

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

Daniela Costa, Laura Vinué, Patricia Poeta, Ana Cláudia Coelho, Manuela Matos, Yolanda Sáenz, Sergio Somalo, Myriam Zarazaga, Jorge Rodrigues, Carmen Torres

▶ To cite this version:

Daniela Costa, Laura Vinué, Patricia Poeta, Ana Cláudia Coelho, Manuela Matos, et al.. Prevalence of extended-spectrum beta-lactamase-producing isolates in faecal samples of broilers. Veterinary Microbiology, 2009, 138 (3-4), pp.339. 10.1016/j.vetmic.2009.03.029 . hal-00514603

HAL Id: hal-00514603 https://hal.science/hal-00514603

Submitted on 3 Sep 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

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

Authors: Daniela Costa, Laura Vinué, Patricia Poeta, Ana Cláudia Coelho, Manuela Matos, Yolanda Sáenz, Sergio Somalo, Myriam Zarazaga, Jorge Rodrigues, Carmen Torres



PII:	S0378-1135(09)00171-0
DOI:	doi:10.1016/j.vetmic.2009.03.029
Reference:	VETMIC 4398
To appear in:	VETMIC
Received date:	29-5-2008
Revised date:	14-3-2009
Accepted date:	19-3-2009

Please cite this article as: Costa, D., Vinué, L., Poeta, P., Coelho, A.C., Matos, M., Sáenz, Y., Somalo, S., Zarazaga, M., Rodrigues, J., Torres, C., Prevalence of extended-spectrum beta-lactamase-producing *Escherichia coli* isolates in faecal samples of broilers, *Veterinary Microbiology* (2008), doi:10.1016/j.vetmic.2009.03.029

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

```
Manuscript
```

1	
2	Prevalence of extended-spectrum beta-lactamase-producing Escherichia coli isolates in
3	faecal samples of broilers
4	
5	Daniela Costa ^{a,d} , Laura Vinué ^d , Patricia Poeta ^{a,c} , Ana Cláudia Coelho ^a , Manuela Matos ^b ,
6	Yolanda Sáenz ^{d,e} , Sergio Somalo ^d , Myriam Zarazaga ^{d,e} , Jorge Rodrigues ^{a,c} , and Carmen
7	Torres ^{d,e*}
8	
9	^a Universidade de Trás-os-Montes e Alto Douro, Departamento de Ciências Veterinárias;
10	and ^b Departamento de Genética e Biotecnología/Instituto de Biotecnología e Bioengenharia,
11	Vila Real, Portugal; ^c Centre of Studies of Animal and Veterinary Sciences, Vila Real,
12	Portugal; ^d Área de Bioquímica y Biología Molecular, Universidad de La Rioja, Logroño,
13	^e Centro de Investigación Biomédica de la Rioja, CIBIR, Logroño, Spain.
14	
15	Running title: E. coli harbouring ESBLs in faeces of poultry in Portugal
16	
17	Corresponding author.
18	Carmen Torres
19	Area de Bioquímica y Biología Molecular
20	Universidad de La Rioja
21	Madre de Dios, 51
22	26006 Logroño
23	Spain
24	FAX: 34-941299721
25	Phone: 34-941299750
26	e-mail: carmen.torres@unirioja.es
27	
28	

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 $bla_{\text{TEM-1b}}$. The $bla_{\text{CTX-M-14}}$ 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 two additional isolates. Three of the 34 cefotaxime-resistant isolates (9%) did not produce 41 ESBLs, and two of them presented mutations at positions -42 (C \rightarrow T), -18 (G \rightarrow A), -1 42 43 $(C \rightarrow T)$, and +58($C \rightarrow T$) 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 aac(3)-II or aac(3)-I + aac(3)-IV genes in all 4 gentamicin-resistant isolates. Different 46 47 combinations of sull, 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 intestinal tract of healthy poultry is a reservoir of ESBL-positive *E. coli* isolates. 50

51

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

53

- 54

55 1. Introduction

Escherichia coli is a common inhabitant of intestinal tract of humans and animals (Sørum and Sunde, 2001; Tannock, 1995), and can be easily disseminated in different ecosystems through the food chain and water (Skurnik et al., 2006). In addition, *E. coli* can become a human pathogen, mainly in immunocompromised patients, and beta-lactams are 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 been reported, and those of the CTX-M type represent an emerging problem in human 67 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

Bage 3 of 21

82 information about the real problem of ESBL in food-producing animals, and valuable help

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.

