replicons were grouped into a single IncF category. Information on the source of origin of the isolates was used to aggregate the empirical frequency distributions (in genes, replicons and strain types) by reservoirs per study and across studies in the total pool of isolates (Table S1 and Figure S2). Isolates from human clinical settings (hospitals, general practitioners and long-term care facilities) were grouped by type of sample (faecal, urine, blood samples and an aggregation of samples from respiratory infections, wounds and other origins); one reservoir represented people in the general population with isolates originating from people in the open community; two reservoirs represented the human farming communities who were in contact with pigs and broilers; animal species constituted independent reservoirs: dogs kept as pets, wild birds and production animals (veal calves, dairy cattle, pigs, broilers and laying hens); meat samples were also classified as independent reservoirs depending on the origin of samples as chicken meat at retail and at the slaughterhouse, turkey meat and beef at retail and veal meat at the slaughterhouse; finally, three environmental reservoirs represented wastewater and surface water for recreational and non-recreational sites (Table S1 and Figure S2). Other possible independent reservoirs reported in the selected studies were excluded to avoid large sampling uncertainties when the final aggregation yielded <25 isolates (e.g. clinical samples from cats and horses, environmental samples from laying hens and meat retail samples other than chicken, beef or turkey).

### Pooled analyses

Visual comparisons of relative frequencies for genes, plasmids and *E. coli* sequence types between the different reservoirs were obtained by bar charts using Sigma Plot (version 13, Systat Software Inc, San José, CA, USA). These relative frequencies formed the basis of the data analysed in the subsequent stages. The relative frequencies were defined based on the total number of ESBL/AmpC genes and/or plasmid replicons instead of the total number of isolates.

Firstly, a pairwise quantification of the associations between the frequency distributions of genes and plasmids in the reservoirs was performed. The proportional similarity index (PSI) was used, which is defined as  $PSI = 1 - 0.5 \sum_k |p_k - q_k|$ , where  $p_k$  is the relative frequency of the gene or plasmid replicon subtype harbouring an ESBL/AmpC gene  $k$  in the first reservoir and  $q_k$  is the relative frequency of subtype  $k$  in the second reservoir.<sup>46</sup> CIs for PSI were calculated using 5000 bootstrap samples.

Secondly, principal component analyses (PCAs) were performed for the pooled collection of genes, for the plasmid replicons and for the combination of replicons and genes.<sup>47</sup> The dimension of the proportion profiles was reduced to the two main components in which each gene and replicon had a different load according to their contribution for differentiation of reservoirs. ESBL/AmpC gene and replicon types with relative frequencies below 0.05 in all reservoirs were excluded to avoid rare occurrences related to the number of samples. Unlike in most PCAs, relative frequencies were not scaled in relation to each other to keep the effect of relatively large variances of particular genes and/or plasmids between reservoirs. Bootstrap samples (5000 times) of the relative frequencies per reservoir were generated to account for sample uncertainty.<sup>48</sup> Biplots of the space represented by the two principal components were constructed and preferences for the

location of genes, replicons or their combination among the bootstrapped samples of the different reservoirs' isolates were displayed. Finally, a rarefaction analysis was performed to evaluate the diversity of genes and plasmid replicons in the pooled collection of isolates per reservoir.<sup>49</sup> All analyses were done in R (version 3.0.2; R Foundation for Statistical Computing, Vienna, Austria).

## Results

### Proximity of reservoirs according to ESBL/AmpC genes and plasmid replicon frequencies

A total of 5808 ESBL/AmpC-EC isolates originating from >27 000 samples collected in 35 studies across 22 reservoirs were compiled (Table S1 and Figure S2). The pooled analyses were done in the total collection of isolates, in a subset of 863 isolates containing information on ESBL/AmpC-gene-carrying plasmid replicons and in a subset of 666 isolates with information on *E. coli* strain types (Table S1 and Figure S2). The prevalence of *E. coli* samples producing ESBL/AmpC across reservoirs was calculated or extracted from the selected publications and varied from low (<5% for human clinical blood samples, people in the general population, pig farmers and retail beef) to high (>60% for wastewater samples, broilers and retail chicken meat) (Table S1 and Figure S3).

