

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

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26 **ABSTRACT**

27 **Background:** Knowledge on the molecular epidemiology of *Escherichia coli* causing *E. coli*
28 bacteremia (ECB) in the Netherlands is almost exclusively based on extended-spectrum beta-
29 lactamase producing *E. coli* isolates (ESBL-PEc) that are responsible for about 10% of all ECB
30 episodes. We determined clonal differences and differences in resistance and virulence gene
31 content between non-ESBL-producing *E. coli* (non-ESBL-PEc) and ESBL-PEc isolates with
32 different epidemiological characteristics.

33 **Materials/methods:** A random selection of non-ESBL-PEc isolates as well as all available
34 ESBL-PEc blood isolates were obtained from two Dutch hospitals between January 2014 and
35 December 2016. For comparative analysis, whole genome sequencing was performed of all
36 isolates to infer the sequence types (STs), serotypes, antibiotic resistance by either acquired
37 genes or chromosomal mutations and virulence gene (VG) scores, based on presence of 49
38 predefined putative pathogenic VG.

39 **Results:** ST73 was most prevalent among the 212 non-ESBL-PEc (N = 26, 12.3%) and ST131
40 among the 69 ESBL-PEc (N = 30, 43.5%). Prevalence of ST131 among non-ESBL-PEc was
41 10.4% (N = 22, *P* value < 0.001 compared to ESBL-PEc). O25:H4 was the most common
42 serotype in non-ESBL-PEc and ESBL-PEc. Median total resistance gene counts were 42 (IQR
43 39 – 45) and 46 (IQR 43 – 50) for non-ESBL-PEc and ESBL-PEc, respectively (*P* value <
44 0.001). Median acquired resistance gene counts were 1 (IQR 1 – 6) and 7 (IQR 4 – 9) for non-
45 ESBL-PEc and ESBL-PEc, respectively (*P* value < 0.001). Median VG scores were 13 (IQR 9 –
46 20) and 12 (IQR 8 – 14) for non-ESBL-PEc and ESBL-PEc isolates, respectively (*P* value =
47 0.002). Resistance gene and VG content varied between different *E. coli* STs.

48 **Conclusions:** We observed differences between non-ESBL-PEc and ESBL-PEc blood isolates
49 in ST distribution, resistance gene and virulence gene content.

50 INTRODUCTION

51 Despite advances in medical healthcare and in contrast to the decline in other infectious
52 diseases, the annual incidence of Gram-negative bacteremia in Europe is increasing [1–4].
53 *Escherichia coli* is the leading causative pathogen in Gram-negative bacteremia and is
54 associated with 30-day mortality up to 18% [1,4–6]. Antibiotic treatment options of *E. coli*
55 bacteremia (ECB) are getting compromised by the pandemic presence of extended-spectrum
56 beta-lactamases (ESBLs) [1–4]; enzymes that confer resistance to antibiotics commonly used
57 for ECB treatment such as third-generation cephalosporins. ESBLs can be exchanged between
58 strains by horizontal gene transfer, such as through carry-over of mobile genetic elements. In
59 some European countries, the incidence of ECB with antibiotic-resistant strains seems to
60 increase faster than ECB caused by susceptible strains [1–4]. The individual patient and
61 financial burden is increased for ECB episodes that are caused by resistant *E. coli*. Yet, ECB
62 due to susceptible strains is far more common and therefore determines the major part of the *E.*
63 *coli* bacteremia disease burden in the population [1–4]. The majority of ECBs is of community
64 onset and is preceded by an infection in the urinary tract, but other sources, such as the
65 hepatic-biliary tract, also comprise important primary foci [4,7]. These clinical characteristics of
66 ECB episodes are important because they can indicate different target populations for
67 prevention. More insight in the molecular epidemiology of ESBL-negative as well as ESBL-
68 positive ECB with different clinical characteristics is needed to help identify key targets for the
69 development of future preventive strategies such as *E. coli* vaccines, which are currently being
70 developed [8]. Up to now, the molecular epidemiology of ECB in the Netherlands has been
71 mainly described in single-center studies [9] and among antimicrobial resistant isolates only
72 [10,11]. Dutch studies combining patient characteristics with high-resolution genetic data of *E.*
73 *coli* isolates are limited, specifically for ECB, with its potential severe clinical consequences.