All the samples were seeded on Levine agar plates supplemented with 2 μg/ml of
cefotaxime and were incubated at 37 °C for 24 h. Two colonies per sample with typical *E. coli* morphology were selected and identified by classical biochemical methods (Gram
staining, catalase, oxidase, indol, Methyl-Red-Voges-Proskauer, citrate and urease), and by
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, trimethoprimsulfamethoxazole (SXT), nalidixic acid, ciprofloxacin and chloramphenicol. Broad-105 106 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

The presence of genes encoding TEM, SHV, OXA-1, CTX-M, and CMY beta-113 114 lactamases was studied by PCR in all broad-spectrum cephalosporin resistant isolates using primers and conditions previously reported (Briñas et al., 2003; Jouini et al., 2007). The 115 obtained DNA amplicons were sequenced on both strands and sequences were compared 116 117 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 also amplified by PCR (Briñas et al., 2003), sequenced, and compared with the same region 119 120 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 sull, 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

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

strands were automatically sequenced by the Applied Biosystem 3730 sequencer (Genome Express, France), using the same set of primers as for the PCR reactions. Sequences obtained were compared with those previously reported for *gyrA* (GenBank accession 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.*

A PCR-based DNA fingerprinting technique which uses primers based on the highly 139 140 conserved repetitive extragenic palindromic (REP) repeated DNA element was used for typing 34 CTX-resistant E. coli isolates. This technique was preferentially used to assess the 141 142 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 single final extension of 16 min at 65°C. PCR end-products were separated on agarose 2% 145 146 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 by148 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 of resistance was demonstrated in the two CTX-resistant isolates obtained from the 157 158 remaining two faecal samples and both of them were included in this study. Consequently, a

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

161

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

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

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

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

In two of the tested faecal samples, different ESBLs were identified in the two *E. coli*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 that included antimicrobial agents belonging to at least four different families, and 10% of 189 190 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 sull, 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

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

204

205 *3.4. REP-PCR analysis and phylogenetic groups*

The REP-PCR technique was applied in order to determine the clonal diversity of the 34 CTX-resistant *E. coli* isolates. Twenty-one different profiles were identified in this series (Table 1), and fifteen profiles were demonstrated among the 26 $bla_{\text{TEM-52}}$ -containing 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.

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

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

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

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

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

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

268

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

276

277 Acknowledgements

This work has been supported in part by the project SAF2006-14207-C02-01 from the Ministry of Education and Science of Spain and by Acções Integradas Luso-Espanholas (E-110/06, HP2005-0052). Laura Vinué was supported by a fellowship of the Spanish Ministry of Education and Science (SAF2006-14207-C02-01).

282

283 Transparency Declarations

284 None to declare

285

286 **References**

- Aarestrup, F.M., Hasman, H., Agerso, Y., Jensen, L.B., Harksen, S., Svensmark, B., 2006.
- First description of *bla_{CTX-M-1}*-carrying *Escherichia coli* isolates in Danish primary food
 production. J. Antimicrob. Chemother. 57, 1258-1259.
- 290 Blanc, V., Mesa, R., Saco, M., Lavilla, S., Prats, G., Miró, E., Navarro, F., Cortés, P.,
- Llagostera, M., 2006. ESBL- and plasmidic class C β-Lactamase-producing *E. coli*strains isolated from poultry, pig and rabbit farms. Vet. Microbiol. 118, 299-304.
- 293 Briñas, L., Moreno, M.A., Zarazaga, M., Porrero, C., Sáenz, Y., García, M, Domínguez, L.,
- 294 Torres, C., 2003. Detection of CMY-2, CTX-M-14, and SHV-12 β-lactamases in
- 295 Escherichia coli fecal-sample isolates from healthy chickens. Antimicrob. Agents
- 296 Chemother. 47, 2056-2058.
- 297 Briñas, L., Moreno, M.A., Teshager, T., Sáenz, Y., Porrero, M.C., Domínguez, L., Torres,
- C., 2005. Monitoring and characterization of extended-spectrum β-lactamases in
 Escherichia coli strains from healthy and sick animals in Spain in 2003. Antimicrob.
 Agents Chemother. 49, 1262-1264.
- 301 Cantón, R., Coque, T.M. 2006. The CTX-M β-lactamase pandemic. Curr. Opin. Microbiol.
 302 9, 466–475.
- Caroff, N., Espaze, E., Gautreau, D., Richet, H., Reynaud, A., 2000. Analysis of the effects
 of -42 and -32 *ampC* promoter mutations in clinical isolates of *Escherichia coli*hyperproducing AmpC. J. Antimicrob. Chemother. 45, 783-788.
- 306 Costa, D., Poeta, P., Briñas, L., Sáenz, Y., Rodrígues, J., Torres, C., 2004. Detection of
- 307 CTX-M-1 and TEM-52 β-Lactamases in *Escherichia coli* strains from healthy pets in
 308 Portugal. J. Antimicrob. Chemother. 54, 960-961.
- 309 Costa, D., Poeta, P., Sáenz, Y., Vinué, L., Rojo-Bezares, B., Jouini, A., Zarazaga, M.,
- 310 Rodríguez, J., Torres C., 2006. Detection of Escherichia coli harbouring extended-