The analysis of the ESBL/AmpC-EC isolate collection provided a summary of the most common gene and plasmid replicon distributions in the different reservoirs (Figures 1 and 2). Genes *bla*<sub>CTX-M-1</sub>, *bla*<sub>CTX-M-14</sub>, *bla*<sub>CTX-M-15</sub>, *bla*<sub>SHV-12</sub> and *bla*<sub>TEM-52</sub> dominated the profile distributions of most reservoirs, except for broilers, human communities living and/or working on broiler farms, chicken meat at slaughterhouses and laying hens. In broilers, people working and/or living on broiler farms, chicken meat and laying hens, *bla*<sub>CMY-2</sub> was frequently observed, while *bla*<sub>CTX-M-15</sub> and *bla*<sub>CTX-M-14</sub> were very rare or absent (Figure 1a). From the subset of 863 *E. coli* isolates with replicon typing information on ESBL/AmpC-harboured plasmids, 812 formed collections of sufficient size to be analysed multivariately (Figure 2). The most common plasmid replicons were IncI1 and IncF, followed by smaller proportions of IncK, IncN, IncB/O and IncX1 (Figure 2a). Remarkably, IncF was absent in the broiler farming community and very low in chicken meat and broilers. Combined with the ESBL/AmpC gene data above, this again hints at a separate reservoir. The relative frequencies of ESBL/AmpC genes in this subset of isolates did not fundamentally differ from those in the complete pool (Figure S4).