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

79 **METHODS**

80 **Study design**

81 Details of the study design, epidemiological data collection and laboratory methods (i.e.
82 phenotypic ESBL detection) are described elsewhere [12]. In short, patients with ECB were
83 retrospectively identified from medical microbiological records in the University Medical Center
84 Utrecht (UMCU), a 1,042-bed tertiary care center and the Amphia Hospital in Breda, an 837-bed
85 teaching hospital. In each hospital, we selected a random sample of 40 isolates of unique
86 patients per year for the years 2014, 2015 and 2016, comprising ~24% of all first bacteremic *E.*
87 *coli* isolates in a year. In addition to this random sample, all ESBL-PEc blood isolates from 2014
88 – 2016 were selected from the two hospitals. Whole genome sequencing (WGS) was performed
89 by The Netherlands National Institute for Public Health and the Environment (RIVM) using the
90 Illumina HiSeq 2500 (BaseClear, Leiden, the Netherlands). De novo assembly was performed
91 using SPAdes genome assembler v.3.6.2 and the quality of assemblies was assessed using
92 QUAST [13]. Only genomes with an estimated genome size between 3 MB and 6 MB and
93 number of contigs not exceeding 1,000 were included in further analyses. Baseline clinical
94 epidemiological characteristics were compared between the non-ESBL-PEc and ESBL-PEc
95 ECB episodes. ESBL-production was defined as confirmed phenotypic ESBL-positivity, unless
96 described otherwise [12]. Baseline characteristics were compared by the Fisher's Exact or
97 Pearson χ^2 test for categorical variables and by Mann-Whitney U test for continuous variables
98 when applicable. A two-tailed *P* value <0.05 was considered statistically significant.

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

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

106 Multi-locus sequence types (STs) were determined using mlst2.0
107 (<https://github.com/tseemann/mlst>) by scanning contig files against the *E. coli* PubMLST typing
108 scheme (updated May 12th, 2018). ST (i.e. clonal) distribution was presented stratified for non-
109 ESBL-PEc and ESBL-PEc isolates and by epidemiological subgroup (i.e. community versus
110 hospital onset; different primary foci of ECB). Genotype (ST) diversity was analysed by
111 Simpson's diversity index [14]. A core-genome (cg) neighbour-joining (NJ) phylogenetic tree
112 was constructed in Seqsphere with the *Escherichia/Shigella* cgMLST v1 scheme developed by
113 Enterobase (<https://enterobase.warwick.ac.uk/species/index/ecoli>), containing 2,513 target
114 genes and visualised using the free online available web-tool Microreact
115 (<https://microreact.org/showcase>) [15]. The cgNJ method reconstructs phylogeny by using a
116 distance matrix that contains the genetic distance between each pair of sequences.

117 **Serotyping**

118 We assigned serotypes by using the web-tool SerotypeFinder 2.0 from the Center for Genomic
119 Epidemiology at the Danish Technical University, Lyngby, Denmark
120 (<https://cge.cbs.dtu.dk/services/SerotypeFinder>) [16]. Simpson's index for serotype diversity
121 was calculated for non-ESBL-PEc and ESBL-PEc isolates. Serotype distribution among non-
122 ESBL-PEc and ESBL-PEc was compared to two current *E. coli* vaccine candidates [8,17],

123 excluding isolates in which no definitive serotype could be defined and the occurrence of
124 serotypes was described by primary focus of ECB

125 **Antimicrobial resistance genes**

126 Abricate (<https://github.com/tseemann/abricate>) version 0.8.13 was used for mass screening of
127 contigs for antimicrobial resistance genes using the ResFinder 3.1.0 database (acquired
128 resistance genes), date of download 24 January 2019, and the Comprehensive Antibiotic
129 Resistance Database (CARD) (all resistance genes), date of download 1 March 2019 [18,19].
130 The thresholds for coverage length and sequence identity were 80% and 95%, respectively. A
131 resistance gene count using each of the databases was made per isolate, which was defined as
132 the total number of resistance genes (using CARD) and the total number of acquired resistance
133 genes (using ResFinder) identified, respectively. In case of double detection of identical
134 resistance genes within a single isolate, they were only counted once. The resistance gene
135 scores were compared between non-ESBL-PEc and ESBL-PEc with the non-parametric
136 Wilcoxon rank sum test (for this comparison only, the scores of the ESBL-PEc isolates were
137 corrected for presence of the ESBL gene). Resistance gene scores were then analysed for non-
138 ESBL-PEc and ESBL-PEc separately and were compared between isolates with different
139 epidemiological characteristics and different STs using Kruskal-Wallis one-way ANOVA. In case
140 of an overall ANOVA P value <0.05 , post-hoc pairwise comparisons were made and the Holm-
141 Bonferroni P value correction was applied to account for multiple testing. For pairwise
142 comparisons, the non-parametric Wilcoxon rank sum test was used.