- 311 spectrum β-lactamases of the CTX-M, TEM and SHV classes in faecal samples of wild
- animals in Portugal. J. Antimicrob. Chemother. 58, 1311-1312.
- Clermont, O., Bonacorsi, S., Bingen, E., 2000. Rapid and simple determination of the
 Escherichia coli phylogenetic group. Appl. Environ. Microbiol. 66, 4555-4558.
- 315 Clinical and Laboratory Standards Institute. Performance standards for antimicrobial
- 316 susceptibility testing: Seventeenth Informational Supplement Approved standard M100-
- 317 S17. CLSI, Wayne, PA, USA, 2007.
- 318 Duan, R.S., Sit, T.H., Wong, S.S., Wong, R.C., Chow, K.H., Mak, G.C., Yam, W.C., Ng,
- 319 L.T., Yuen, K.Y., Ho, P.L., 2006. Escherichia coli producing CTX-M beta-lactamases
- in food animals in Hong Kong. Microb. Drug. Resist. 12, 145-148.
- 321 Girlich, D., Poirel, L., Carattoli, A., Kempf, I., Lartigue, M.F., Bertini, A., Nordmann, P.,
- 322 2007. Extended-spectrum-betalactamse CTX-M-1 in *Escherichia coli* in healthy poultry
 323 in France. Appl. Environ. Microbiol. 73, 4681-4685.
- Jouini, A., Vinué, L., Slama, K.B., Sáenz, Y., Klibi, N., Hammami, S., Boudabous, A.,
- 325 Torres, C., 2007. Characterization of CTX-M and SHV extended-spectrum beta-326 lactamases and associated resistance genes in *Escherichia coli* strains of food samples in
- 327 Tunisia. J. Antomicrob. Chemother. 60, 1137-41.
- Kim, T,E,, Jeong, Y,W,, Cho, S,H,, Kim, S,J,, Kwon, H,J., 2007. Chronological study of
 antibiotic resistances and their relevant genes in Korean avian pathogenic *Escherichia coli* isolates. J. Clin. Microbiol. 45, 3309-15.
- 331 Kojima, A., Ishii, Y., Ishihara, K., Esaki, H., Asai, T., Oda, C., Tamura, Y., Takahashi, T.,
- 332 Yamaguchi, K., 2005. Extended-spectrum-β-lactamase-producing Escherichia coli
- 333 strains isolated from farm animals from 1999 to 2002: report from the Japanese
- 334 Veterinary Antimicrobial Resistance Monitoring Program. Antimicrob. Agents
- 335 Chemother. 49, 3533-3537.
- 336 Liu, J.H., Wei, S.Y., Ma, J.Y., Zeng, Z.L., Lü, D.H., Yang, G.X., Chen, Z.L., 2007.
- 337 Detection and characterization of CTX-M and CMY-2 β-lactamases among *Escherichia*