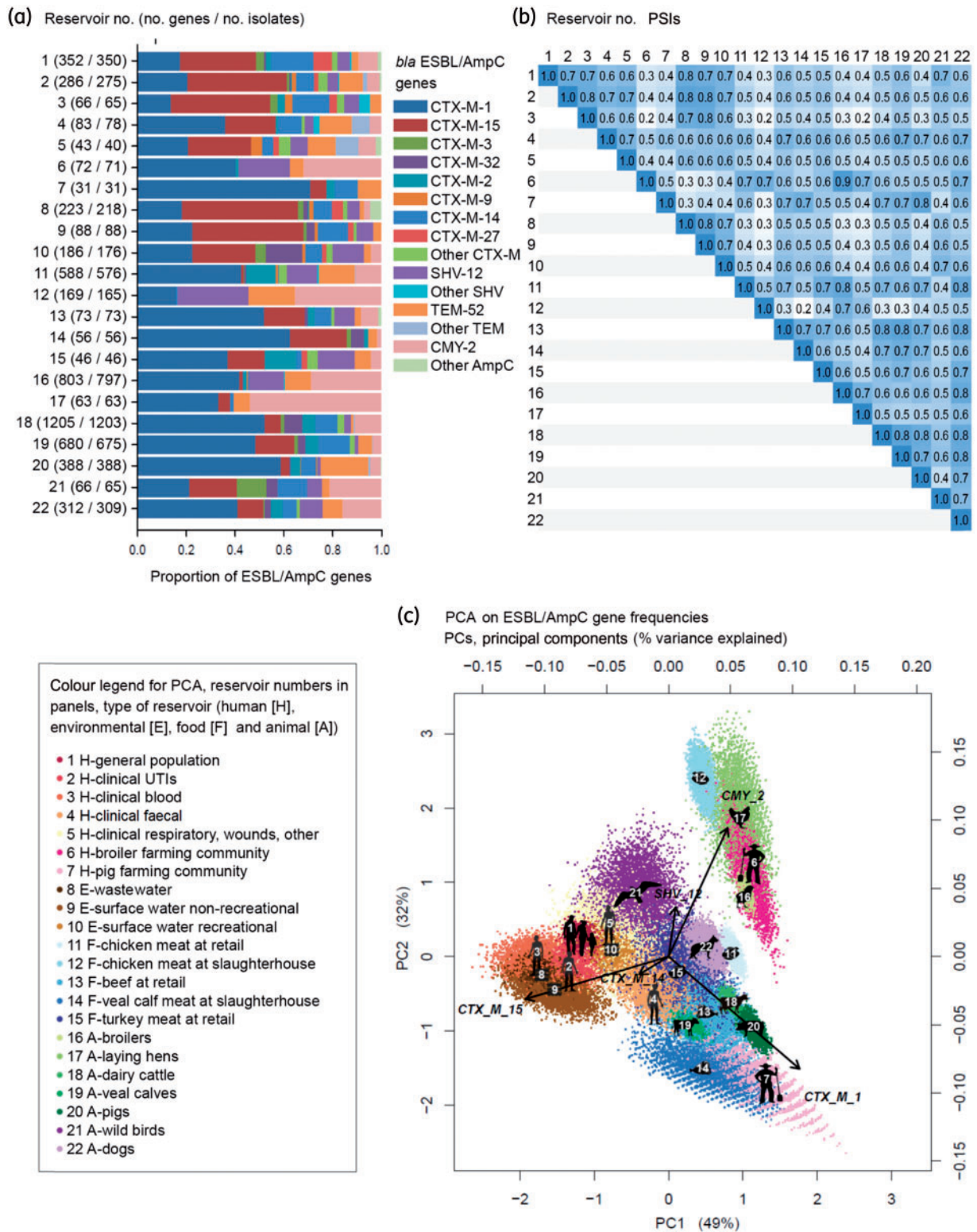
PSIs (Figure 1b) showed that most livestock or food-associated reservoirs did not have a high level of similarity with the ESBL/AmpC gene distributions from humans (average PSI <0.5), with the exception of livestock farmers, in whom the gene distributions were very similar to those of their animals (PSI of 0.8 for pig farming and 0.9 for broiler farming). The general and clinical human populations had relatively similar ESBL/AmpC gene profiles to water samples (PSI 0.6–0.8), wild birds (mainly ducks and waders), dogs, veal calves, pigs and beef (PSI 0.5–0.7). The least similar gene distributions to the human general and clinical populations were those of farming communities, chicken meat at slaughterhouses, broilers and laying hens (average PSI 0.3–0.4). The PSIs for plasmid replicon profiles in the subset of 812 isolates (Figure 2b) were very close to those determined for the ESBL/AmpC genes. CIs for the PSIs are provided in Tables S2 and S3 and Figure S3.

PCAs on the ESBL/AmpC gene and replicon distributions visualized the genetic relatedness of ESBL/AmpC-EC across all reservoirs (Figure 1c and Figure 2c and d). For ESBL/AmpC genes (Figure 1c), the first principal component (explained variance 49%) was determined by the distinct abundance of *bla*<sub>CTX-M-15</sub> (in human clinical populations, people in the general population and water sources), versus a higher occurrence of *bla*<sub>CTX-M-1</sub> and/or *bla*<sub>CMY-2</sub> (in meat samples, animals and people in farming communities). The second component (explained variance 32%) separated these latter reservoirs according to the relative abundance of *bla*<sub>CTX-M-1</sub>, *bla*<sub>CMY-2</sub> and *bla*<sub>SHV-12</sub>. In dairy cattle, veal calves, pigs, the pig farming community, beef at retail and veal calf meat at the slaughterhouse, *bla*<sub>CTX-M-1</sub> dominated the distributions; *bla*<sub>CMY-2</sub> and/or *bla*<sub>SHV-12</sub> were more frequently present in laying hens and chicken meat at the slaughterhouse. Broilers and the broiler farming community had a more intermediate position on the vertical axis due to their abundance of *bla*<sub>CMY-2</sub>, *bla*<sub>CTX-M-1</sub> and *bla*<sub>SHV-12</sub>, and absence of *bla*<sub>CTX-M-14</sub> and *bla*<sub>CTX-M-15</sub>. Genes from wild birds clustered relatively close to the human general and clinical populations. Genes from dogs, turkey meat and human clinical faecal isolates clustered close to the centre of the two component loadings.