143 **Virulence genes**

144 The presence of putative virulence factor genes (VG) was identified using abricate version
145 0.8.13 for BLAST against the VFDB database (<http://www.mgc.ac.cn/VFs>), date of download 8
146 February 2019, with minimal coverage length and sequence identity 80% and 95% [20]. We
147 searched for 49 putative VG that were previously described as extra-intestinal pathogenic *E. coli*

148 (ExPEC)-associated VG [21–25]. If any of the predefined VG were not included in VFDB,
149 BLAST against the *ecoli_VF_collection* database was performed (date 8 February 2019), a
150 repository that contains known VG from VFDB supplemented with additional *E. coli* VG that
151 have been reported in literature [26]. The *kpsM*, *afa/dra* and *sfa/foc* operons were considered
152 present if any of the corresponding genes or allelic variants were identified. A virulence score
153 was made per isolate and was defined as the total number of pre specified VG, adjusted for
154 multiple detection of the *afa/dra* (Afa/Dr adhesins), *pap* (P fimbrial adhesins), *sfa/foc* (S and F1C
155 fimbrial adhesins) and *kpsM* (group 2 and III capsule) operons, as described previously [23]. If a
156 VG was detected multiple times within a single isolate (i.e. with different quality measures), it
157 was only counted once. These virulence scores were then compared between isolates with
158 different epidemiological characteristics and between different STs using Kruskal-Wallis one-
159 way ANOVA. In case of an overall ANOVA *P* value <0.05, post-hoc pairwise comparisons were
160 made with the non-parametric Wilcoxon rank sum test and the Holm-Bonferroni *P* value
161 correction was applied to account for multiple testing.

162 **RESULTS**

163 **Patient characteristics**

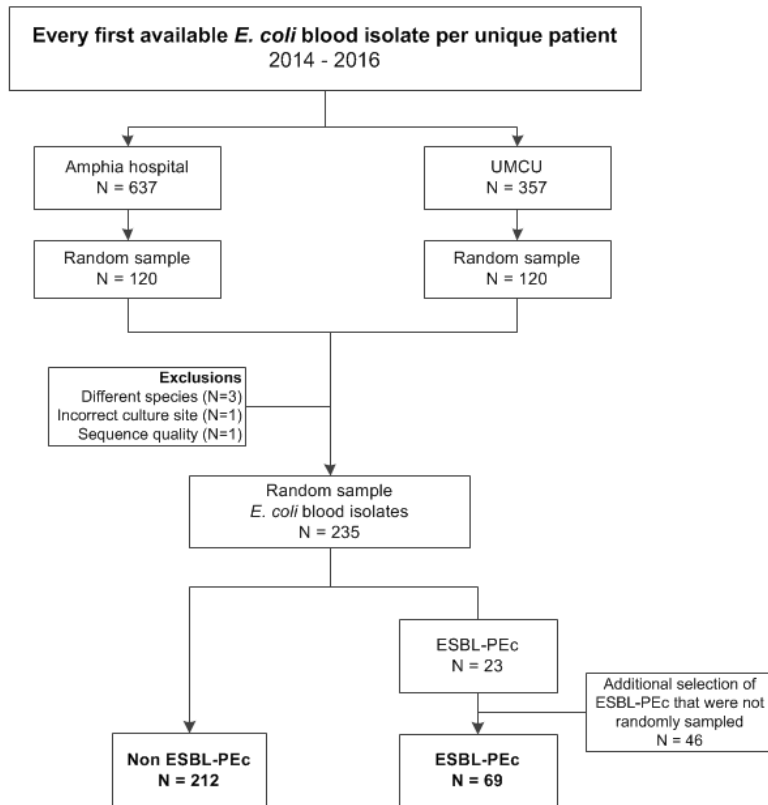
164 The isolate collection consisted of 212 phenotypic non-ESBL-PEc and 69 ESBL-PEc blood
165 isolates (Fig. 1). Distribution of age, sex, onset of infection and primary foci were comparable
166 between non-ESBL-PEc and ESBL-PEc bacteremia episodes (Table 1). As compared to non-
167 ESBL-PEc, ECB episodes with ESBL-PEc were less often of community onset (63.8% versus
168 81.1%, *P* value = 0.003). Crude 30-day and 1-year mortality was higher in ECB episodes
169 caused by ESBL-PEc (27.5% and 50.7%, respectively) in comparison with ECB episodes
170 caused by non-ESBL-PEc (11.3% and 29.2%, respectively).

