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Prevalence of extended-spectrum beta-lactamase-producing *Escherichia coli* isolates in faecal samples of broilers

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Running title: *E. coli* harbouring ESBLs in faeces of poultry in Portugal

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29 **Abstract**

30

31 Seventy-six faecal samples were obtained from broilers at slaughterhouse level in
32 Portugal. Samples were inoculated on cefotaxime-supplemented Levine agar plates.
33 Cefotaxime-resistant *Escherichia coli* isolates were recovered from 32 samples (42.1%),
34 obtaining a total of 34 *E. coli* isolates (one or two isolates per sample). Susceptibility to 16
35 antibiotics was studied by disk diffusion method, and 85% of the isolates presented a
36 phenotype of multi-resistance that included antimicrobial agents of at least four different
37 families. Extended-spectrum-beta-lactamases (ESBL) of the TEM and CTX-M groups were
38 detected in 31 ESBL-positive *E. coli* isolates. Twenty-six isolates harboured the *bla*_{TEM-52}
39 gene and two of them also harboured *bla*_{TEM-1b}. The *bla*_{CTX-M-14} gene was identified in 3
40 isolates (in association with *bla*_{TEM-1b} in one of them), and *bla*_{CTX-M-32} was demonstrated in
41 two additional isolates. Three of the 34 cefotaxime-resistant isolates (9%) did not produce
42 ESBLs, and two of them presented mutations at positions -42 (C→T), -18 (G→A), -1
43 (C→T), and +58(C→T) of the promoter/attenuator region of *ampC* gene. *tet(A)* and/or
44 *tet(B)* genes were detected in all 34 tetracycline-resistant isolates, *aadA* in all 26
45 streptomycin-resistant isolates; *cmlA* in 3 of 6 chloramphenicol-resistant isolates, and
46 *aac(3)-II* or *aac(3)-I* + *aac(3)-IV* genes in all 4 gentamicin-resistant isolates. Different
47 combinations of *sul1*, *sul2* and *sul3* genes were demonstrated among the 22 trimethoprim-
48 sulfamethoxazole-resistant isolates. Amino acid changes in GyrA and ParC proteins were
49 identified in all 18 ciprofloxacin-resistant isolates. The results of this study indicate that the
50 intestinal tract of healthy poultry is a reservoir of ESBL-positive *E. coli* isolates.

51

52 **Keywords:** TEM-52, CTX-M-14, CTX-M-32, broilers

53

54

55 1. Introduction

56 *Escherichia coli* is a common inhabitant of intestinal tract of humans and animals
57 (Sørum and Sunde, 2001; Tannock, 1995), and can be easily disseminated in different
58 ecosystems through the food chain and water (Skurnik et al., 2006). In addition, *E. coli* can
59 become a human pathogen, mainly in immunocompromised patients, and beta-lactams are
60 frequently used for treatment of infections caused by this microorganism.

61 In the last years, the important increase of the prevalence of extended-spectrum beta-
62 lactamases (ESBLs) among clinical *E. coli* isolates in human medicine is a cause of great
63 concern, and this mechanism of resistance is implicated in failures in the treatment of
64 infectious diseases (Paterson and Bonomo, 2005; Livermore, 2008; Cantón et al., 2006).
65 ESBLs confer resistance to penicillins, first-, second- and third-generation cephalosporins,
66 as well as to aztreonam, but not to cefoxitin or carbapenems. Different types of ESBLs have
67 been reported, and those of the CTX-M type represent an emerging problem in human
68 health, being considered as “the CTX-M pandemic” (Cantón et al., 2006; Rossolini et al.,
69 2008).