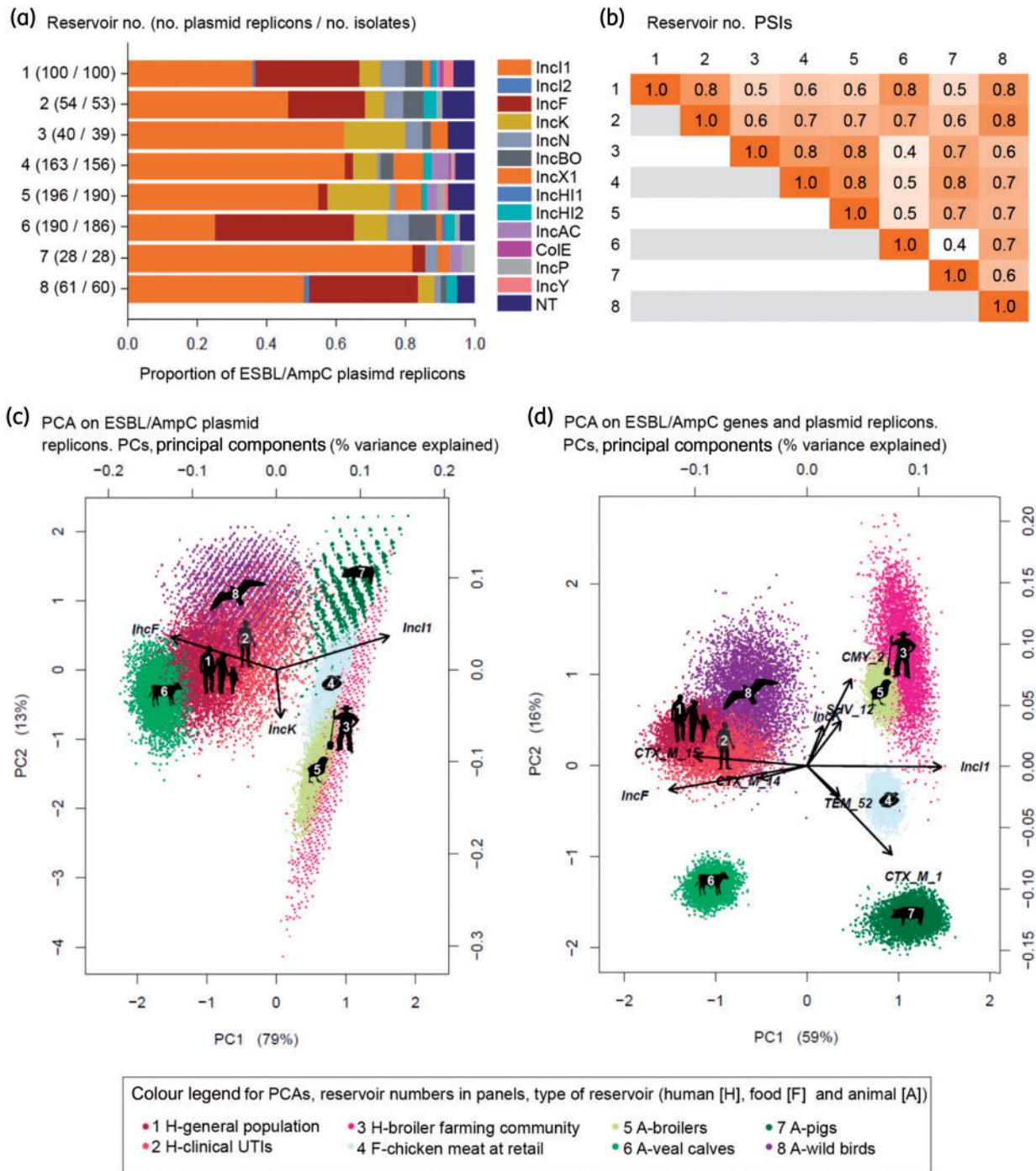
In the subset of eight reservoirs with sufficient replicon data ( $n = 812$  isolates) the PCA showed proximities between reservoirs comparable with those seen for gene profiles (Figure 2c). The first component explained most variance (79%) and separated reservoirs with high abundance of IncF replicons (e.g. veal calves) from those with higher abundance of IncI1 (e.g. pigs). The second component (explained variance 13%) defined reservoirs with a distinct presence of IncK plasmids. A PCA including genes from the complete pool of isolates ( $n = 5808$ ), together with the replicons from the subset of 812 isolates (Figure 2d), yielded comparable proximities, despite an increased variability within individual reservoirs which resulted in lower explained variance (69% for the two components). As a sensitivity analysis, another gene–replicon combined PCA was produced with gene frequencies only from isolates containing replicon typing. Again, a very similar pattern was shown (Figure S5).