171

172 **Clonal distribution**

173 Among non-ESBL-PEc, ST73 was the most frequently observed ST (12.3%), followed by ST131
174 (10.4%). Isolates of ST73, 95, 127, 141, 80 and 1193 were solely identified among non-ESBL-
175 PEc (Fig. 2). ST131 was dominant among ESBL-PEc (N = 30, 43.5%) and prevalence was
176 higher than among non-ESBL-PEc (P value < 0.001). Simpson's index for ST diversity was
177 95.6% (95% CI 94.4% – 96.8%) and 80.6% (95% CI 70.9% – 90.4%) for non-ESBL-PEc and
178 ESBL-PEc, respectively. The occurrence of different STs did not differ between nosocomial and
179 community onset ECB (S1 Figure and S2 Table). ST131 was the dominant ST among ESBL-
180 positive ECB episodes with a primary urinary (63%) and hepatic-biliary focus (57%), which was
181 higher as compared to other primary foci of ESBL-positive ECB (i.e. 21% among primary
182 hepatic-biliary focus, see S3 Figure and S4 Table). The NJ-phylogenetic tree of all isolates can
183 be found in the Supporting Information (S5 Figure).

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



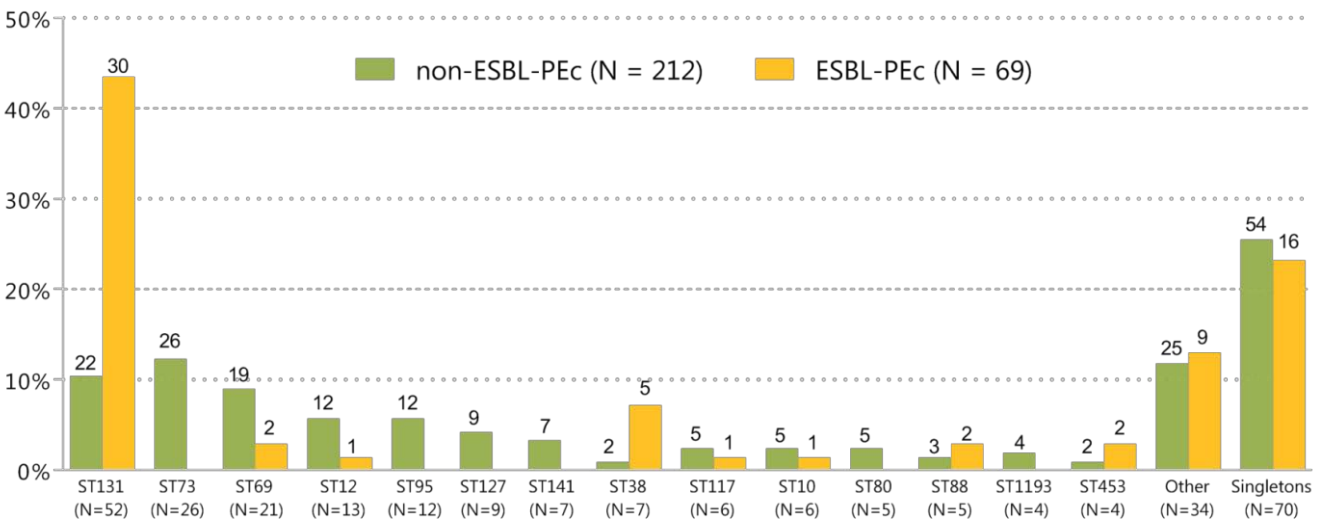
186 **Table 1.** Baseline epidemiological characteristics of *E. coli* bacteremia episodes

| | Non-ESBL-PEc ^a N = 212 | | ESBL-PEc ^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) | <i>0.003</i> |
| 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) | <i>0.001</i> |
| 1-year | 62 | (29.2) | 35 | (50.7) | <i>0.001</i> |

ECB, *E. coli* bacteremia; ESBL, extended-spectrum beta-lactamase; ESBL-PEc, ESBL-producing *E. coli*; ICU, intensive care unit; IQR, interquartile range; non-ESBL-PEc, non-ESBL-producing *E. coli*
^aESBL-positivity based on phenotype.

^bP value of comparison between non-ESBL-PEc versus ESBL-PEc, 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.

187 **Figure 2.** ST distribution among non-ESBL-PEc versus ESBL-PEc^a in order of frequency^b



188 ESBL-PEc, ESBL-producing *E. coli*; non-ESBL-PEc, non-ESBL-producing *E. coli*; ST, sequence type

189 ^aESBL-positivity based on phenotype.