70 Different reports have alerted in the last few years about the dissemination of ESBL-
71 positive *E. coli* among the intestinal microbiota of healthy food-producing animals or food
72 products in different countries of Europe (Aarestrup et al., 2006; Blanc et al., 2006; Briñas
73 et al., 2003, 2005; Girlich et al., 2007; Smet et al., 2008), Asia (Duan et al., 2006; Kojima et
74 al., 2005; Liu et al., 2007), or Africa (Jouini et al., 2007). These resistant bacteria could
75 enter the food chain, representing a problem for food safety. Previous studies carried out in
76 Portugal reported the dissemination of ESBL-containing *E. coli* isolates in the faecal
77 microbiota of dogs and also of wild animals, specially in birds of prey (Costa et al., 2004,
78 2006), and very recently also in chickens and swine (Machado et al., 2008). The purpose of
79 our work was to analyse the faecal carriage of ESBL-containing *E. coli* isolates in broilers at
80 slaughterhouse level in Portugal, to identify the type of ESBLs, and to detect the presence of
81 other antimicrobial resistance markers in these bacteria. This study provides more

82 information about the real problem of ESBL in food-producing animals, and valuable help
83 to control this emerging problem and to track its future evolution.

84

85 **2. Material and methods**

86 *2.1. Bacteria included in this study*

87 Seventy-six faecal samples from broilers were collected in a slaughterhouse located in
88 Northern Portugal, which received animals from different regions of the country. The
89 samples were obtained during 20 visits to the slaughterhouse from February to June 2004.
90 Faecal samples were obtained from different flocks that contained from 2,000 to 10,000
91 animals. Fresh dropping faecal samples were recovered from the crates, just before culling
92 the animals.

93 All the samples were seeded on Levine agar plates supplemented with 2 µg/ml of
94 cefotaxime and were incubated at 37 °C for 24 h. Two colonies per sample with typical
95 *E. coli* morphology were selected and identified by classical biochemical methods (Gram
96 staining, catalase, oxidase, indol, Methyl-Red-Voges-Proskauer, citrate and urease), and by
97 the API 20E system (BioMérieux, La Balme Les Grottes, France).

98

99 *2.2 Antimicrobial susceptibility testing*

100 Antimicrobial susceptibility was performed by the agar disk diffusion method
101 following the Clinical and Laboratory Standards Institute recommendations (CLSI, 2007),
102 and a total of 16 antimicrobial agents were tested: ampicillin, amoxicillin-clavulanic acid
103 (AMC), cefoxitin, cefotaxime (CTX), ceftazidime (CAZ), imipenem, aztreonam,
104 gentamicin, tobramycin, amikacin, streptomycin, tetracycline, trimethoprim-
105 sulfamethoxazole (SXT), nalidixic acid, ciprofloxacin and chloramphenicol. Broad-
106 spectrum cephalosporin-resistant isolates were selected and one isolate per sample was kept

107 for further studies (two isolates of the same sample were kept for further studies if they
108 presented different antimicrobial resistance phenotypes). The double disk synergy test with
109 cefotaxime or ceftazidime in the proximity to amoxicillin-clavulanic acid was used for the
110 screening of ESBL.

111

112 2.3. Characterization of antimicrobial resistance genes

113 The presence of genes encoding TEM, SHV, OXA-1, CTX-M, and CMY beta-
114 lactamases was studied by PCR in all broad-spectrum cephalosporin resistant isolates using
115 primers and conditions previously reported (Briñas et al., 2003; Jouini et al., 2007). The
116 obtained DNA amplicons were sequenced on both strands and sequences were compared
117 with those included in the GeneBank database in order to identify the specific beta-
118 lactamase gene. The promoter and attenuator region of the chromosomal *ampC* gene was
119 also amplified by PCR (Briñas et al., 2003), sequenced, and compared with the same region
120 of the *E. coli* K12 *ampC* gene, in order to analyse the mutations, associated to the
121 overexpression of the *ampC* gene. In addition, the presence of *tet(A)*, *tet(B)*, *tet(C)*, *tet(D)*
122 and *tet(E)* genes was studied by PCR in tetracycline-resistant isolates (Sáenz et al., 2004).
123 The following genes were also studied by PCR (Sáenz et al., 2004): *aadA1* and *aadA2* (in
124 streptomycin-resistant isolates), *aac(3)-I*, *aac(3)-II* and *aac(3)-IV* (in gentamicin-resistant
125 isolates), *cmlA* (in chloramphenicol-resistant isolates), and *sul1*, *sul2* and *sul3* (in SXT-
126 resistant isolates). Positive and negative PCR controls from the bacterial collection of the
127 University of La Rioja, Spain, were used in all assays.

