Optimizing triage and treatment strategies in urinary tract infection

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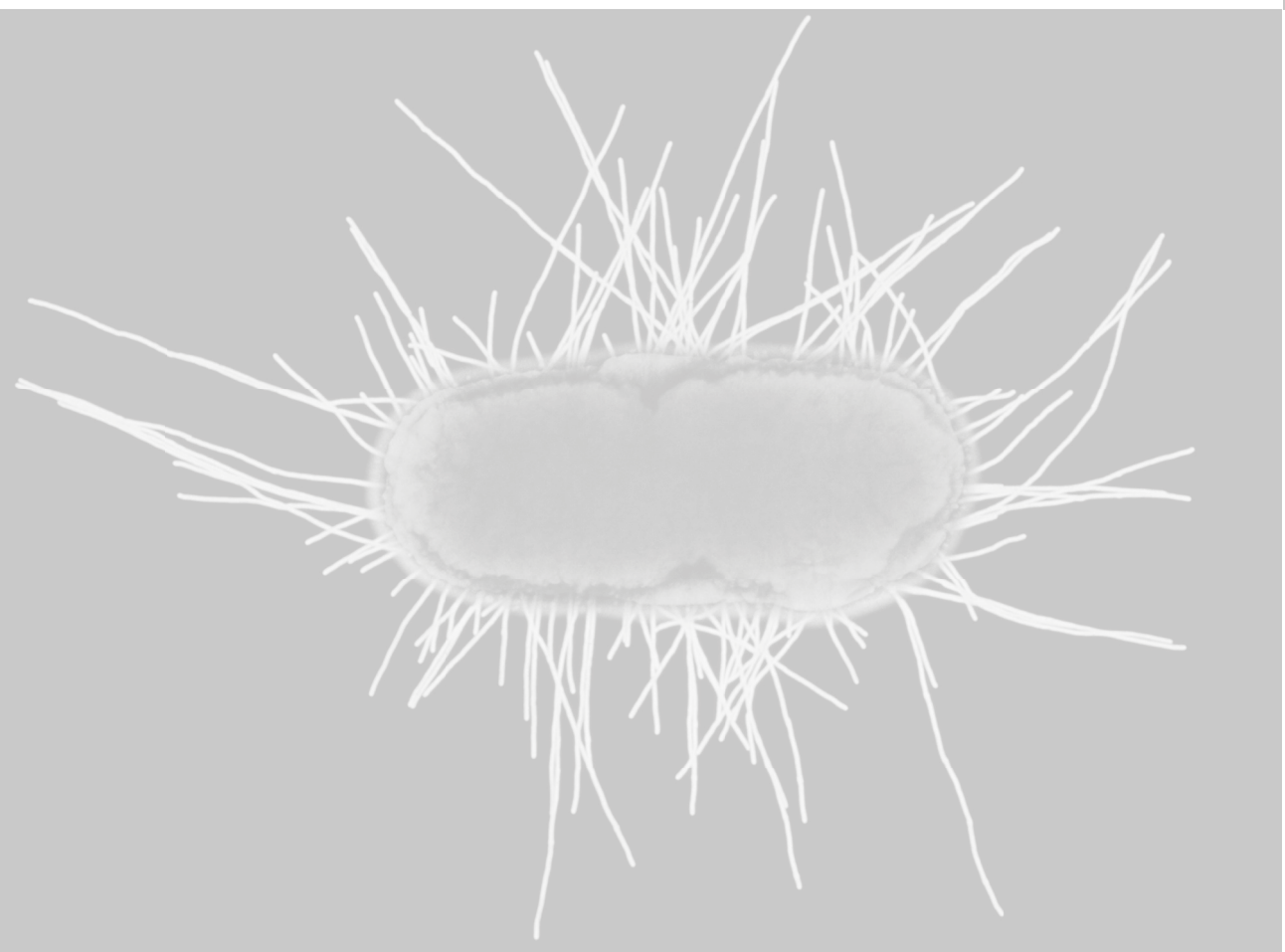

CHAPTER 8

Comparative virulotyping of extended-spectrum cephalosporin-resistant *E. coli* isolated from broilers, humans on broiler farms and in the general population and UTI patients

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ABSTRACT

During the last decade extended-spectrum cephalosporin (ESC)-resistant *Escherichia coli* from food producing animals, especially from broilers, have become a major public health concern because of the potential transmission of these resistant bacteria or their plasmid-encoded resistance genes to humans.