190 ^bMissing STs and STs that occurred ≤ 3 times are grouped in "Other". STs that only occurred once are grouped in

191 "Singletons". The height of each individual bars represents the proportion of the ST within the group of non-ESBL-

192 PEc and ESBL-PEc, respectively. The numbers represent the absolute numbers of occurrence.

193

194 **Serotypes**

195 The most common serotype was O25:H4 and was identified in 19 (9.0%) non-ESBL-PEc and 24
196 (34.8%) ESBL-PEc isolates, which largely reflected the prevalence of ST131 in each group
197 (Table 2). Multiple serotypes only occurred among non-ESBL-PEc, such as O6:H1 and O6:H31.
198 ST73 was most often of serotype O6:H1 (16 / 26, 61.5%). Simpson's index for serotype diversity
199 was 96.7% (95% CI 95.8% – 97.6%) and 83.8% (95% CI 76.9% – 90.6%) for non-ESBL-PEc
200 and ESBL-PEc, respectively. The distribution of O:serotypes per primary focus of ECB was
201 mostly in line with the distribution of the most important STs per primary focus (i.e. ST131,
202 ST73) (S6 Table).

203 53 (25.0%) non-ESBL-PEc and 25 (36.2%) ESBL-PEc isolates belonged to either O1,
204 O2, O6 or O25, the serotypes of the 4-valent *E. coli* vaccine that has reached phase 2
205 development stage [8,28], whereas the majority of non-ESBL-PEc (N = 113; 53.3%) and ESBL-
206 PEc isolates (N = 35; 50.7%) belonged to one of the O:serotypes of the new 10-valent
207 conjugant *E. coli* vaccine (ExPEC-10V) that is currently in development [17].

208 **Antimicrobial resistance genes**

209 In total, 110 unique resistance genes were identified with CARD and 69 unique acquired
210 resistance genes were identified with ResFinder 3.1.0 (see S7 Table). ESBL-genes were
211 detected in 65 (94.2%) of 69 *E. coli* blood isolates with phenotypic ESBL production. *bla*_{CTX-M-15}
212 was the most prevalent ESBL gene (N = 28, 43.1%), followed by *bla*_{CTX-M-9} (N = 14, 21.5%) and
213 *bla*_{CTX-M-27} (N = 9, 13.8%). Assemblies of the phenotypic ESBL-PEc isolates in which no ESBL-
214 gene was identified (N = 4) were individually uploaded on the DTU Resfinder 3.1.0 website
215 (date 11 March 2019, thresholds for coverage length 80% and sequence identity 95%); these
216 isolates remained genotypically ESBL-negative. One of these isolates was positive for *bla*_{CMY-2}
217 (AmpC gene).

