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Extended-spectrum beta-lactamase (ESBL)-producing and non-ESBL-producing *Escherichia coli* isolates causing bacteremia in the Netherlands (2014 – 2016) differ in clonal distribution, antimicrobial resistance gene and virulence gene content

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ABSTRACT

Background: Knowledge on the molecular epidemiology of *Escherichia coli* causing *E. coli* bacteremia (ECB) in the Netherlands is mostly based on extended-spectrum beta-lactamase-producing *E. coli* (ESBL-Ec). We determined differences in clonality and resistance and virulence gene (VG) content between non-ESBL-producing *E. coli* (non-ESBL-Ec) and ESBL-Ec blood isolates with different epidemiological characteristics.

Materials/methods: A random selection of non-ESBL-Ec isolates as well as all available ESBL-Ec blood isolates was obtained from two Dutch hospitals between 2014 and 2016. Whole genome sequencing was performed to infer sequence types (STs), serotypes, acquired antibiotic resistance genes and VG scores, based on presence of 49 predefined putative pathogenic VG.

Results: ST73 was most prevalent among the 212 non-ESBL-Ec (N=26, 12.3%) and ST131 among the 69 ESBL-Ec (N=30, 43.5%). Prevalence of ST131 among non-ESBL-Ec was 10.4% (N=22, *P* value < 0.001 compared to ESBL-Ec). O25:H4 was the most common serotype in both non-ESBL-Ec and ESBL-Ec. Median acquired resistance gene counts were 1 (IQR 1 – 6) and 7 (IQR 4 – 9) for non-ESBL-Ec and ESBL-Ec, respectively (*P* value < 0.001). Among non-ESBL-Ec, acquired resistance gene count was highest among blood isolates from a primary gastro-intestinal focus (median 4, IQR 1 – 8). Median VG scores were 13 (IQR 9 – 20) and 12 (IQR 8 – 14) for non-ESBL-Ec and ESBL-Ec, respectively (*P* value = 0.002). VG scores among non-ESBL-Ec from a primary urinary focus (median 15, IQR 11 – 21) were higher compared to non-ESBL-Ec from a primary gastro-intestinal (median 10, IQR 6 – 13) or hepatic-biliary focus (median 11, IQR 5 – 18) (*P* values = 0.007 and 0.036, respectively). VG content varied between different *E. coli* STs.

Conclusions: Non-ESBL-Ec and ESBL-Ec blood isolates from two Dutch hospitals differed in clonal distribution, resistance gene and VG content. Also, resistance gene and VG content differed between non-ESBL-Ec from different primary foci of ECB.

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INTRODUCTION

Despite advances in medical healthcare and in contrast to the decline in other infectious diseases, the annual incidence of Gram-negative bacteremia in Europe is increasing [1–4]. *Escherichia coli* is the leading causative pathogen in Gram-negative bacteremia and is associated with 30-day mortality up to 18% [1,4–6]. Antibiotic treatment options of *E. coli* bacteremia (ECB) are getting compromised by the pandemic presence of extended-spectrum beta-lactamases (ESBLs) [1–4]; enzymes that confer resistance to antibiotics commonly used for ECB treatment such as third-generation cephalosporins. Genes encoding ESBLs are encoded on mobile genetic elements and can be exchanged between strains by horizontal gene transfer. In some European countries, the incidence of ECB with antibiotic-resistant strains seems to increase faster than ECB caused by susceptible strains [1–4]. The individual patient and financial burden is increased for ECB episodes that are caused by resistant *E. coli*. Yet, ECB due to susceptible strains is far more common and therefore determines the major part of the ECB disease burden in the population [1–4]. The majority of ECBs is of community onset and is preceded by an infection in the urinary tract, but other sources, such as the hepatic-biliary tract, also comprise important primary foci [4,7]. These clinical characteristics of ECB episodes are important because they can indicate different target populations for prevention. More insight in the molecular epidemiology of ESBL-negative as well as ESBL-positive ECB with different clinical characteristics is needed to help identify key targets for the development of future preventive strategies such as *E. coli* vaccines, which are currently being developed [8]. Up to now, the molecular epidemiology of ECB in the Netherlands has been mainly described in single-center studies [9] or among antimicrobial resistant isolates only [10]. Dutch studies combining patient characteristics with high-resolution genetic data of *E. coli* isolates are limited, specifically for ECB, with its potential severe clinical consequences.

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In this study, we aimed to analyze the current population structure of ECB in the Netherlands, with special attention to differences in antimicrobial resistance and virulence gene content and clonal and serotype distribution between isolates with different clinical epidemiological characteristics and between non-ESBL-producing *E. coli* (non-ESBL-Ec) and ESBL-producing *E. coli* (ESBL-Ec) blood isolates.