The objective of this study was to compare ESC-resistant *E. coli* isolates from broilers (n = 149), humans in contact with these broilers (n = 44), humans in the general population (n = 63), and patients with a urinary tract infection (UTI) (n = 10) with respect to virulence determinants, phylogenetic groups and extended-spectrum b-lactamase (ESBL)/plasmidic-AmpC (pAmpC) genes. The most prevalent ESBL/pAmpC genes among isolates from broilers and individuals on broiler farms were $bla_{\text{CTX-M-1}}$, $bla_{\text{CMY-2}}$ and $bla_{\text{SHV-12}}$. In isolates from humans in the general population $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ were found most frequently, whereas in UTI isolates blaCTX-M-15 predominated. The marker for enteroaggregative *E. coli*, *aggR*, was only identified in a broiler and human isolates from the general population. The extraintestinal virulence genes *afa* and *hlyD* were exclusively present in human isolates in the general population and UTI isolates. Multivariate analysis, based on ESBL/pAmpC resistance genes, virulence profiles and phylogenetic groups, revealed that most UTI isolates formed a clearly distinct group. Isolates from broilers and humans associated with broiler farms clustered together. In contrast, isolates from the general population showed some overlap with the former two groups but primarily formed a separate group. These results indicate than transmission occurs between broilers and humans on broiler farms, but also indicate that the role of broilers as a source of foodborne transmission of ESC-resistant *E. coli* to the general population and subsequently causative agents of human urinary tract infections is likely relatively small.

INTRODUCTION

Escherichia coli is generally considered a beneficial commensal of the gastrointestinal tract of humans and animals. Certain *E. coli* strains, however, can cause community-acquired infections, including urinary tract infections (UTIs).[1]

Acute, uncomplicated UTI is one of the most common bacterial infections seen in general practice with an incidence of 70 per 1000 women each year in the Netherlands [1]. Between 70–95% of UTIs are caused by uropathogenic *E. coli* (UPEC) [2]. UPEC belong to the broader group of extraintestinal pathogenic *E. coli* (ExPEC), which possess specific virulence traits allowing them to colonize environments other than the gastrointestinal tract, such as the urogenital tract.[3] Some ExPEC isolates from humans have similar virulence factors as *E. coli* isolated from food-producing animals and therefore it has been postulated that a proportion of human UTI is caused by ExPEC strains originating from these animals. Recent studies have suggested that animals and food may be a source of antimicrobial-resistant extraintestinal pathogenic *E. coli* isolates.[4-7]

The prevalence of extended-spectrum b-lactamase (ESBL)- and pAmpC b-lactamase-producing *E. coli* among Dutch broilers is high and broilers may therefore form a reservoir from where spread of these resistant bacteria or ESBL/pAmpC encoding resistance genes to humans may occur.[8] [9, 10] Transmission of extended-spectrum cephalosporin (ESC)-resistant *E. coli* from broilers to humans through the food chain has been proposed [11] [12, 13]. It has been demonstrated that close contact between humans and broilers on broiler farms increased the risk of carrying ESC-resistant *E. coli*.[9]

Transmission of ESC-resistant *E. coli* or their resistance genes between animals and humans may form a direct public health threat when dealing with pathogenic types. The aim of this study was to compare ESBL/pAmpC resistance genes, virulence determinants and phylogenetic groups from ESC- resistant E. coli isolates from broilers, humans working or living on broiler farms, humans in the general Dutch population and patients with urinary tract infection (UTI) in order to investigate potential public health implications.