128

129 2.4. Characterization of the mechanism of ciprofloxacin resistance

130 The quinolone-resistance-determining region (QRDR) of the *gyrA* gene, as well as the
131 analogous region of the *parC* gene, was amplified by PCR in all ciprofloxacin-resistant
132 *E. coli* isolates (Sáenz et al., 2003). Amplified fragments were purified (Qiagen), and both

133 strands were automatically sequenced by the Applied Biosystem 3730 sequencer (Genome
134 Express, France), using the same set of primers as for the PCR reactions. Sequences
135 obtained were compared with those previously reported for *gyrA* (GenBank accession
136 number X06373) and *parC* genes (M58408 with the modification included in L22025).

137

138 *2.5. REP-PCR clonal analysis and identification of phylogenetic groups.*

139 A PCR-based DNA fingerprinting technique which uses primers based on the highly
140 conserved repetitive extragenic palindromic (REP) repeated DNA element was used for
141 typing 34 CTX-resistant *E. coli* isolates. This technique was preferentially used to assess the
142 diversity of the isolates due to its easy methodology. REP-PCR was performed using Rep-F,
143 IIIGCGCCGICATCAGGC and Rep-R, ACGTCTTATCAGGCCTAC primers under the
144 following conditions: 30 cycles of 1 min at 94°C, 1 min at 40°C and 8 min at 65°C, with a
145 single final extension of 16 min at 65°C. PCR end-products were separated on agarose 2%
146 w/v gels and the gel images were analyzed visually comparing all the isolates.

147 The identification of the major phylogenetic groups of the isolates was determined by
148 PCR (Clermont et al., 2000).

149

150 **3. Results**

151 Results showed that CTX-resistant *E. coli* isolates were present in 32 out of the 76
152 samples of our study, representing 42.1% of the total faecal samples. Two *E. coli* isolates
153 per sample were recovered from these in 32 positive samples and the phenotype of
154 antimicrobial resistance was analysed in all of them. An identical phenotype of resistance
155 was demonstrated in the two CTX-resistant isolates obtained from 30 of these samples and
156 one isolate from each one was kept for further studies. Nevertheless, a different phenotype
157 of resistance was demonstrated in the two CTX-resistant isolates obtained from the
158 remaining two faecal samples and both of them were included in this study. Consequently, a

159 total of 34 CTX-resistant *E. coli* isolates were included in this study for beta-lactamases
160 characterization (Table 1).

161

162 3.1. Beta-lactamases detected in CTX-resistant *E. coli* isolates

163 The screening for ESBL production was performed in all 34 CTX-resistant *E. coli*
164 isolates and results were positive in 31 of them, recovered from 29 of the 76 tested faecal
165 samples (38.2%). The genes encoding ESBLs of the TEM and CTX-M groups were
166 detected in these 31 ESBL-positive *E. coli* isolates. Twenty-six isolates harboured the
167 *bla*_{TEM-52c} gene, 3 isolates the *bla*_{CTX-M-14a} gene, and 2 additional isolates the *bla*_{CTX-M-32}
168 gene. The *bla*_{TEM-1b} gene was also detected in four of these 31 ESBL-positive isolates.

169 Three of our 34 CTX-resistant isolates presented a negative ESBL-screening test (9%)
170 and they showed resistance to cefoxitin and amoxicillin-clavulanic acid. The
171 promoter/attenuator region of the chromosomal *ampC* gene was amplified in these isolates
172 and the amplicons were sequenced. Mutations at positions -42 (C→T), -18 (G→A), -1
173 (C→T), and +58 (C→T) were detected in two of these isolates (Table 2). The third CTX-
174 resistant ESBL-negative *E. coli* isolate did not present important mutations in *ampC* gene
175 (at positions -42 or -32), and all PCRs performed to detect acquired ESBLs or CMY-type
176 beta-lactamase were negative (Table 1 and 2).

177 The presence of mutations in the promoter/attenuator region of the chromosomal
178 *ampC* gene was also studied in four cefoxitin-resistant and ESBL-positive *E. coli* isolates
179 (AV-48.1A, AV-65.1A, AV-5.1A and AV-8.1A) (Table 2). It is interesting to underline that
180 the specific C→T mutation at position -42 was identified in two of the three TEM-52-
181 containing isolates, but not in the CTX-M-14a-containing one.