METHODS

Study design

Details of the study design, clinical epidemiological data collection and laboratory methods (i.e. phenotypic ESBL detection) are described elsewhere [11]. In short, patients with ECB were retrospectively identified from medical microbiological records in the University Medical Center Utrecht (UMCU), a 1,042-bed tertiary care center and the Amphia Hospital in Breda, an 837-bed teaching hospital. In each hospital, we selected a random sample of 40 isolates of unique patients per year for the years 2014, 2015 and 2016, comprising ~24% of all first bacteremic *E. coli* isolates in a year. In addition to this random sample, all ESBL-Ec blood isolates from 2014 – 2016 were selected from the two hospitals. Whole genome sequencing (WGS) was performed by The Netherlands National Institute for Public Health and the Environment (RIVM) using the Illumina HiSeq 2500 (BaseClear, Leiden, the Netherlands). All generated raw reads were submitted to the European Nucleotide Archive (ENA) of the European Bioinformatics Institute (EBI) under the study accession number PRJEB35000. De novo assembly was performed using SPAdes genome assembler v.3.6.2 and the quality of assembles was assessed using QUAST [12]. Only genomes with an estimated genome size between 3 MB and 6 MB and number of contigs not exceeding 1,000 were included in further analyses. Baseline clinical epidemiological characteristics were compared between the non-ESBL-Ec and ESBL-Ec ECB episodes. ESBL-production was defined as confirmed phenotypic ESBL-positivity, unless described otherwise [11]. Baseline characteristics were compared by the Fisher's Exact or Pearson χ^2 test for

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105 categorical variables and by Mann-Whitney U test for continuous variables when applicable. A
106 two-tailed *P* value <0.05 was considered statistically significant.

107 This study does not fall under the scope of the Medical Research Involving Human
108 Subjects Act. The Medical Research Ethics Committee of the UMCU has therefore waived the
109 need for official approval by the Ethics Committee (IRB number 18/056) and informed consent
110 was not obtained. All statistical analyses were performed with Statistical Package for Social
111 Sciences V.25.0 (SPSS, Chicago, Illinois, USA) and R Version 3.4.1. Boxplots were made with
112 R packages *ggplot2* and *ggpubr* and bar charts were made with Graphpad Prism Version 8.0.1.

113 **Multi-locus sequence types (MLST)**

114 Multi-locus sequence types (STs) were determined using mlst2.0
115 (https://github.com/tseemann/mlst) by scanning contig files against the *E. coli* PubMLST typing
116 scheme (updated May 12th, 2018). Clonal (i.e. ST) distribution was presented stratified for non-
117 ESBL-Ec and ESBL-Ec isolates and by epidemiological subgroup (i.e. community versus
118 hospital onset; different primary foci of ECB). Genotype (ST) diversity was analysed by
119 Simpson's diversity index [13].

120 **Serotyping**

121 We assigned serotypes by using the web-tool SerotypeFinder 2.0 from the Center for Genomic
122 Epidemiology at the Danish Technical University, Lyngby, Denmark
123 (https://cge.cbs.dtu.dk/services/SerotypeFinder) [14]. Simpson's index for serotype diversity was
124 calculated for non-ESBL-Ec and ESBL-Ec isolates. Serotype distribution among non-ESBL-Ec
125 and ESBL-Ec was compared to two current *E. coli* vaccine candidates [8,15], excluding those
126 isolates in which no definitive serotype could be defined, and the occurrence of serotypes was
127 described by primary focus of ECB.

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129 **Antimicrobial resistance genes**

130 Abricate (https://github.com/tseemann/abricate) version 0.8.13 was used for mass screening of
131 contigs for antimicrobial resistance genes using the ResFinder 3.1.0 database (acquired
132 resistance genes only), date of download 24 January 2019 [16]. The thresholds for coverage
133 length and sequence identity were 80% and 95%, respectively. A resistance gene count was
134 made per isolate, which was defined as the total number of identified acquired resistance
135 genes. In case of double detection of identical resistance genes within a single isolate, they
136 were only counted once. The resistance gene counts were compared between non-ESBL-Ec
137 and ESBL-Ec with the non-parametric Wilcoxon rank sum test (for this comparison only, the
138 scores of the ESBL-Ec isolates were corrected for presence of the ESBL gene). Resistance
139 gene scores were then analysed for non-ESBL-Ec and ESBL-Ec separately and were compared
140 between isolates with different epidemiological characteristics and different STs using Kruskal-
141 Wallis one-way ANOVA. In case of an overall ANOVA *P* value <0.05, post-hoc pairwise
142 comparisons were made and the Holm-Bonferroni *P* value correction was applied to account for
143 multiple testing. For pairwise comparisons, the non-parametric Wilcoxon rank sum test was
144 used.

145 **Virulence genes**

146 The presence of putative virulence factor genes (VG) was identified using abricate version
147 0.8.13 for BLAST against the VFDB database (http://www.mgc.ac.cn/VFs), date of download 8
148 February 2019, with minimal coverage length and sequence identity 80% and 95% [17]. We
149 searched for 49 putative VG that were previously described as extra-intestinal pathogenic *E. coli*
150 (ExPEC)-associated VG [18–22]. If any of the predefined VG were not included in VFDB,
151 BLAST against the ecoli_VF_collection database was performed (date 8 February 2019), a
152 repository that contains known VG from VFDB supplemented with additional *E. coli* VG that
153 have been reported in literature [23]. The *kpsM*, *afa/dra* and *sfa/foc* operons were considered