### Rarefaction analysis of ESBL/AmpC genes and replicon types

Rarefaction analysis per reservoir showed that the number of isolates in the analysis was enough to yield correct estimates for the diversity (i.e. most rarefaction curves approached a plateau for collected sample sizes).<sup>50</sup> Rarefaction curves showed that sampling 40–80 isolates was sufficient to capture most of the molecular diversity (in genes and associated replicons) across reservoirs. The people in the general population, waste/surface water, dairy cattle, dogs, veal calves and human clinical urinary tract infection (UTI) isolates had the most diverse ESBL/AmpC gene pool (~8–9 different genes) (Figure 3a). The least diverse gene pool was found in chicken meat at the slaughterhouse, broilers, laying hens and the farming community in direct contact with pigs and poultry (~4 different genes). The diversity in plasmid replicon types associated with these genes was highest in people in the general population and in chicken meat at retail (~6 different types) (Figure 3b).



**Figure 1.** Pooled analysis of the ESBL/AmpC genes from 5808 *E. coli* isolates in human, animal, food and environmental reservoirs in the Netherlands. (a) Proportion of gene types over total number of genes collated per reservoir. (b) Pairwise PSI for gene types between reservoirs. Cells are shaded gradually according to PSI values [from 0 (no similarity in gene distribution) to 1 (identical distribution)]. (c) PCA on the bootstrapped samples of ESBL/AmpC gene frequencies per reservoir. Only the most discriminatory genes are plotted. Higher dispersion of point clouds indicates less confidence in the clustering and vice versa.