182 In two of the tested faecal samples, different ESBLs were identified in the two *E. coli*
183 isolates recovered from each of them. Two isolates from one faecal sample were positive for

184 CTX-M-32 and TEM-52, respectively. Two isolates of the other faecal sample contained
185 CTX-M-14a and TEM-52, respectively.

186

187 3.2. Associated antimicrobial resistance markers in CTX-resistant isolates

188 Most of our CTX-resistant isolates (85%) presented a phenotype of multi-resistance
189 that included antimicrobial agents belonging to at least four different families, and 10% of
190 them showed resistance to 6 different antimicrobial families. A wide variety of resistance
191 genes were detected among our isolates (Table 1). The *tet(A)* or *tet(B)* genes were detected
192 in all 33 tetracycline-resistant isolates grouped as follows: *tet(A)* in 11 isolates, *tet(B)* in 12,
193 and *tet(A)+tet(B)* in the remaining five isolates. The *aadA* gene was identified in all 26
194 streptomycin-resistant isolates; the *cmlA* gene in 3 of 6 chloramphenicol-resistant isolates,
195 and the *aac(3)-II* or *aac(3)-I + aac(3)-IV* genes in all 4 gentamicin-resistant isolates.
196 Different combinations of *sul1*, *sul2* and *sul3* genes were demonstrated among the 22 SXT-
197 resistant isolates, and 50% of them harboured two of these genes.

198

199 3.3. Characterization of the mechanism of ciprofloxacin resistance

200 Eighteen of our ESBL-positive *E. coli* isolates exhibited ciprofloxacin-resistance and
201 two amino acid changes were identified in GyrA protein (S83L+D87N in 16 isolates;
202 S83L+D87G in 1 isolate; S83L+D87Y in 1 isolate), and one additional amino acid change
203 in ParC protein (S80I in 18 isolates) (Table 1).

204

205 3.4. REP-PCR analysis and phylogenetic groups

206 The REP-PCR technique was applied in order to determine the clonal diversity of the
207 34 CTX-resistant *E. coli* isolates. Twenty-one different profiles were identified in this series
208 (Table 1), and fifteen profiles were demonstrated among the 26 *bla*_{TEM-52}-containing
209 isolates, indicating that many different clones were disseminated in the poultry population.

210 Fifty-per cent of the 34 CTX-resistant isolates belonged to the B₁ phylogenetic group, and
211 none of them were included in the B₂ group (Table 1). Figure 1 shows the number of ESBL-
212 positive *E. coli* isolates distributed according to their phylogenetic group. Seventy-seven-per
213 cent of TEM-52-containing isolates belonged to phylogenetic groups A or B₁.

214

215 **4. Discussion**

216 Almost forty per cent of the faecal samples of broilers recovered in 2004 at
217 slaughterhouse level in Portugal revealed ESBL-containing *E. coli* isolates. This result
218 should arouse concern because these animals enter the food chain and they could transfer
219 these resistant bacteria to humans. This percentage is higher than that recently found by
220 Machado et al (2008) in faecal samples of healthy chickens (10%), but slightly lower than
221 the prevalence found on chicken carcasses (60%). High prevalence of CTX-resistant or
222 ESBL-producing *E. coli* isolates in faecal samples of food-producing animals has also been
223 found in other European countries (Smet et al., 2008; Moreno et al., 2007; Blanc et al.,
224 2006). The wide dissemination of ESBL-positive *E. coli* among faecal isolates of healthy
225 food-producing animals is a problem of food safety and it is important to analyse the factors
226 that could contribute to this situation. One possible origin of this problem might be the use
227 of broad-spectrum cephalosporins in broiler production, although these antimicrobial agents
228 are not widely used in this type of farming. Another possible reason would be the use in
229 broilers of other antimicrobial agents (quinolones, tetracycline, sulfamides, trimethoprim, or
230 aminoglycosides, among others) that could co-select ESBL-positive bacteria, that harbour
231 their resistance genes in the same mobile structures. Other possible factor would be the high
232 concentration of animals in flocks, which facilitates the transmission of resistant bacteria
233 among animals. It is difficult to know the specific contribution of each factor to the global
234 problem of the wide dissemination of ESBL in the animal ecosystem, and all these factors
235 should be studied.