2. MATERIAL AND METHODS

2.1. Isolate origin

The ESC-resistant broiler isolates (n = 149) originated from two studies on conventional (n = 98) and organic (n = 51) broiler farms in The Netherlands.[9, 10] Broiler isolates were included when ESC-resistant *E. coli* from individuals living and/or working on these farms were also available. Multiple broiler isolates from one farm were selected based on as many different resistance genotype and phylogenetic group as possible with a maximum of three isolates with an identical ESBL/pAmpC gene and phylogenetic group per farm. ESC-resistant isolates from individuals living and/or working on these farms (n = 44) originating from conventional (n = 35) or organic (n = 9) broiler farms were also included.[9, 10] In addition, ESC-resistant isolates from individuals in the general

population living in areas with either a high and low broiler density (n = 63) were investigated.[14] Isolates from febrile UTIs (n = 10) were obtained from two hospitals in the Netherlands.

2.2. Molecular characterization

The ESBL/pAmpC genes and phylogenetic groups of all isolates from broilers and humans, except for those from febrile UTIs, had been determined in previous studies.[9, 10, 14] The phylogenetic groups of the UTI isolates were investigated according to Doumith et al. and they were subgrouped as described by Escobar-Páramo et al.[28, 15] The ESBL or pAmpC encoding genes of the UTI isolates were characterized as described by van Hoek et al. (2015).[14] Multi locus sequence typing (MLST) had been conducted for 130 isolates, while the ESC-resistance plasmids had been identified for 115.[9, 10, 14] MLST of the UTI isolates was performed according to Wirth et al.[16]

Analysis of the virulence factors was performed by PCR targeting the aggregative virulence regulator (*aggR*) of enteroaggregative *E. coli* (EAEC) as described by Boisen et al.[17] In addition, several markers were selected to identify ExPEC; i.e. afimbrial adhesion (*afa*), type 1 fimbriae (*fimH*), F1C fimbriae (*focG*), cytolytic protein toxin (*hlyD*), increased serum survival (*iss*), iron acquisition system (*iutA*), group 2 polysaccharide capsule (*kpsMII*), P fimbriae (*papA*) and S fimbriae (*sfaS*). The PCR primers and protocols applied to identify these ExPEC genes were described by Franz et al.,[18] with the exception of *fimH* and *iss*. The *fimH* PCR originated from Dias et al.,[19] while the following two primers were used to amplify a nearly complete *iss* gene; iss_39F: 50-cgctctggcaatgcttattac-30 and iss_285R: 50-ttccagcggagtataaatgcc-30. Both primers were also used to sequence this virulence determinant by BaseClear B.V. (Leiden, the Netherlands). The obtained alleles were typed according to Johnson et al.[4]

2.3. Statistical analysis

Differences in frequencies of ESC-genotypes, phylogenetic groups and virulence markers among groups was evaluated using chi-squared tests (x2) on contingency tables with a significance level of P = 0.05. Univariate analysis of variance was performed for inference on differences in average numbers of virulence markers between ESBL-genotypes and phylogenetic groups. Analyses were performed in IBM SPSS Statistics version 19. Principal component analysis and construction of a scatterplot was calculated from the ESBL-genotypes, the presence/absence of the different virulence genes, and the origin of the isolate in SAS studio 3.4.

Table 1 ESC genotypes of all isolates studied.

Resistance genotype	Broiler isolates (n = 149 (%))[c]	Human isolates, broiler farm (n = 44 (%))	Human isolates, general population (n = 63 (%))	UTI isolates (n = 10 (%))	Total (n = 266 (%))
CTX-M-1 62	62 (42)	12 (27)	16 (25)	1 (10)	91 (34)
CMY-2	55 (37)	18 (41)	3 (4.8)		76 (29)
SHV-12	17 (11)	7 (16)	2 (3.2)		26 (9.8)
CTX-M-15			13 (21)	6 (60)	19 (7.1)
TEM-52	12 (8.1)	2 (4.5)	1 (1.6)		15 (5.6)
Promoter mutation		4 (9.1)	9 (14)		13 (4.9)
CTX-M-14	1 (0.7)		9 (14)	1 (10)	11 (4.1)
CTX-M-2	2 (1.3)	1 (2.3)	3 (4.8)		6 (2.3)
CTX-M-3			2 (3.2)		2 (0.8)
CTX-M-24			2 (3.2)		2 (0.8)
CTX-M-32	1 (0.7)		1 (1.6)		2 (0.8)
CTX-M group 8[a]				1 (10)	1 (0.4)
CTX-M-9	1 (0.7)				1 (0.4)
CTX-M-27			1 (1.6)		1 (0.4)
DHA-1			1 (1.6)		1 (0.4)
Unknown[b]				1 (10)	1 (0.4)
Total	**151**	**44**	**63**	**10**	**266**