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present if any of the corresponding genes or allelic variants were identified. A virulence score was made per isolate and was defined as the total number of pre specified VG, adjusted for multiple detection of the *afa/dra* (Afa/Dr adhesins), *pap* (P fimbrial adhesins), *sfa/foc* (S and F1C fimbrial adhesins) and *kpsM* (group 2 and III capsule) operons, as described previously [20]. If a VG was detected multiple times within a single isolate (i.e. with different quality measures), it was only counted once. These virulence scores were then compared between isolates with different epidemiological characteristics and between different STs using Kruskal-Wallis one-way ANOVA. In case of an overall ANOVA *P* value <0.05, post-hoc pairwise comparisons were made with the non-parametric Wilcoxon rank sum test and the Holm-Bonferroni *P* value correction was applied to account for multiple testing.

RESULTS

Patient characteristics

The isolate collection consisted of 212 phenotypic non-ESBL-Ec and 69 ESBL-Ec blood isolates (Fig. 1). Distribution of age, sex, onset of infection and primary foci were comparable between non-ESBL-Ec and ESBL-Ec bacteremia episodes (Table 1). As compared to non-ESBL-Ec, ECB episodes with ESBL-Ec were less often of community onset (63.8% versus 81.1%, *P* value = 0.003). Crude 30-day and 1-year mortality were higher in ECB episodes caused by ESBL-Ec (27.5% and 50.7%, respectively) compared to ECB episodes caused by non-ESBL-Ec (11.3% and 29.2%, respectively) (both *P* values = 0.001).

Clonal distribution

Among non-ESBL-Ec, ST73 was the most frequently observed ST (N = 26, 12.3%), followed by ST131 (N = 22, 10.4%). Isolates of ST73, 95, 127, 141, 80 and 1193 were solely identified among non-ESBL-Ec (Fig. 2). ST131 was dominant among ESBL-Ec (N = 30, 43.5%) and prevalence was higher than among non-ESBL-Ec (*P* value < 0.001). Simpson's index for clonal diversity was 95.6% (95% CI 94.4% – 96.8%) and 80.6% (95% CI 70.9% – 90.4%) for non-

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179 ESBL-Ec and ESBL-Ec, respectively. The occurrence of different STs did not differ between
180 nosocomial and community onset ECB (S1 Appendix). ST131 was the dominant ST among
181 ESBL-positive ECB episodes with a primary urinary (63%) and gastro-intestinal focus (57%),
182 which was higher as compared to other primary foci of ESBL-positive ECB (i.e. 21% among
183 primary hepatic-biliary focus, see S1 Appendix).