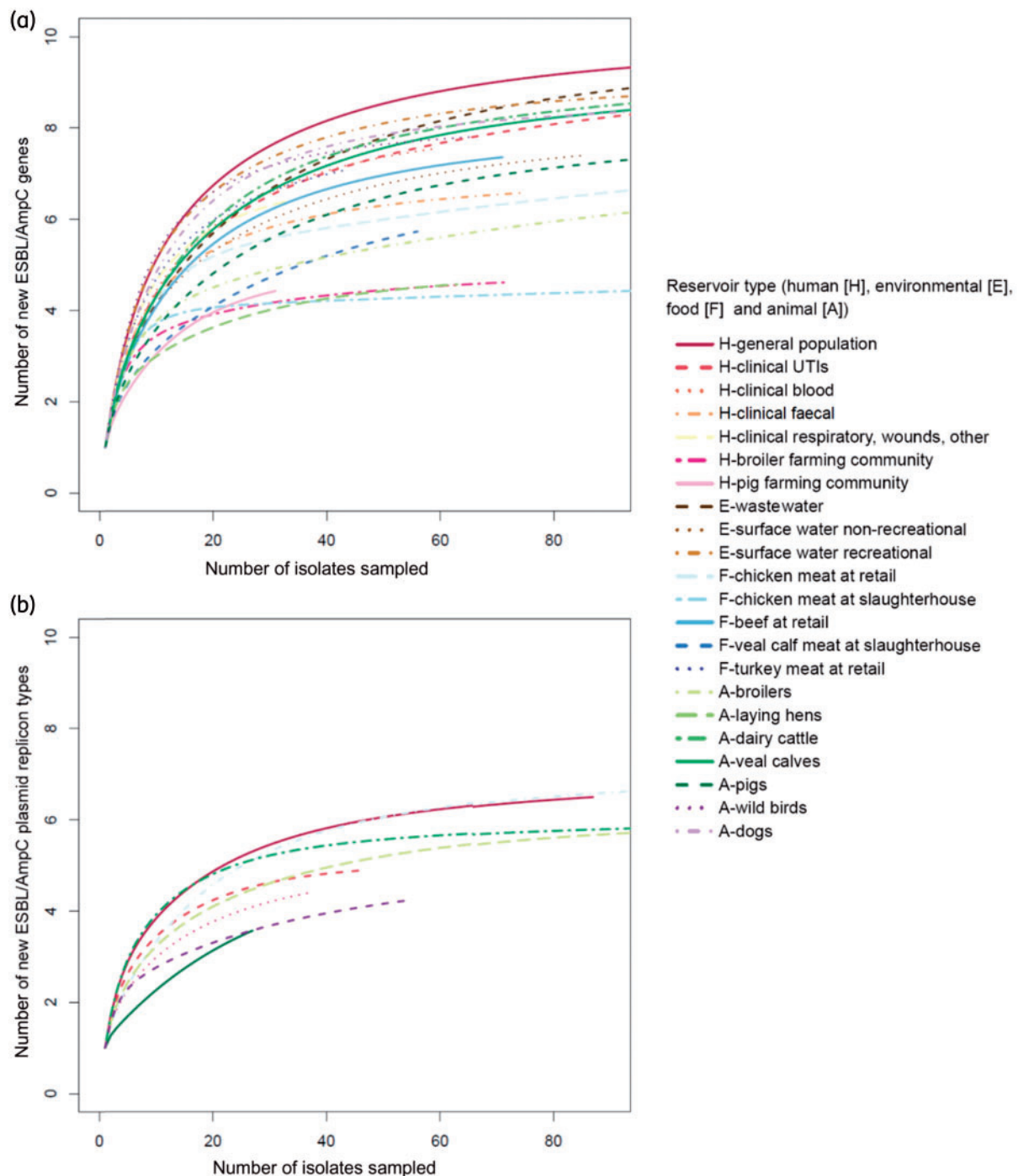


**Figure 2.** Pooled analysis of the plasmid replicons associated with production of ESBL/AmpC in 812 *E. coli* isolates from human, animal, food and environmental reservoirs in the Netherlands. (a) Proportion of replicon types over total number of replicons collated per reservoir. (b) Pairwise PSI for replicon types between reservoirs. Cells are shaded gradually according to PSI values [from 0 (no similarity in gene distribution) to 1 (identical distribution)]. (c) PCA on the bootstrapped samples of replicon frequencies per reservoir. Only the most discriminatory replicons are plotted. Higher dispersion of point clouds indicates less confidence in the clustering and vice versa. (d) PCA on the bootstrapped samples of replicon frequencies and gene frequencies (from the complete isolate pooled collection,  $n = 5808$ ) per reservoir. Only the most discriminatory replicons and genes are plotted. Higher dispersion of point clouds indicates less confidence in the clustering and vice versa.