Note: [a]Unfortunately the sequencing reaction failed to identify the allele. [b]This UTI isolate, displaying an AmpC phenotype had an unknown resistance genotype because the CMY and DHA PCR screening was negative. Presumably this isolate has a promoter mutation, but this was not further investigated. [c]Two broiler isolates harboured two ESBL genes, i.e. $bla_{CTX\text{-}M\text{-}1}$ & $bla_{CTX\text{-}M\text{-}9}$ and $bla_{CTX\text{-}M\text{-}1}$ & $bla_{SHV\text{-}12}$, consequently, the total number of genes is larger than the number of isolates.

3. RESULTS AND DISCUSSION

3.1. Distribution of ESBL/pAmpC genes, phylogenetic groups and virulence genes

Fourteen different ESBL/pAmpC genes were identified among the 266 *E. coli* investigated (Table 1). The most prevalent ESBL/ pAmpC genes among isolates from broilers were $bla_{CTX\text{-}M\text{-}1}$ (42%), $bla_{CMY\text{-}2}$ (37%) and $bla_{SHV\text{-}12}$ (11%). The ESBL/pAmpC gene distribution among *E. coli* isolates from humans living/working on broiler farms resembled those from the broilers; the most common genes were $bla_{CMY\text{-}2}$ (41%), $bla_{CTX\text{-}M\text{-}1}$ (27%) and $bla_{SHV\text{-}12}$ (16%). In contrast, the gene distribution among isolates from individuals in the general population was different: $bla_{CTX\text{-}M\text{-}1}$ (25%), $bla_{CTX\text{-}M\text{-}15}$ (21%) and $bla_{CTX\text{-}M\text{-}14}$ (14%) were the predominant genotypes. The diversity in ESBL/pAmpC genes among human isolates from the general population was larger than among those from humans associated with broiler farms and broilers. Interestingly, $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}14}$ were only observed among UTI isolates (60% and 10%, respectively) and isolates obtained from the

general population (21% and 14%, respectively), but not in isolates from humans associated with broiler farms. In addition, $bla_{CTX\text{-}M\text{-}15}$ was also absent among broiler isolates, while $bla_{CTX\text{-}M\text{-}14}$ was found in only one broiler isolate (0.7%) (Table 1).

All phylogenetic groups and subgroups were found among isolates from broilers, humans on broiler farms and individuals in the general population (Table 2). Notable was the predominance of phylogenetic group B2 among UTI isolates, however, this might partly be due to the small number investigated here. The fact that all phylogenetic groups and subgroups were found among isolates from broilers, humans on broiler farms and individuals in the general population indicates that ESC-resistance is acquired by *E. coli* from all phylogenetic groups. Consequently, this complicates source attribution of ESC-resistance as horizontal transfer of ESBL/ pAmpC genes among *E. coli* seems to be a common event.

Screening for the presence of ten virulence genes revealed that the F1C and S fimbriae determinants *focG* and *sfaS*, respectively, were not identified in any of the investigated isolates, while the type 1 fimbrial gene *fimH* was shown to be present in nearly all isolates (92%, Table 3). The extraintestinal virulence markers *iss, iutA*, *kpsMII*, and *papA* were found in all groups of isolates investigated, whereas *afa* and *hlyD* were exclusively observed among human isolates from the general population and UTI isolates (Table 3). The aggregative virulence regulator *aggR* was also demonstrated in only two groups of isolates studied, i.e. in four human isolates from the general population and one broiler isolate. Isolates were classified as potential ExPECs based on the presence of two or more of the ExPEC-defining markers *afa*, *focG* and/or *sfaS*, *iutA*, *kpsMII* and *papA*.[20]

Table 2 Phylogenetic (sub)group distribution of the ESC-resistant *E. coli* isolates.