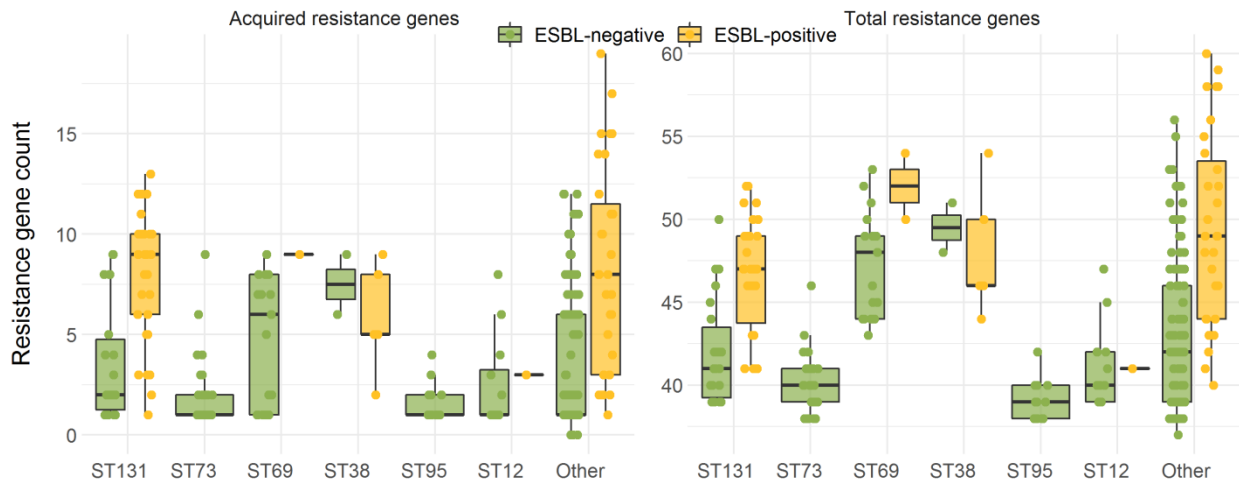
218 The median total resistance gene count was 42 (IQR 39 – 45) for non-ESBL-PEc and 46
219 (IQR 43 – 50) for ESBL-PEc isolates (P value < 0.001). The median acquired resistance gene
220 count for non-ESBL-PEc versus ESBL-PEc was 1 (IQR 1 – 6) versus 7 (IQR 4 – 9) (P value <
221 0.001). Among non-ESBL-PEc, total and acquired resistance gene counts were not different
222 between community and hospital-onset ECB episodes (S8 Figure and S9 Table). Among non-
223 ESBL-PEc, there were statistically significant differences in resistance gene count for different
224 primary foci of ECB, but absolute differences were small (S8 Figure). The median acquired
225 resistance gene count of non-ESBL-PEc isolates from ECB with a primary hepatic-biliary focus
226 was 1 (IQR 1 – 1), whereas for a primary urinary focus this was 2 (IQR 1 – 6) (P value \leq 0.001),
227 primary gastro-intestinal focus this was 4 (IQR 1 – 8) (P value \leq 0.01) and unknown primary
228 focus this was 1 (IQR 1 – 6) (P value \leq 0.0001) (S9 Table). Among ESBL-PEc isolates, there
229 were no statistical significant differences in total and acquired resistance gene counts between
230 community and hospital-onset ECB or different primary foci of ECB (S8 Figure and S9 Table).
231 Among non-ESBL-PEc, there was heterogeneity in total resistance gene count between almost
232 all dominant STs; this was not the case for acquired resistance gene count (Fig. 3 and S10
233 Table). No statistically significant differences were observed among ESBL-PEc isolates of
234 different STs (S10 Table).

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

| | Non-ESBL-PEc N = 212 (%) | ESBL-PEc^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) |

ESBL, extended-spectrum beta-lactamase; ESBL-PEc, ESBL-producing *E. coli*, non-ESBL-PEc, non-ESBL-producing *E. coli*

237 **Figure 3. Resistance gene count per ST, stratified for ESBL-positivity^a**



238

239 ESBL, extended-spectrum beta-lactamase; ST, sequence type

240 ^aESBL-positivity based on phenotype.

241 Boxplots display median resistance gene count and inter quartile range (IQR); and every dot represents a single
242 isolate. Results of the CARD database are depicted in the left graph and included all resistance genes. The results of
243 the ResFinder 3.1.0 database are depicted in the right graph and only included acquired resistance genes. Only STs
244 that occurred >5% within ESBLs or non-ESBLs were grouped into main groups, the rest was categorized as "Other".
245 Results of the pairwise comparisons can be found in S10 Table.

246

247 Virulence genes

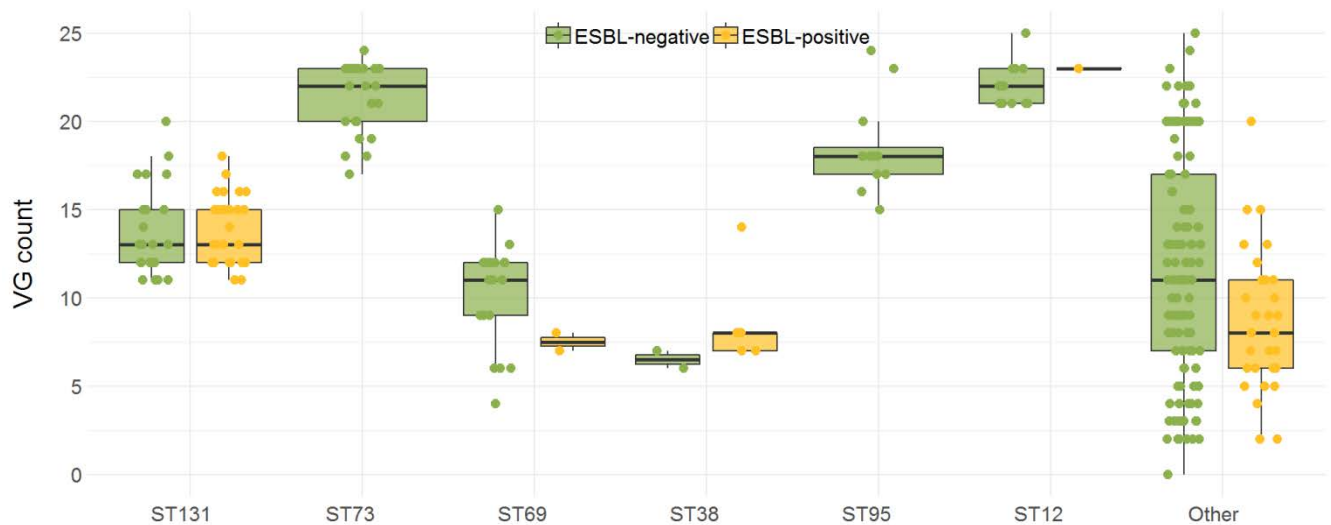
248 Of the 49 predefined ExPEC-associated VG, 44 (89.8%) were detected in at least one *E. coli*
249 blood isolate (S11 Table). The median VG score was 13 (IQR 9 – 20) for non-ESBL-PEc and 12
250 (IQR 8 – 14) for ESBL-PEc blood isolates (P value = 0.002). In one non-ESBL-PEc isolate no
251 predefined ExPEC-associated VG was detected, while a maximum VG score of 25 was found in
252 two non-ESBL-PEc isolates.