**Sensitivity analyses**

The number of isolates and information available per reservoir varied over the years (Figure S6). As a result, potential shifts in gene

and replicon frequency distributions over time may have been overlooked. Most isolates (97.8%) were collected between 2008 and 2015 (Figure S6), long after major changes were recorded in



**Figure 3.** Rarefaction analysis for ESBL/AmpC genes from the complete pooled collection of 5808 *E. coli* isolates (a) and for replicon types in the subset of 812 isolates with replicon typing information (b).

the epidemiology of ESBL/AmpCs—namely, the emergence of the CTX-M group of enzymes around the year 2000,<sup>3</sup> and the emergence of AmpC plasmid-mediated  $\beta$ -lactamases in the poultry chain around the year 2006.<sup>32,51</sup> In a sensitivity analysis, gene and plasmid replicon distributions per time period remained essentially unaltered across reservoirs (Table S4 and Table S5).

### MLST types

A relatively low proportion of *E. coli* isolates in the collection were genotyped (MLST) (Table S1 and Figure S2). A detailed description of MLST types found in this subset is provided (Figure S7), but multivariate analysis was not appropriate given the low number of isolates and reservoirs to compare. ST131 was most commonly

found in human UTIs (41%) followed by faecal isolates from the general population (16%), the broiler farming community (10%), broilers (6%) and dogs (5%); ST10 was present in all reservoirs (<10%) except for dogs. Other common MLST types were ST117 (17% in the broiler farming community), ST57 (38% in veal calves) and ST617 (38% in non-recreational surface water).

## Discussion

The analysis of a large collection of isolates from different reservoirs showed that ESBL-AmpC gene distributions from all human, animal, meat and environmental reservoirs have a certain level of similarity (i.e. most ESBL/AmpC gene subtypes are found in each individual reservoir). However, an important observation is that most livestock or food-associated reservoirs did not show a high level of similarity in their gene profiles compared with humans from the general and clinical populations. This suggests that livestock reservoirs including poultry and poultry meat are not major contributors to ESBL/AmpC occurrence in humans, as has been suggested earlier by several research groups.<sup>6,16,52</sup> Analogous findings are available for *Salmonella* Typhimurium DT104, demonstrating that this bacterium and its resistance genes are largely maintained within animal and human populations separately with limited transmission (although for *Salmonella*, vertical clonal dissemination dominates the transmission profile).<sup>53</sup> Moreover, a quantitative microbial risk assessment of human exposure to ESBL/AmpC-EC through consumption of contaminated meat is indicative of low exposure through this route.<sup>54</sup> Surprisingly, despite the high prevalence of ESBL/AmpC-EC in poultry, consumption of poultry meat contributed less than 20% to the total exposure through meat consumption. In contrast, owing to the consumption of raw meat, beef products contributed more than 75% to the total exposure through meat.<sup>54</sup> It was remarkable that, in our study, the gene distribution in chicken meat at retail was distant from the reservoirs of broilers and chicken meat at the slaughterhouse. The reason behind this inconsistency might arise from meat from different countries where different ESBL types might be predominant (data on imports were not available)<sup>44</sup> or, although never investigated, cross-contamination of meat by humans might be happening during processing or at retail.

A high similarity was found in ESBL/AmpC gene distributions from humans in the general and clinical populations, which can be explained by the frequent contact between individuals from these niches. People in the general population showed the highest ESBL/AmpC gene and replicon diversities, indicating that this population is a receiving reservoir with numerous sources. We could not investigate the specific causes behind this high diversity in our study, but transmission routes in the community are complex, involving patients recently discharged from hospitals or non-domestic sources such as international travellers that might be contributing to a more diverse pool of genes.<sup>55</sup> A high similarity in gene distributions was found in isolates from farmers and their animals, which is the result of intensive and direct contact as has previously been shown in studies in the pig and poultry sectors.<sup>19,21,28,37</sup> Diversity in ESBL/AmpC genes among people in farming communities was lower (comparable to their animals) than in people from the general and clinical populations. This gives additional support to the hypothesis that direct contact is the most important route in the exchange of ESBL genes between reservoirs

in farming communities. Additionally, living in close proximity to livestock production farms without occupational exposure to animals has not been associated with an increased ESBL/AmpC-EC carriage risk.<sup>42,56</sup> A major research question that could be answered by longitudinal studies is whether this ESBL/AmpC-EC carriage in farmers is transient or a more permanent colonization.