Phylogenetic (sub)group[a]	Broiler isolates (n = 149 (%))	Human isolates, broiler farm (n = 44 (%))	Human isolates, general population (n = 63 (%))	UTI isolates (n = 10 (%))	Total (n = 266 (%))
A	**46 (31)**	**17 (39)**	**20 (32)**	**1 (10)**	**84 (32)**
A_0	29 (20)	9 (21)	5 (7.9)	-	43 (16)
A_1	17 (11)	8 (18)	15 (24)	1 (10)	41 (15)
B1	**33 (22)**	**6 (14)**	**16 (25)**	**-**	**55 (21)**
B2	**19 (13)**	**9 (21)**	**10 (16)**	**7 (70)**	**45 (17)**
$B2_2$	1 (0.7)	3 (6.8)	2 (3.2)	1 (10)	7 (2.6)
$B2_3$	18 (12)	6 (14)	8 (13)	6 (60)	38 (14)
D	**51 (34)**	**12 (27)**	**17 (27)**	**2 (20)**	**82 (31)**
D_1	10 (6.7)	4 (9.1)	6 (9.5)	-	20 (7.5)
D_2	41 (28)	8 (18)	11 (18)	2 (20)	62 (23)
Total	**149**	**44**	**63**	**10**	**266**

Note: [a]In bold letters phylogenetic groups are indicated, in normal font the subgroups.

Table 3 Prevalence of the virulence genes studied.

Gene	Broiler isolates (n = 149 (%))	Human isolates, broiler farm (n = 44 (%))	Human isolates, general population (n = 63 (%))	UTI isolates (n = 10 (%))	Total (n = 266 (%))
afa	-	-	9 (14)	1 (10)	10 (3.8)
aggR	1 (0.7)	-	4 (6.3)	-	5 (1.9)
fimH	134 (90)	44 (100)	56 (90)	10 (100)	244 (92)
focG	-	-	-	-	-
hlyD	-	-	2 (3.2)	6 (60)	8 (3.0)
iss	121 (81)	35 (80)	34 (54)	8 (80)	198 (74)
iutA	110 (74)	24 (55)	37 (59)	10 (100)	181 (68)
kpsMII	48 (32)	13 (30)	23 (37)	8 (80)	92 (35)
papA	2 (1.3)	2 (4.5)	9 (14)	7 (70)	20 (7.5)
sfaS	-	-	-	-	-
Total	**149**	**44**	**63**	**10**	**266**

Note: A minus sign indicates that a gene was not identified in any of the isolates tested.

Table 4 Matrix of the significant differences in the frequencies of ESBL/pAmpC-genes, phylogenetic groups and virulence markers between the investigated groups of isolates.

	Broiler	Human_broiler farm	Human_general population	UTI
Broiler	-	**Pmut (x2 = 14.5, P = 0.002)**	**CTX-M-14 (x2 = 17.9, P < 0.001)** **CTX-M-15 (x2 = 32.2, P < 0.001)** **Pmut (x2 = 21.9, P < 0.001)** **afa(x2 = 21.9, P < 0.001)** **aggR(x2 = 6.1, P = 0.029)** **papA(x2 = 14.8, P < 0.001)** CMY-2 (x2 = 23.5, P < 0.001) iss (x2 = 18.8, P < 0.001) iutA (x2 = 5.4, P = 0.024)	**B2 (x2 = 22.5, P < 0.001)** **CTX-M-15 (x2 = 92.9, P < 0.001)** **hlyD(x2 = 92.9, P < 0.001)** **kpsMII(x2 = 9.4, P = 0.004)** **papA(x2 = 82.7, P < 0.001)** CMY-2 (x2 = 5.6, P = 0.016)
Human_ broiler farm	NA	-	**CTX-M-14 (x2 = 6.5, P = 0.011)** **CTX-M-15 (x2 = 9.7, P = 0.001)** **afa(x2 = 6.5, P = 0.011)** CMY-2 (x2 = 23.3, P < 0.001) SHV-12 (x2 = 6.0, P = 0.027) iss (x2 = 10.2, P = 0.002)	**B2 (x2 = 8.9, P = 0.006)** **CTX-M-15 (x2 = 28.5, P < 0.001)** **hlyD(x2 = 28.5, P < 0.001)** **iutA(x2 = 6.6, P = 0.01)** **kpsMII(x2 = 8.1, P = 0.009)** **papA(x2 = 24.0, P < 0.001)** CMY-2 (x2 = 6.6, P = 0.01)
Human_ general population	NA	NA	-	**B2 (x2 = 14.45, P = 0.001)** **CTX-M-15 (x2 = 7.1, P = 0.015) hlyD(x2 = 29.0, P < 0.001) iutA(x2 = 6.6, P = 0.001) kpsMII(x2 = 6.9, P = 0.014) papA(x2 = 16.0, P = 0.001)**
UTI	NA	NA	NA	-