184 **Figure 1.** Flowchart of selection of *E. coli* blood isolates

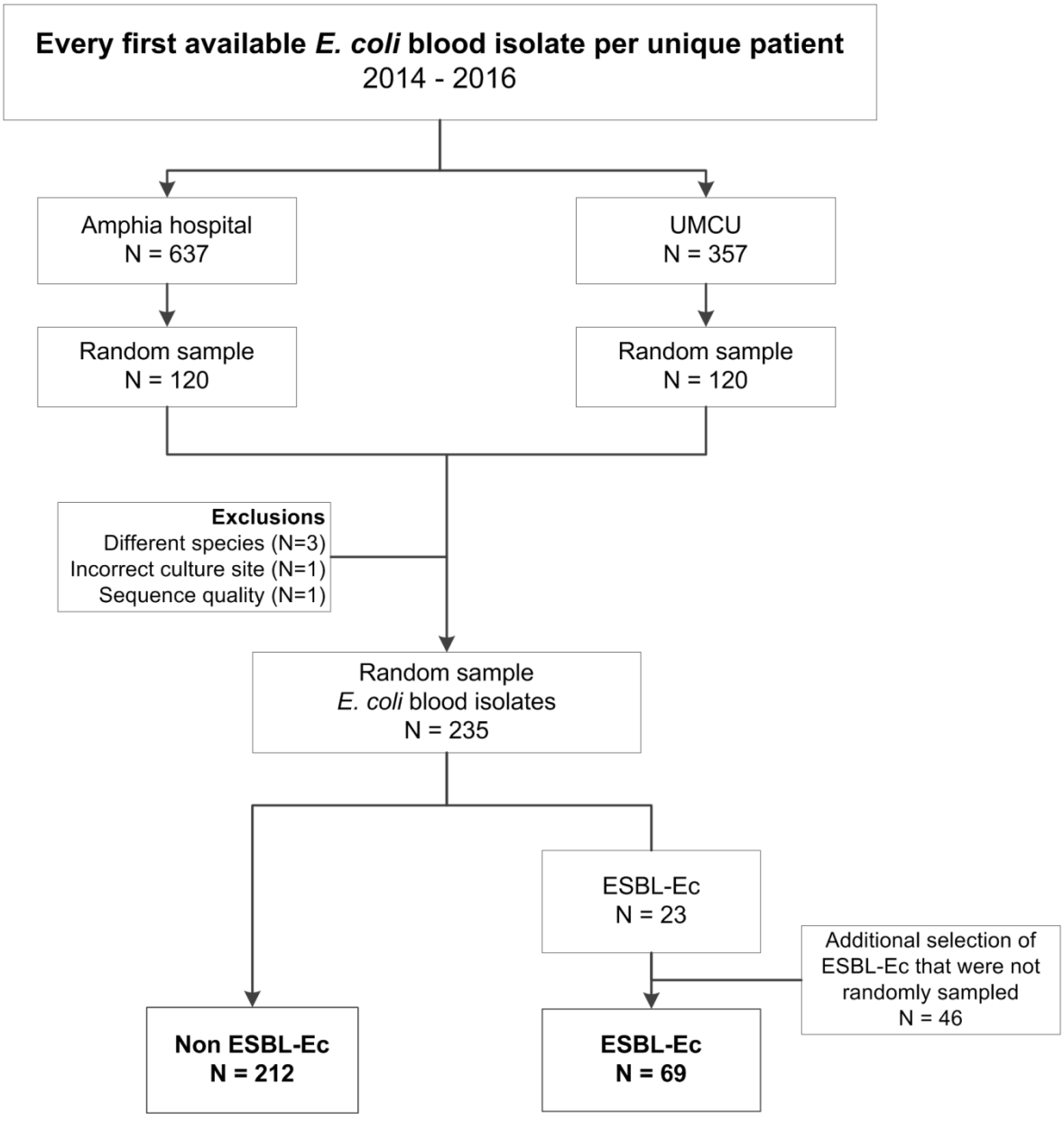

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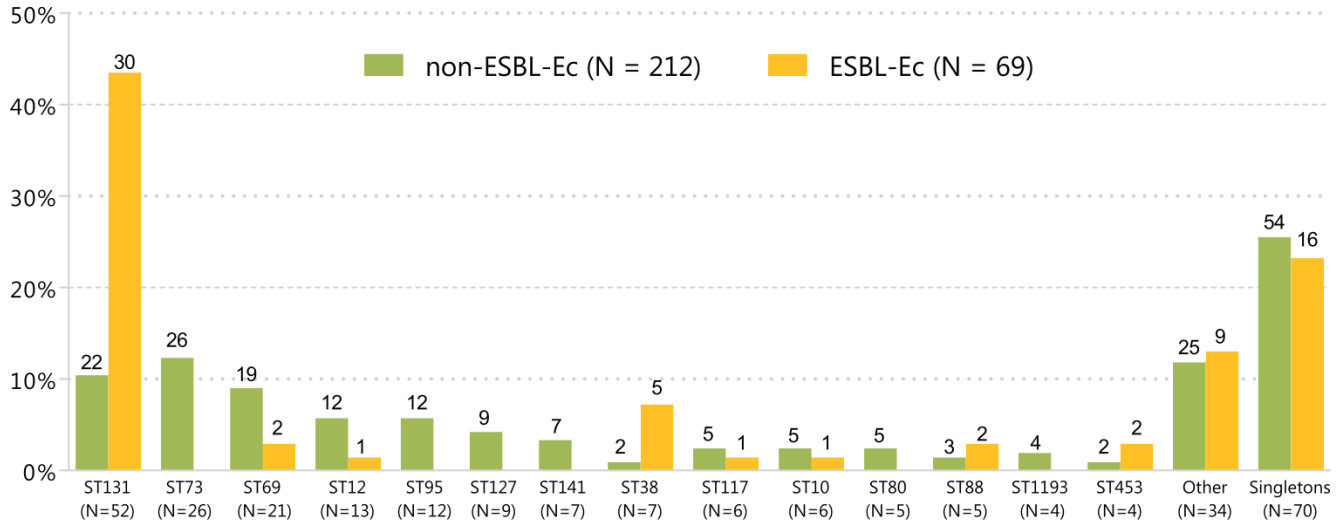
Table 1. Baseline epidemiological characteristics of *E. coli* bacteremia episodes

	Non-ESBL-Ec[a] N = 212		ESBL-Ec[a] N = 69		*P* value[b]
Median age, years (IQR)	69	(59 – 77)	69	(56 – 76)	0.802
Female sex (%)	102	(48.1)	32	(46.4)	0.802
Community onset (%)	172	(81.1)	44	(63.8)	*0.003*
Primary focus of ECB (%)					
Urinary tract	103	(48.6)	30	(43.5)	0.785
Hepatic-biliary	46	(21.7)	14	(20.3)	
Gastro-intestinal	23	(10.8)	7	(10.1)	
Other	10	(4.7)	5	(7.2)	
Unknown	30	(14.2)	13	(18.8)	
Urinary catheter (%)	69	(32.5)	28	(40.6)	0.223
Ward (%)					
Non-ICU	182	(85.8)	58	(84.1)	0.714
ICU	30	(14.2)	11	(15.9)	
Mortality (%)					
30-day	24	(11.3)	19	(27.5)	*0.001*
1-year	62	(29.2)	35	(50.7)	*0.001*

ECB, *E. coli* bacteremia; ESBL, extended-spectrum beta-lactamase; ESBL-Ec, ESBL-producing *E. coli*; ICU, intensive care unit; IQR, interquartile range; non-ESBL-Ec, non-ESBL-producing *E. coli*
[a]ESBL-positivity based on phenotype.
[b]*P* value of comparison between non-ESBL-Ec versus ESBL-Ec, calculated with Pearson's χ^2, Fisher's exact, or Mann-Whitney U test when applicable. *P* values in italic represent *P* values <0.05.