236 Recent reports in the last years also refer a relatively high frequency of the presence of
237 ESBL-producing *E. coli* isolates in samples of healthy humans (Miró et al., 2005;
238 Moubareck et al., 2005; Pallecchi et al., 2007; Valverde et al., 2004; Vinué et al., 2008),
239 although the reported percentages are lower than those found in the poultry samples of this
240 study. An important issue to be elucidated is whether the presence of ESBL-producing
241 bacteria in the intestinal tract of healthy animals and humans has occurred in an independent
242 way, or by contrast, both ecosystems interrelated in their evolution.

243 It is important to note that 84% of the ESBL-positive *E. coli* isolates recovered in this
244 study harboured the TEM-52 enzyme and a wide diversity of clones were detected among
245 them. A high prevalence of TEM-52 and a high heterogeneity of PFGE patterns were also
246 reported in *E. coli* isolates recovered from healthy chickens, healthy swine and chicken meat
247 products in Portugal (Machado et al., 2008).

248 Mutations in the promoter and attenuator regions of the chromosomal *ampC* gene of
249 some *E. coli* isolates were found in our study, even associated to ESBL genes. The C→T
250 mutation at position -42, associated with hyperproduction of AmpC beta-lactamase (Mulvey
251 et al., 2005; Caroff et al., 2000), was found in the 57% of our cefoxitin-resistant isolates.
252 The G→A mutation at position -18 was detected in 71% of cefoxitin-resistant isolates;
253 some authors indicate that this mutation could play an important role in the expression of the
254 chromosomal *ampC* gene (Caroff et al., 2000; Smet et al., 2008).

255 Most of the CTX-resistant *E. coli* isolates were also resistant to tetracycline and
256 nalidixic acid (>90%), and a high percentage of them showed resistance to streptomycin
257 (76%) and trimethoprim/sulfamethoxazole (65%). The association of beta-lactam resistance
258 with tetracycline, nalidixic acid, streptomycin and trimethoprim resistance has also been
259 previously reported (Smet et al., 2008). The presence of two amino acid changes in GyrA
260 (S83L and D87N or D87Y or D87G) and one in ParC (S80I) were responsible for the
261 ciprofloxacin resistance detected in eighteen isolates of this study. The substitutions S83L,

262 D87N and S80I were the most frequently detected (88.2%), and these results are similar to
263 those of other reports (Kim et al., 2007).

264 In our study we have detected a high diversity of *E. coli* clones disseminated in the
265 intestinal microbiota of poultry population. Future studies will be performed with the ESBL-
266 positive isolates to find possible associations among serotypes, virulence factors, resistance
267 mechanisms and phylogenetic groups that will be useful for epidemiological research.

268

269 As a conclusion, in this study a high prevalence of ESBLs has been detected in faecal
270 samples of poultry collected at slaughterhouse level in Northern Portugal. TEM-52 beta-
271 lactamase was specially frequent among these isolates. TEM-52 is as well the ESBL of the
272 TEM class more frequently detected in human infections in Portugal (Machado et al., 2007).
273 More studies should be carried out in the future in order to track the evolution of this type of
274 resistance among faecal *E. coli* and to analyse the relationship between human and animal
275 ESBL-containing *E. coli* isolates.

276

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282

283 **Transparency Declarations**

284 None to declare

285

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395

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Table 1. Characteristics of the 34 CTX-resistant *E. coli* isolates of poultry origin recovered from a slaughterhouse in Northern Portugal.