253 For non-ESBL-PEc and ESBL-PEc isolates, there was no significant difference in the VG
254 score between isolates that caused community or hospital onset ECB (S13 Table). Non-ESBL-
255 PEc isolates that caused ECB with a primary gastro-intestinal focus (median 10, IQR 6 – 13)
256 and hepatic-biliary focus (median 11, IQR 5 – 18) had lower VG scores as compared to isolates
257 with a primary focus in the urinary tract (median 15, IQR 11 – 21) (P value = 0.007 and P value
258 = 0.036, respectively, see S12 Figure and S13 Table). Among non-ESBL-PEc and ESBL-PEc,
259 there were no statistical significant differences in VG scores between isolates of patients without

260 versus with a urinary catheter, between patients alive or deceased after 30 days or between
261 patients admitted to the intensive care unit (ICU) versus a non-ICU ward (data not shown).

262 There was heterogeneity in VG scores between non-ESBL-PEc of different STs, this
263 was less pronounced for ESBL-PEc isolates (Fig. 4 and S14 Table). ESBL-negative ST38 had
264 the lowest average VG score (median 7, IQR 6 – 7) and ESBL-positive ST12 had the highest
265 VG score (median 23, IQR 23 – 23). Median VG score of both ESBL-negative and ESBL-
266 positive ST131 isolates was 13 (IQR 12 – 15). All pairwise comparisons between ESBL-
267 negative STs yielded Holm-Bonferroni adjusted P values < 0.05 , except for the comparison
268 ST12 versus ST73 and all pairwise comparisons that included ST38.

269 **Figure 4.** ExPEC-associated VG score in different STs, stratified for ESBL-positivity^a



270
271 ESBL, extended-spectrum beta-lactamase; ST, sequence type

272 ^aESBL-positivity based on phenotype.

273 Boxplots display median VG score and inter quartile range (IQR); and every dot represents a single isolate. Only STs
274 that occurred $>5\%$ within ESBLs or non-ESBLs were grouped into main groups, the rest was categorized as "Other".
275 Results of pairwise comparisons can be found in S14 Table.

276

277 DISCUSSION

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

281 The ST distribution among ESBL-PEc blood isolates was less diverse as compared to
282 non-ESBL-PEc. This was mainly caused by the predominance of ST131 within ESBL-PEc, as
283 has been described before [29,30]. In contrast, ST73, a ST that is known for its susceptible
284 antibiotic profile [29], was only identified among non-ESBL-PEc blood isolates. The association
285 between ESBL phenotype and STs in *E. coli*, which is repeatedly found, implies that the
286 molecular backbone of strains can increase (or decrease) its propensity to acquire and
287 subsequently maintain plasmids carrying ESBL genes. A recent large-scale study that
288 compared the pan-genomes of invasive *E. coli* isolates, including ST131 and ST73, suggested
289 that due to ongoing adaptation to long term human intestinal colonisation and consequent
290 evolutionary gene selection, ST131 might have become able to reduce the fitness costs of long
291 term plasmid maintenance [31,32]. Interestingly, in our study, isolates that belonged to ST73
292 had low resistance gene content but relatively high VG score as compared to other STs.
293 Furthermore, the average VG score among non-ESBL-PEc was slightly higher in comparison to
294 ESBL-PEc blood isolates, which demonstrates that ESBL-positivity in *E. coli* is not necessarily
295 related to an increased VG content. In case we would assume that VG content is associated
296 with virulent potential, i.e. the ability of a strain to cause invasive disease, then these findings do
297 not support the theory that increased virulence of resistant strains causes the increased
298 incidence of resistant ECB as compared to sensitive ECB. This theory has been suggested for
299 other pathogens, such as MRSA [1,33,34]. Still, results of the current study show that molecular
300 characteristics of ESBL-PEc cannot be merely generalized to non-ESBL-PEc blood isolates,
301 highlighting the importance of not preselecting on ESBL-positivity when investigating the
302 molecular epidemiology of ECB.