Figure 2. ST distribution among non-ESBL-Ec versus ESBL-Ec[a] in order of frequency[b]



ESBL-Ec, ESBL-producing *E. coli*; non-ESBL-Ec, non-ESBL-producing *E. coli*; ST, sequence type
[a]ESBL-positivity based on phenotypic ESBL production.
[b]Missing STs and STs that occurred ≤3 times are grouped in "Other". STs that only occurred once are grouped in "Singletons". The height of each individual bars represents the proportion of the ST within the group of non-ESBL-Ec and ESBL-Ec, respectively. The numbers represent the absolute numbers of occurrence.

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Serotypes

The most common serotype O25:H4 was identified in 19 (9.0%) non-ESBL-Ec and 24 (34.8%) ESBL-Ec isolates, which largely reflected the prevalence of ST131 in each group (Table 2). Multiple serotypes only occurred among non-ESBL-Ec, such as O6:H1 and O6:H31. ST73 was most often of serotype O6:H1 (16 / 26, 61.5%). Simpson's index for serotype diversity was 96.7% (95% CI 95.8% – 97.6%) and 83.8% (95% CI 76.9% – 90.6%) for non-ESBL-Ec and ESBL-Ec, respectively. Non-ESBL-Ec and ESBL-Ec isolates from ECB episodes with a primary focus in the urinary tract were most often of O serotype O6 (15 / 103, 14.6%) and O25 (17 / 30, 56.7%), respectively (S2 Appendix). For ECB episodes with a primary focus in the hepatic-biliary tract, O25 was the most prevalent O serotype among non-ESBL-Ec (7 / 46, 15.2%) and O8 (4 / 14, 28.6%) among ESBL-Ec isolates (S2 Appendix).

53 (25.0%) non-ESBL-Ec and 25 (36.2%) ESBL-Ec isolates belonged to either O1, O2, O6 or O25, the serotypes of the 4-valent *E. coli* vaccine that has reached phase 2 development stage [8,24], whereas the majority of non-ESBL-Ec (N = 113; 53.3%) and ESBL-Ec isolates (N = 35; 50.7%) belonged to one of the O serotypes of the new 10-valent conjugant *E. coli* vaccine (ExPEC-10V) that is currently in development [15].

Antimicrobial resistance genes

In total, 69 unique acquired resistance genes were identified (S3 Appendix). ESBL-genes were detected in 65 (94.2%) of 69 *E. coli* blood isolates with phenotypic ESBL production. $bla_{\text{CTX-M-15}}$ was the most prevalent ESBL gene (N = 28, 43.1%), followed by $bla_{\text{CTX-M-9}}$ (N = 14, 21.5%) and $bla_{\text{CTX-M-27}}$ (N = 9, 13.8%). Assemblies of the phenotypic ESBL-Ec isolates in which no ESBL-gene was identified with the used bioinformatics pipeline (N = 4) were individually uploaded on the DTU Resfinder 3.1.0 website (date 11 March 2019, thresholds for coverage length 80% and sequence identity 95%); these isolates remained genotypically ESBL-negative. One of these isolates was positive for $bla_{\text{CMY-2}}$ (AmpC gene).

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The median acquired resistance gene count for non-ESBL-Ec versus ESBL-Ec was 1 (IQR 1 – 6) versus 7 (IQR 4 – 9) (*P* value < 0.001). Among non-ESBL-Ec, acquired resistance gene counts were not different between community and hospital-onset ECB episodes (S3 Appendix). Among non-ESBL-Ec, there were statistically significant differences in resistance gene count for different primary foci of ECB, but absolute differences were small: median resistance gene count from ECB with a primary hepatic-biliary focus was 1 (IQR 1 – 1), whereas for a primary urinary focus this was 2 (IQR 1 – 6) (*P* value ≤0.001), for a primary gastro-intestinal focus this was 4 (IQR 1 – 8) (*P* value ≤0.01 for comparison to hepatic-biliary focus) and for an unknown primary focus this was 2 (IQR 1 – 7) (*P* value ≤0.0001 for comparison to hepatic-biliary focus) (S3 Appendix). Among ESBL-Ec isolates, there were no statistical significant differences in acquired resistance gene counts between community and hospital-onset ECB or different primary foci of ECB (S3 Appendix). There were no statistically significant differences observed in resistance gene count among non-ESBL-Ec or ESBL-Ec isolates of different clonal background (Fig. 3 and S3 Appendix).