| <i>E. coli</i> isolate | ESBL-test | ESBL detected | REP-PCR pattern ^a | Phylogenetic group | Phenotype of resistance to non-beta-lactams ^a | Antimicrobial resistance genes detected: | Amino acid changes in: | |
|------------------------|-----------|---------------|------------------------------|--------------------|--|---|------------------------|------|
| | | | | | | | GyrA | ParC |
| AV-7.1 | + | CTX-M-14a | 17 | A | STR-TET-NAL | <i>aadA</i> , <i>tet(B)</i> | | |
| AV-13.1A | + | CTX-M-14a | 20 | B ₁ | STR-TET-NAL-CIP-SXT-CHL | <i>aadA</i> , <i>tet(B)</i> , <i>sul1</i> | S83L+D87G | S80I |
| AV-8.1A | + | CTX-M-14a | ND | D | STR-TET-NAL-CIP-SXT-CHL-GEN-TOB | <i>aadA</i> , <i>tet(A)</i> , <i>sul1</i> , <i>sul2</i> , <i>cmlA</i> , <i>aac(3)-II</i> , <i>bla</i> _{TEM-1b} | S83L+D87N | S80I |
| AV-11-1A | + | CTX-M-32 | 18 | B ₁ | STR-TET-NAL-CIP-SXT | <i>aadA</i> , <i>tet(B)</i> , <i>sul2</i> , <i>sul3</i> , | S83L+D87N | S80I |
| AV-12-1B | + | CTX-M-32 | 19 | B ₁ | STR-TET-NAL-CIP-SXT | <i>aadA</i> , <i>tet(A)</i> , <i>tet(B)</i> , <i>sul2</i> | S83L+D87N | S80I |
| AV-1.1A | + | TEM-52 | 8 | B ₁ | STR-TET-NAL-CIP-SXT-CHL | <i>aadA</i> , <i>tet(A)</i> , <i>sul1</i> | S83L+D87N | S80I |
| AV-2.1A | + | TEM-52 | 9 | A | STR-TET-NAL | <i>aadA</i> , <i>tet(A)</i> | | |
| AV-3.1A | + | TEM-52 | 3 | A | STR-TET-NAL | <i>aadA</i> , <i>tet(A)</i> | | |
| AV-6.1A | + | TEM-52 | 2 | B ₁ | TET-NAL-CIP-SXT-CHL | <i>tet(A)</i> , <i>sul1</i> , <i>sul2</i> , <i>cmlA</i> | S83L+D87N | S80I |
| AV-12.1A | + | TEM-52 | 1 | B ₁ | TET-NAL-CIP-SXT | <i>tet(A)</i> , <i>tet(B)</i> , <i>sul2</i> | S83L+D87N | S80I |
| AV-13.1B | + | TEM-52 | 6 | A | STR-TET-NAL-CIP | <i>aadA</i> , <i>tet(B)</i> | S83L+D87N | S80I |
| AV-14.1A | + | TEM-52 | 1 | B ₁ | STR-TET-NAL-CIP-SXT | <i>aadA</i> , <i>tet(A)</i> , <i>sul2</i> , <i>sul3</i> | S83L+D87N | S80I |
| AV-15.1A | + | TEM-52 | 15 | D | STR-TET-NAL-CIP-SXT | <i>aadA</i> , <i>tet(B)</i> , <i>sul2</i> , <i>bla</i> _{TEM-1b} | S83L+D87N | S80I |
| AV-18.1A | + | TEM-52 | 15 | D | STR-TET-NAL-CIP-SXT | <i>aadA</i> , <i>tet(B)</i> , <i>sul2</i> , <i>bla</i> _{TEM-1b} | S83L+D87N | S80I |
| AV-16.1A | + | TEM-52 | 1 | B ₁ | STR-TET-NAL-CIP | <i>aadA</i> , <i>tet(A)</i> | S83L+D87N | S80I |
| AV-19.1A | + | TEM-52 | 12 | A | TET | <i>tet(B)</i> | | |
| AV-20.1A | + | TEM-52 | 12 | A | TET | <i>tet(A)</i> | | |
| AV-21.1A | + | TEM-52 | 6 | A | TET-NAL-SXT | <i>tet(A)</i> , <i>tet(B)</i> , <i>sul1</i> , <i>sul2</i> | | |