Note: Pmut indicates a promoter mutation. The bold versus normal letters display in which particular group of isolates the genetic characteristic occurs at a significant higher frequency.

Potential ExPECs were identified in 33% of the broiler isolates, 27% of the isolates from humans associated with broiler farms, 44% of isolates from humans in the general population, and 90% of the UTI isolates. These percentages indicate that a considerable fraction of these isolates combine pathogenic potential with ESC-resistance.

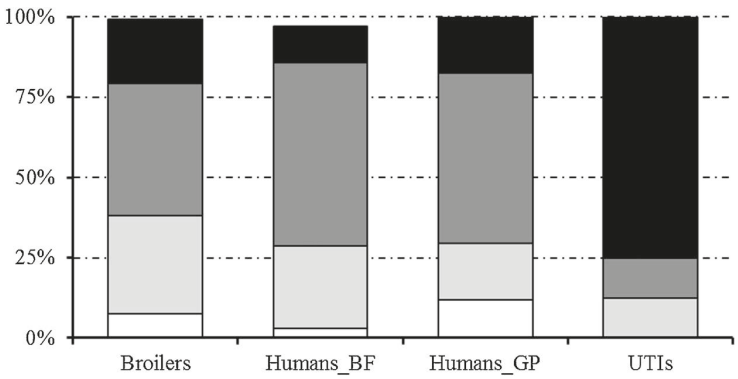
Based on the detection of the *aggR* gene, five ESC-resistant *E. coli* isolates (1 broiler and 4 human isolates) could be classified as EAEC. The general consensus is that humans are the only natural reservoir of EAEC but transmission to other hosts can occur via contaminated food and/or water.[29] Therefore, the EAEC broiler isolate most likely is a transient event and not indicative for a reservoir. Recently, seven out of 170 ESC-resistant *E. coli* from wastewater (2/88) and surface water (5/82) were identified to be positive for *aggR*.[18] Whether these isolates originated from humans or other sources like broilers remains unknown. EAEC are known to cause diarrhoea but have also been associated with UTIs, possibly due to a combination of EAEC (*aggR*) and ExPEC virulence factors like *iutA*.[30, 31] Two EAEC from this study (one broiler and one human general population isolates) were also classified as potential ExPECs.[20]

The increased serum survival gene iss has been recognized for its role in ExPEC virulence due to its association with an increase in complement resistance.[4] In the current study, the prevalence of *iss* was significantly higher among broiler isolates (81%) than among human isolates (45%). In addition, the percentage of *iss*-positive isolates obtained from humans living and/or working on a broiler farm (80%) was significantly higher ($x2 = 10.2$, $P = 0.002$) compared to human isolates from the general population (54%) (see Table 3 and 4). Sequence analysis of the iss amplicons revealed a different distribution of the iss types in broiler and healthy human isolates versus UTI isolates (Fig.1). Most genes could be differentiated in the three known iss allele types, but in 14 isolates the bacteriophage I gene bor was found. This determinant encodes for an outer-membrane lipoprotein involved in serum resistance, which is believed to be the precursor of the iss alleles.[4] Broiler and human (non UTI) isolates predominantly contained iss type 1 and 2, with the latter allele found most often, whereas nearly all iss positive UTI isolates harboured type 3 (Fig. 1). This corresponds with the findings of Johnson et al. (2008) where type 3 occurred frequently among ExPEC isolates. These results imply that different ESC-resistant *E. coli* populations reside in broilers compared to people with an UTI, and therefore clonal transmission of ESC-resistant *E. coli* from broilers to humans does not seem to play a significant role in the epidemiology of human UTIs.