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235 **Table 2.** Serotype distribution among *E. coli* blood isolates, stratified for ESBL-positivity

	Non-ESBL-Ec **N = 212** (%)	**ESBL-Ec**[a] **N = 69** (%)
O25:H4 (%)	19 (9.0)	24 (34.8)
O6:H1 (%)	16 (7.5)	-
O2/O50:H6 (%)	10 (4.7)	-
O6:H31 (%)	9 (4.2)	-
O15:H18 (%)	7 (3.3)	2 (2.9)
O17/O44/O77:H18 (%)	8 (3.8)	-
O4:H5 (%)	7 (3.3)	1 (1.4)
O75:H5 (%)	8 (3.8)	-
O8:H9	5 (2.4)	2 (2.9)
O16:H5 (%)	3 (1.4)	3 (4.3)
O86:H18	1 (0.5)	4 (5.8)
O4:H1 (%)	5 (2.4)	-
O1:H7	4 (1.9)	-
O117:H4	4 (1.9)	-
O2/O50:H1	4 (1.9)	-
O23:H16	2 (0.9)	2 (2.9)
O25:H1	4 (1.9)	-
O18/O18ac:H7	3 (1.4)	-
O2/O50:H7	3 (1.4)	-
O45:H7	3 (1.4)	-
O75:H7	3 (1.4)	-
O8:H17	3 (1.4)	-
O9:H17	-	2 (2.9)
O9/O104:H9	-	2 (2.9)
O13/O135:H4	2 (0.9)	-
O18:H1	2 (0.9)	-
O18:H5	2 (0.9)	-
O22:H1	2 (0.9)	-
O24:H4	2 (0.9)	-
O8:H10	2 (0.9)	-
O8:H25	2 (0.9)	-
O8:H30	2 (0.9)	-
Singletons	45 (21.2)	13 (18.8)
Unknown	20 (9.4)	14 (20.3)

[a]ESBL-positivity based on phenotypic ESBL production.
ESBL, extended-spectrum beta-lactamase; ESBL-Ec, ESBL-producing *E. coli*, non-ESBL-Ec, non-ESBL-producing *E. coli*

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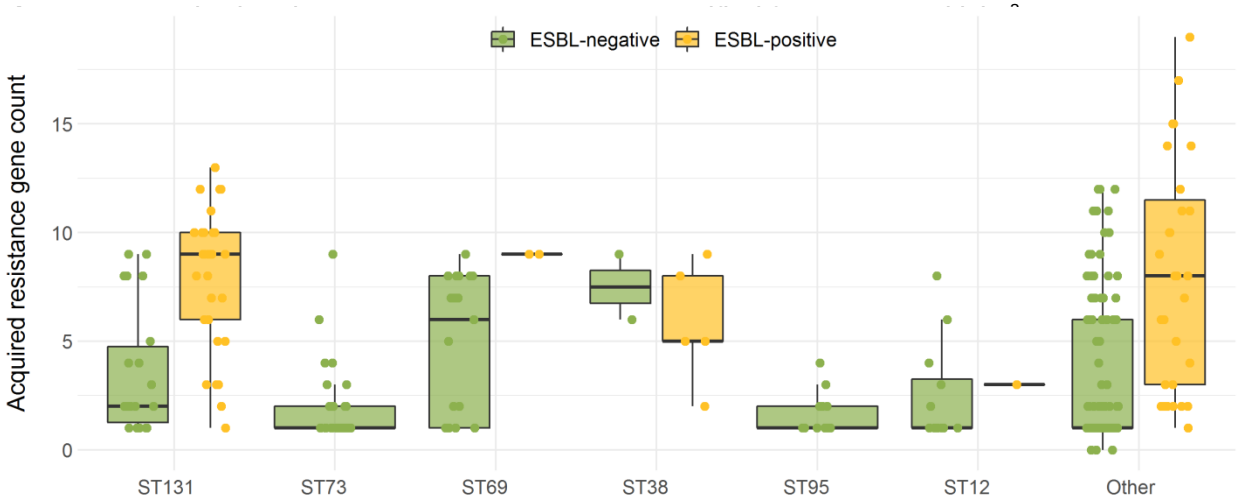

[a]ESBL-positivity based on phenotypic ESBL production.
Boxplots display median resistance gene count and inter quartile range (IQR); every dot represents a single isolate. Only STs that occurred >5% within non-ESBL-Ec or ESBL-Ec were grouped into main groups, the rest was categorized as "Other". Results of the pairwise comparisons between STs can be found in S3 Appendix.

Virulence genes

Of the 49 predefined ExPEC-associated VG, 44 (89.8%) were detected in at least one *E. coli* blood isolate (S4 Appendix). The median VG score was 13 (IQR 9 – 20) for non-ESBL-Ec and 12 (IQR 8 – 14) for ESBL-Ec blood isolates (*P* value = 0.002). In one non-ESBL-Ec isolate no predefined ExPEC-associated VG was detected, while a maximum VG score of 25 was found in two non-ESBL-Ec isolates.