| | | | | | | | | |
|----------|---|--------|-----|----------------|--------------------------|---|-----------|------|
| AV-23.1A | + | TEM-52 | 13 | D | STR-TET-NAL-SXT-GEN | <i>tet(B), sul2, aac(3)-II</i> | | |
| AV-24.1A | + | TEM-52 | 7 | D | STR-TET-NAL-CIP-SXT | <i>aadA, tet(B), sul2</i> | S83L+D87N | S80I |
| AV-52.1B | + | TEM-52 | 1 | B ₁ | STR-TET-NAL-CIP-SXT | <i>aadA, tet(B), sul2</i> | S83L+D87N | S80I |
| AV-36.1A | + | TEM-52 | 12a | A | STR-TET-NAL-SXT-GEN-TOB- | <i>aadA, tet(B), sul1, sul2, aac(3)-I, aac(3)-IV</i> | | |
| AV-43.1A | + | TEM-52 | 1 | B ₁ | STR-TET-NAL-CIP-SXT | <i>aadA, tet(A), tet(B), sul2</i> | S83L+D87N | S80I |
| AV-44.1A | + | TEM-52 | ND | A | STR-TET-NAL-CIP-SXT | <i>aadA, tet(A), sul2, sul3</i> | S83L+D87N | S80I |
| AV-50.1A | + | TEM-52 | 4 | B ₁ | STR-TET-NAL | <i>aadA, tet(A)</i> | | |
| AV-66.1A | + | TEM-52 | 14 | D | TET | <i>tet(A)</i> | | |
| AV-69.1A | + | TEM-52 | ND | B ₁ | STR-TET-NAL-SXT | <i>aadA, tet(A), sul1, sul2</i> | | |
| AV-73.1A | + | TEM-52 | ND | A | TET-NAL-CIP | <i>tet(B)</i> | S83L+D87Y | S80I |
| AV-5.1A | + | TEM-52 | 5 | D | STR-TET-NAL-CIP-SXT-CHL | <i>aadA, tet(B), sul2, sul3</i> | S83L+D87N | S80I |
| AV-65.1B | + | TEM-52 | 10 | B ₁ | STR-TET-NAL-SXT | <i>aadA, tet(A), tet(B), sul1, sul2</i> | | |
| AV-48.1A | + | TEM-52 | 11 | B ₁ | NAL | <i>bla_{TEM-1b}</i> | | |
| AV-68.1B | - | none | 16 | B ₁ | STR-TET-NAL-SXT | <i>tet(B), sul2, bla_{TEM-1b}</i> | | |
| AV-75.1A | - | none | 16 | B ₁ | STR-TET-NAL-SXT | <i>aadA, tet(B), sul1, sul2, bla_{TEM-1b}</i> | | |
| AV-9.1A | - | none | 21 | B ₁ | STR-TET-CHL-GEN-TOB | <i>aadA, tet(B), cmlA, aac(3)-II</i> | | |

^a TET, tetracycline; NAL, nalidixic acid; CIP, ciprofloxacin; STR, streptomycin; SXT; trimethoprim/sulfamethoxazole, GEN, gentamicin; TOB, tobramycin; CHL, chloramphenicol; ND: no determined.

Table 2. Mutations in promoter/attenuator region of *ampC* gene in the 7 cefoxitine- and amoxicillin-clavulanic acid-resistant *E. coli* isolates detected in this study^a.

| <i>E. coli</i> | ESBL gene | Mutations in <i>ampC</i> promoter region |
|----------------|-----------|---|
| AV-68.1B | none | -42 (C→T), -18 (G→A), -1 (C→T), +58(C→T) |
| AV-75.1A | none | -42 (C→T), -18 (G→A), -1 (C→T), +58(C→T) |
| AV-9.1A | none | -18 (G→A), -1 (C→T), and +58(C→T) |
| AV-48.1A | TEM-52 | -42 (C→T), -18 (G→A), -1 (C→T), +58(C→T) |
| AV-65.1A | TEM-52 | -42 (C→T), -18 (G→A), -1 (C→T), +58(C→T) |
| AV-5.1A | TEM-52 | No mutations |
| AV-8.1A | CTX-M-14a | No mutations |

^aThe CMY encoding gene was negative by PCR in all these isolates

5 **Caption**

Figure 1. Phylogenetic groups identified in the *E. coli* isolates harbouring different ESBLs

10

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Figure 1