Statistical analysis of the molecular characteristics revealed that $bla_{CTX\text{-}M\text{-}15}$ was strongly associated with the presence of virulence genes; *aggR* ($x2 = 21.5$, $P = 0.003$), *afa* ($x2 = 16.9$, $P = 0.003$), *hlyD* ($x2 = 22.8$, $P = 0.001$), *kpsMII* ($x2 = 10.4$, $P = 0.002$) and *papA* ($x2 = 10.4$, $P = 0.008$). The predominance of $bla_{CTX\text{-}M\text{-}15}$ among ESC-resistant *E. coli* with certain virulence factors in the general population and the occurrence of isolates with this CTX-M allelic variant together with similar virulence factors among clinical human isolates in the Netherlands as well as globally suggests that healthy humans can be a source of these bacteria for vulnerable persons.[13, 21, 22] The occurrence of $bla_{CTX\text{-}M\text{-}15}$ among ESBL-producing *E. coli* is partially associated with the spread of the pathogenic ST131 clone, causing urinary tract and bloodstream infections worldwide.[23-25] In this study 18 *E. coli* ST131 were found, however, only five of them carried $bla_{CTX\text{-}M\text{-}15}$, one human

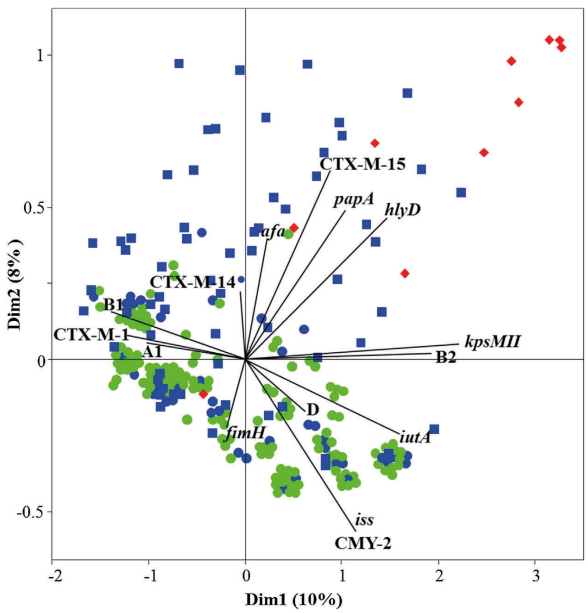
isolate from the general population and four among the UTI isolates tested. The other ST131 isolates obtained from broilers and healthy humans predominantly had different resistance genes, i.e. bla_{CMY-2} and $bla_{CTX-M-1}$ which is indicative of another clonal line.

Fig. 1. Prevalence of the different iss alleles among iss-positive isolates.



Humans_BF represent isolates from humans living and/or working on a broiler farm, Humans_GP indicate isolates from humans in the general population. Because some sequences failed not all prevalences add up to 100% (see Supplementary file). White bars represent bor, light grey iss type 1, dark grey iss type 2, and black iss type 3.

Fig. 2. Principal component analysis scatterplot calculated from the ESBL-genotypes, the presence/absence of the different virulence genes, and the origin of the isolate.



The analysis successfully separated out different clusters primarily based on isolate origin. The length and direction of the lines represent those components which show a contribution to the separation of the first two dimensions (i.e., the explained variance). Green circles represent broiler isolates and blue circles human isolates from individuals living/working on those broiler farms.[9, 10] Blue squares indicate human isolates from the general population[14] and red diamonds the UTI isolates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Multivariate analysis of isolates from different sources