High molecular similarities between human and environmental reservoirs suggest a contribution of human wastewater to ESBL/AmpC-EC presence in surface water.<sup>57</sup> The exposure of humans to contaminated surface water, for instance resulting from recreational swimming, requires further quantitative evaluation. The ESBL/AmpC genes in wild birds clustered close to humans. These animals may have acquired their ESBL/AmpC-EC through contaminated surface water. Such birds may act as vectors or even reservoirs for local dissemination.<sup>58-60</sup> These observations are illustrative of the spread of ESBL/AmpC-EC to the environment and wildlife beyond the human and domestic animal populations exposed to antimicrobials and show that more studies directed at human exposure to ESBL/AmpC-EC in the environment are needed.<sup>61</sup>

Limitations of the current study material should be taken into consideration when results are interpreted. Details about the structure of some populations, especially those related to acquisition of non-domestic bacteria or genes (e.g. through importation of foods or international travel), were not available. All studies were scrutinized to determine whether they would contribute to a representative pool of ESBL/AmpC genes or associated plasmid replicons for each reservoir; however, selected studies were heterogeneous in terms of temporality, design and microbiological methods. In order to gain statistical power with more observations, isolates from longitudinal studies (e.g. dogs, pigs, veal calves and dairy cows) were pooled in the collection independently of the sampling time they were obtained. This may have led to a somewhat reduced variance because observations were not fully independent.<sup>22,36</sup> Some of the studies (deliberately) did not include identification of plasmid-mediated AmpC genes in the reservoirs, but it is anticipated that AmpC occurrence in these reservoirs is relatively low.<sup>12</sup> Unfortunately, replicon and MLST data were limited and specific gene-replicon or gene-replicon-MLST combinations could not be evaluated at the isolate level because of the nature of the collected data and the heterogeneity in methods used across studies. An important issue in ESBL/AmpC-EC epidemiology is that representation of some reservoirs in the scientific literature might be biased, for instance because human clinical samples are routinely analysed for ESBL/AmpC-EC, but not published in the peer-reviewed literature. It is also possible that some reservoirs have received disproportional attention compared with others. The latter is for instance the case for broilers, chicken meat or dairy cattle. This requires more unbiased large sampling efforts for studies that focus on attribution of different sources of ESBL/AmpC-EC. These aspects should be considered in future microbial risk assessments.

These findings demonstrate the complex dynamics involved in the transmission of ESBL/AmpC-EC and show distinguishable epidemiological cycles between certain reservoirs, such as livestock (and meat) and humans in general and clinical populations. Information used in this study was mostly based on traditional molecular typing and WGS may provide a more refined picture in terms of relatedness between the bacterial host, all plasmids present and ESBL/AmpC genes. Likewise, although molecular

relatedness has been established for some reservoirs, observational epidemiological or mathematical modelling studies are still required to understand the exact mode of transmission or its relative contribution in the case of multiple modes of transmission. To explore this multi-directionality, a combination of strong epidemiological designs, involving longitudinal studies with repeated sampling on the same individual or animal, and high-resolution molecular techniques, are required. The fact that the ESBL/AmpC epidemiology is driven by interactions between a variety of genes, plasmids and strains is a further complication that needs to be considered more explicitly in future studies.

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## Transparency declarations

None to declare.

## Supplementary data

Figures S1 to S7 and Tables S1 to S5 are available as [Supplementary data](#) at JAC Online.

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