For non-ESBL-Ec and ESBL-Ec isolates, there was no significant difference in the VG score between isolates that caused community or hospital onset ECB (S4 Appendix). Non-ESBL-Ec isolates that caused ECB with a primary gastro-intestinal focus and hepatic-biliary focus had lower VG scores (median 10, IQR 6 – 13 and median 11, IQR 5 – 18, respectively) as compared to isolates with a primary focus in the urinary tract (median 15, IQR 11 – 21) (*P* value = 0.007 and *P* value = 0.036, respectively) (see S4 Appendix). Among non-ESBL-Ec and ESBL-Ec, there were no statistical significant differences in VG scores between isolates of patients without versus with a urinary catheter, between patients alive or deceased after 30

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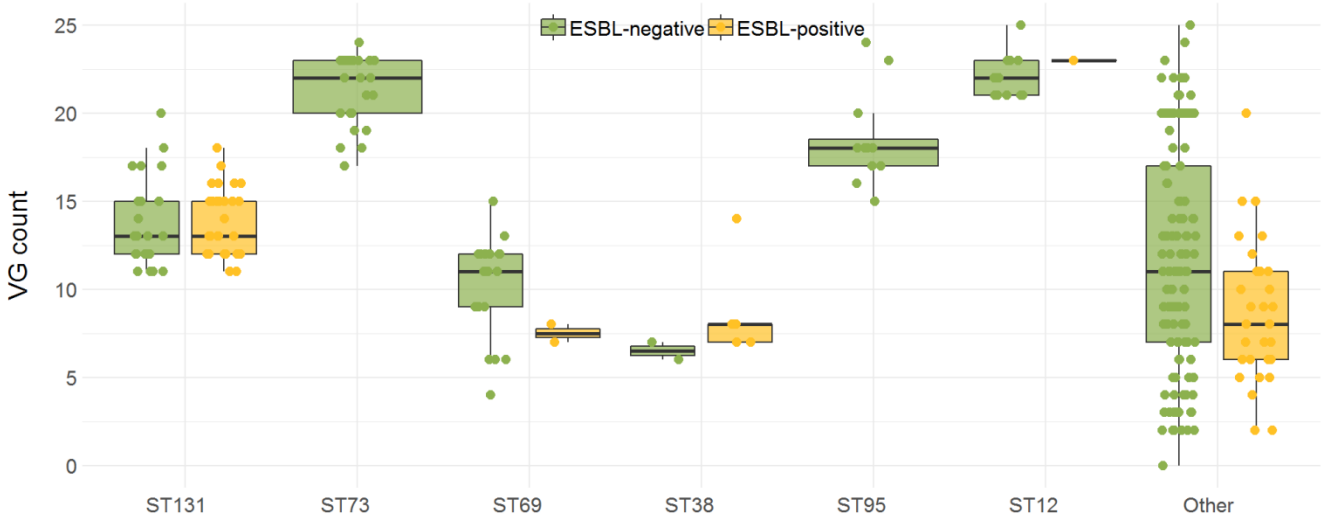
256 days or between patients admitted to the intensive care unit (ICU) versus a non-ICU ward (S4
257 Appendix).

258 There was heterogeneity in VG scores between non-ESBL-Ec of different STs, this was
259 less pronounced for ESBL-Ec isolates (Fig. 4 and S4 Appendix). ESBL-negative ST38 had the
260 lowest average VG score (median 7, IQR 6 – 7) and ESBL-positive ST12 had the highest VG
261 score (median 23, IQR 23 – 23). Median VG score of both ESBL-negative and ESBL-positive
262 ST131 isolates was 13 (IQR 12 – 15). All pairwise comparisons between ESBL-negative STs
263 yielded Holm-Bonferroni adjusted *P* values < 0.05, except for the comparison ST12 versus
264 ST73 and all pairwise comparisons that included ST38.

265

266 **Figure 4.** ExPEC-associated VG score in different STs, stratified for ESBL-positivity[a]



267
268 [a]ESBL-positivity based on phenotypic ESBL production.
269 Boxplots display median VG score and inter quartile range (IQR); every dot represents a single isolate. Only STs that
270 occurred >5% within non-ESBL-Ec or ESBL-Ec were grouped into main groups, the rest was categorized as "Other".
271 Results of pairwise comparisons between STs can be found in S4 Appendix.

272

273 **DISCUSSION**

274 In this study, we found that ESBL-producing *E. coli* blood isolates were different from non-
275 ESBL-producing *E. coli* causing bacteraemia in terms of clonal distribution, serotype distribution,
276 antimicrobial resistance gene count and VG scores.

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In line with previous research, the clonal distribution among ESBL-Ec blood isolates was less diverse as compared to non-ESBL-Ec [25–27]. This was mainly caused by the predominance of ST131 within ESBL-Ec, as has been described before [28,29]. In contrast, ST73, a ST that is known for its susceptibility to antibiotics [28], was only identified among non-ESBL-Ec blood isolates. The association between ESBL phenotype and STs in *E. coli*, which is repeatedly found, implies that the genetic make-up of strains contributes to the ability to acquire and subsequently maintain plasmids carrying ESBL genes. Indeed, a recent large-scale study that compared the pan-genomes of invasive *E. coli* isolates, including ST131 and ST73, suggested that due to ongoing adaptation to long term human intestinal colonisation and consequent evolutionary gene selection, ST131 might have become able to reduce the fitness costs of long term plasmid maintenance [30,31]. It has been hypothesized that this is also true for other *E. coli* lineages that are associated with multi-drug resistance. Reducing the fitness costs of replicating plasmids encoding multi-drug resistance will result in having competitive advantage over other intestinal strains [32].