303 One of our hypotheses was that the distributions of STs, resistance gene and VG
304 content would differ between ECB episodes of community and hospital onset and between
305 different primary foci, as a possible result of different levels of sub specialization of intestinal *E.*
306 *coli* strains. However, we did not observe large differences when assessing epidemiological

307 subgroups, but found that these differences in molecular content mostly depended on
308 phenotypic ESBL-production and STs. This confirms the findings from a recent study that was
309 performed in Scotland [35]. In that study, there were combinations of virulence genes as well as
310 a particular accessory gene composition that differentiated between STs rather than between
311 epidemiological factors. The association between ST69 and community onset ECB, as found in
312 the Scottish study, was not identified in the current study. Other differences were the large
313 proportion of *E. coli* isolates from ECB episodes that were deemed hospital-acquired (62%) as
314 compared to our study (18.4% for ESBL-negative and 36.2% for ESBL-positive ECB) and in that
315 study, analyses were not stratified for ESBL-positivity. More studies that combine clinical
316 characteristics with molecular characteristics of ECB are important, because these data help to
317 further elucidate the role of host-specific factors versus strain-specific factors in the
318 pathogenesis of ECB. Since different determinants of ECB might indicate different targets for
319 surveillance or infection-prevention, a thorough understanding of the molecular epidemiology is
320 needed to reduce the occurrence of this invasive infectious disease with potential severe clinical
321 consequences.

322 We identified serotype O25:H4 as the most prevalent serotype causing ESBL-negative
323 as well as ESBL-positive ECB in the Netherlands, followed by O6:H1. The serotype distribution
324 among non-ESBL-PEc was more heterogeneous as compared to ESBL-PEc, similar to the
325 differences in clonal diversity between these two groups. A large recent European surveillance
326 study that included 1,110 *E. coli* blood isolates from adults between 2011 and 2017 showed that
327 there is heterogeneity in serotype distribution among different countries, which highlights the
328 need for country specific data [17]. Furthermore, we showed that the coverage of the new
329 potential 10-valent vaccine was higher as compared to the 4-valent vaccine and was actually
330 doubled for non-ESBL-PEc bacteremia. The findings of the current study can be used for future
331 studies and can help further evaluation and implementation of *E. coli* vaccines.

332 Strengths of the current study are the multicenter design and combination of
333 epidemiological characteristics and highly discriminatory genetic data. There are also important
334 limitations. Firstly, *E. coli* is a heterogeneous species, of which the seven MLST genes only
335 constitute a small proportion of the entire gene content. Because we also only investigated
336 presence of a small fraction of the genes that are commonly part of the accessory genome,
337 such as virulence and acquired resistance genes, but did not assess the entire accessory gene
338 pool, we could have missed genomic differences between isolates that are reflected in the
339 accessory gene pool only. Secondly, we selected *E. coli* isolates from a tertiary care center and
340 teaching hospital from the Netherlands from two different regions, which we considered to be
341 representative of the Netherlands. The description of strains that were identified here might not
342 be entirely generalizable to other countries since there could be differences between circulating
343 *E. coli* strains, dependent on local population characteristics and antimicrobial resistance levels.
344 Thirdly, many pairwise comparisons between subgroups were performed, which increases the
345 risk of false-positive findings (i.e. type I errors). Even though we applied a strict p-value
346 correction for multiple testing, this naturally does not eliminate the risk of false-positive findings.
347 The analyses on resistance gene and VG content should therefore be viewed as hypothesis
348 generating.

349 In conclusion, there are molecular differences between non-ESBL-PEc and ESBL-PEc
350 blood isolates that reach beyond their phenotypic ESBL positivity. Future genomic research of
351 *E. coli* should preferably focus on *E. coli* without preselection on ESBL-positivity, to limit the risk
352 of inferring characteristics of resistant *E. coli* to the *E. coli* population as a whole. Furthermore,
353 more studies are needed to better understand repeatedly found associations between gene
354 content and STs, which could aid the development of targeted preventive interventions.

355

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