Table 4 shows the ESBL/pAmpC genes, virulence markers and phylogenetic groups that differ significantly in frequency between isolates from broilers, humans living and/or working on a broiler farm and humans from the general population. Isolates from humans in the general population varied from broiler isolates with respect to the frequency of ESBL/pAmpC genes *bla*$_{CMY-2}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15}$ and virulence determinants *afa, aggR, iss, iutA* and *papA* (Table 1 and 4). The prevalence of certain genes, i.e. *afa*, *bla*$_{CMY-2}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15}$, *bla*$_{SHV-12}$, and *iss*, differed significantly between the isolates from humans on broiler farms and those from the general population. The ESBL gene *bla*$_{CTX-M-15}$, virulence markers *hlyD, kpsMII, papA*, and phylogenetic group B2 were strongly associated with UTI isolates and the frequency of occurrence among UIT isolates was different compared to the non-UTI groups (Table 4).

Principal component analysis based on the ESBL/pAmpC genes, virulence determinants and phylogenetic groups revealed that most UTI isolates were distinct from the other three groups investigated (Fig. 2). Isolates from broilers and humans living and/ or working on a broiler farm clustered together. In contrast, isolates from humans in the general population showed some overlap with the former two groups but also formed a separate cluster in the same dimension as the UTI isolates. The separation of UTI isolates and those of humans in the general population from the broiler and broiler farm related human ones was mainly due to the higher frequency of *afa*, *bla*$_{CTX-M-15}$, *hlyD, kpsMII*, *papA*, and phylogenetic group B2 (Fig. 2). Within the groups of broiler isolates and isolates from humans living and/or working on a broiler farm two clusters could be identified. One group was primarily defined by *bla*$_{CTX-M-1}$ or *bla*$_{CTX-M-14}$ and phylogenetic (sub)group B1 or A1, whereas the second one was characterized by ESC-resistance gene *bla*$_{CMY-2}$, phylogenetic group D and virulence determinant *iss* or *iutA*.

Currently, much effort is directed towards determining the contribution of various livestock reservoirs to human colonization and infection with ESC-resistant bacteria. Recent studies demonstrated a high prevalence of ESC-resistance among *E.coli* isolates from broilers[8-10] and high similarity of the b-lactamase encoding genes and *E. coli* genotypes in humans, poultry, and retail poultry products,[11-13] suggesting that broilers are an important source of these bacteria or resistance genes for humans. Poultry meat has been postulated to be a source for human ExPEC based on a certain similarity in phylogenetic backgrounds and virulence genes.[4, 26] The current results showed a more nuanced view. Multivariate analysis displayed a high level of similarity between ESC-resistant *E. coli* from broilers and from humans living and/or working on a broiler farm, confirming that contact with broilers is a risk factor for carriage of these bacteria.[9] In contrast, isolates from humans in the general population and clinical (UTI) isolates differed considerable from broiler isolates with respect to the frequency of ESC-resistance genes as well as virulence genes. Although there is some overlap between broiler isolates and isolates causing UTI, the results challenge the opinion of some researchers that poultry is an important reservoir for human infections by indicating a less strong link between the broiler reservoir and the human general population and clinical cases of urinary tract infection. This is best illustrated

by the relatively high frequency of the ESC-resistance gene $bla_{CTX\text{-}M\text{-}15}$ and virulence genes *afa* and *hlyD* and their absence among broiler isolates.

In order to make a valid estimation of the relative importance of poultry with respect to transmission of ESC-resistance and ExPEC to humans, some major hurdles have to be resolved. First, the level of genetic similarity between isolates and their plasmids from different hosts needs to be studied at a much higher resolution, which can be achieved by employing whole-genome-sequencing (WGS) including plasmid analysis. We propose a multilevel genotyping approach where comparative high resolution typing of strains, plasmids and genes is combined in order to obtain a more complete picture of the complex ESBL attribution problem. Second, conceptual frameworks are necessary to provide means to quantify the frequency and directionality of transmission. Third, improvements are essential in experimental design of studies aiming at source attribution of ESC-resistance and ExPEC.[2] Key is that poultry should not a priori be considered as the main reservoir. *E. coli* with the same resistance genes and virulence factors have been found in several potential reservoirs like other food-producing animals, but also horses and companion animals,[5, 22, 27] therefore, these should not be neglected. In addition, the human general population itself should not be underestimated as a major reservoir.

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