We hypothesized that the clonal distribution and resistance gene and VG content would differ between ECB episodes of community and hospital onset and between different primary foci, as a result of adaptive evolution of intestinal *E. coli*. We observed some statistical significant differences in resistance gene count and VG scores among non-ESBL-Ec from different primary foci of ECB, such as higher VG scores of blood isolates from a primary urinary focus as compared to isolates from a primary focus in the gastro-intestinal or hepatic-biliary tract. However, absolute differences in median gene counts were small and the clinical significance remains unclear. In the current study, we found that differences in molecular content mostly depended on phenotypic ESBL-production and STs. This confirms the findings from a recent study that was performed in Scotland [33]. In that study, there were combinations of VGs as well as a particular accessory gene composition that differentiated between STs rather than between epidemiological factors. The association between ST69 and community

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303 onset ECB, as found in the Scottish study, was not identified in the current study. Other
304 differences were the large proportion of *E. coli* isolates from ECB episodes that were deemed
305 hospital-acquired (62%) as compared to our study (18.4% for ESBL-negative and 36.2% for
306 ESBL-positive ECB) and in that study, analyses were not stratified for ESBL-positivity.

307 Interestingly, in our study, isolates that belonged to ST73 had low resistance gene
308 content but relatively high VG scores as compared to other STs. Furthermore, the average VG
309 score among non-ESBL-Ec was slightly higher in comparison to ESBL-Ec blood isolates, which
310 demonstrates that ESBL-positivity in *E. coli* is not necessarily related to an increased VG
311 content. These findings do not support the theory that increased virulence of resistant strains
312 causes the increased incidence of resistant ECB as compared to sensitive ECB. This theory has
313 been suggested for other pathogens, such as MRSA [1,34,35].

314 We identified serotype O25:H4 as the most prevalent serotype causing ESBL-negative
315 as well as ESBL-positive ECB in The Netherlands, followed by O6:H1. The serotype distribution
316 among non-ESBL-Ec was more heterogeneous as compared to ESBL-Ec, similar to the
317 differences in clonal diversity between these two groups [36]. A large recent European
318 surveillance study that included 1,110 *E. coli* blood isolates from adults between 2011 and 2017
319 showed that there is heterogeneity in serotype distribution among different countries, which
320 highlights the need for country specific data, such as provided in the current study [15]. We
321 showed that the coverage of the new potential 10-valent vaccine was higher as compared to the
322 4-valent vaccine and was actually doubled for non-ESBL-Ec bacteraemia. Findings of the
323 current study can be used for future studies and can help further evaluation and implementation
324 of *E. coli* vaccines.

325 Strengths of the current study are the multicenter design and combination of
326 epidemiological characteristics and highly discriminatory genetic data. There are also important
327 limitations. Firstly, *E. coli* is a heterogeneous species, of which the seven MLST genes only
328 constitute a small proportion of the entire gene content. Because we also only investigated a

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small fraction of the genes that are commonly part of the accessory genome, such as VGs and acquired resistance genes, we may have missed genomic differences that could have importantly contributed to ecological specialization in the different clinically relevant primary foci. Secondly, we selected *E. coli* isolates from a tertiary care center and teaching hospital from the Netherlands from two different regions, which we considered to be representative of the Netherlands. The description of strains that were identified here might not be entirely generalizable to other countries since there could be differences between circulating *E. coli* strains, dependent on local population characteristics and antimicrobial resistance levels. Thirdly, many pairwise comparisons between subgroups were performed, which increases the risk of false-positive findings (i.e. type I errors). Even though we applied a strict *P* value correction for multiple testing, this naturally does not eliminate the risk of false-positive findings. The analyses on resistance gene and VG content should therefore be viewed as hypothesis generating.

In conclusion, associations between clinical characteristics of ECB episodes and molecular content of *E. coli* isolates were limited. However, we did identify important differences in clonality, serotypes, antimicrobial resistance genes and VG scores between non-ESBL-Ec and ESBL-Ec blood isolates that reached beyond their phenotypic ESBL-positivity. Future studies that aim to describe the molecular epidemiology of ECB should therefore preferably focus on *E. coli* without preselection on ESBL-positivity, to limit the risk of inferring characteristics of resistant *E. coli* to the *E. coli* population as a whole. Furthermore, a more thorough understanding of the molecular epidemiology of ECB and specifically studies that further disclose targets for surveillance or infection-prevention will help to reduce the occurrence of this invasive infectious disease with its severe potential consequences.

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AUTHOR CONTRIBUTIONS

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P.C.J. Bruijning-Verhagen	Conceptualization, formal analysis, methodology, supervision, validation, writing – review & editing
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A.C. Schürch	Methodology, software, resources, supervision, validation, writing – review & editing
R.J.L. Willems	Methodology, software, resources, supervision, writing – review & editing
M.J.M. Bonten	Conceptualization, formal analysis, funding acquisition, resources, supervision, writing – review & editing
J.A.J.W. Kluytmans	Conceptualization, formal analysis, resources, supervision, writing – review & editing

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SUPPORTING INFORMATION

S1 Appendix

S2 Appendix

S3 Appendix

S4 Appendix