- 338 *coli* isolates from farm animals in Guangdong Province of China. Int. J. Antimicrob.
- Agents. 29, 576-581.
- 340 Livermore, D.M., 2008. Defining an extended-spectrum beta-lactamase. Clin Microbiol
 341 Infect. 14 Suppl 1,3-10.
- 342 Machado, E., Coque, T.M., Cantón, R., Sousa, J.C., Peixe. L., 2008. Antibiotic resistance
- 343 integrons and extended-spectrum beta-lactamases among Enterobacteriaceae isolates
- recovered from chickens and swine in Portugal. J. Antimicrob. Chemother. 62, 296-302.
- 345 Machado, E., Coque, T.M., Cantón, R., et al., 2007. High diversity of extended-spectrum β-
- 346 lactamases (ESBL) among clinical isolates of *Enterobacteriaceae* from Portugal. J.
- 347 Antimicrob. Chemother. 60, 1370-4.
- 348 Miró, E., Mirelis, B., Navarro, F., Rivera, A., Mesa, R.J., Roig, M.C., Gómez, L., Coll, P.,
- 349 2005. Surveillance of extended-spectrum β-Lactamases from clinical samples and faecal
 350 carries in Barcelona, Spain. J. Antimicrob. Chemother. 56, 1152-1155.
- 351 Moreno, M.A., Teshager, T., Porrero, M.C., García, M., Escudero, E., Torres, C.,
- 352 Domínguez, L., 2007. Abundance and phenotypic diversity of *Escherichia coli* isolates
- 353 with diminished susceptibility to expanded-spectrum cephalosporins in faeces from
- healthy food animals after slaughter. Vet. Microbiol. 120, 363-369.
- 355 Moubareck, C, Daoud, Z., Hakimé, N.I., Hamzé, M., Mangeney, N., Matta, H., Mokhbat,
- 356 J.E., Rohbanm R., Sarkism D.K., Doucet-Populaire, F., 2005. Countrywide spread of
- 357 community- and hospital-acquired extended-spectrum beta-lactamase (CTX-M-15)-
- producing Enterobacteriaceae in Lebanon. J Clin Microbiol. 43, 3309-3313.
- 359 Mulvey, M.R., Bryce, E., Boyd, D.A., Ofner-Agostini, M., Land, A.M., Simor, A.E., Paton,
- S., 2005. Molecular characterization of cefoxitin-resistant *Escherichia coli* from
 Canadian hospitals. Antimicrob Agents Chemother. 49, 358-65.
- 362 Pallecchi, L., Bartoloni, A., Fiorelli, C., Mantella, A., Di Maggio, T., Gamboa, H., Gotuzzo,
- 363 E., Kronvall, G., Paradisi, F., Rossolini, G.M., 2007. Rapid dissemination and diversity

- 364 of CTX-M extended-spectrum beta-lactamase genes in commensal Escherichia coli
- 365 isolates from healthy children from low-resource settings in Latin America. Antimicrob.
- 366 Agents Chemother. 51, 2720-2725.
- 367 Paterson, D.L., Bonomo, R.A., 2005. Extended-spectrum beta-lactamases: a clinical update.
- 368 Clin. Microbiol. Rev. 18, 657-686.
- Rossolini, G. M., D'Andrea, M.M., Mugnaioli, C., 2008. The spread of CTX-M-type
 extended-spectrum beta-lactamases. Clin. Microbiol. Infect. 14, 33-41.
- 371 Sáenz, Y., Briñas, L., Domínguez, E., Ruiz, J., Zarazaga, M., Vila, J., Torres, C., 2004.
- 372 Mechanisms of resistance in multiple-antibiotic-resistant Escherichia coli strains of
- human, animal, and food origins. Antimicrob. Agents Chemother. 48, 3996-4001.
- Sáenz, Y., Zarazaga, M., Briñas, L., Ruiz-Larrea, F., Torres, C., 2003. Mutations in *gyrA*and *parC* genes in nalidixic acid-resistant *Escherichia coli* strains from food products,
 humans and animals. J. Antimicrob. Chemother. 51, 1001-1005.
- 377 Skurnik, D., Ruimy, R., Andremont, A., Amorin, C., Rouquet, P., Picard, B., Denamur, E.,
- 378 2006. Effect of human vicinity on antimicrobial resistance and integrons in animal
 379 faecal *Escherichia coli*. J. Antimicrob. Chemother. 57, 1215-1219.
- 380 Smet, A., Martel, A., Persoons, D., Dewulf, J., Heyndrickx, M., Catry, B., Herman, L.,
- 381 Haesebrouck, F., Butaye, P., 2008. Diversity of extended-spectrum beta-lactamases and
- class C beta-lactamases among cloacal *Escherichia coli* isolates in Belgian broiler
 farms. Antimicrob. Agents Chemother. 52, 1238-1243.
- 384 Sørum, H., Sunde, M., 2001. Resistance to antibiotics in the normal flora of animals. Vet.
 385 Res. 32, 227-241.
- 386 Tannock, G.W., 1995. Normal microflora. An introduction to microbes inhabiting the387 human body. Chapman and Hall, London.
- 388 Valverde, A., Coque, T.M., Sánchez-Moreno, M.P., Rollán, A., Baquero, F., Cantón, R.,
- 389 2004. Dramatic increase in prevalence of fecal carriage of extended-spectrum β -

- 390 Lactamase-producing *Enterobacteriaceae* during nonoutbreak situations in Spain. J.
- 391 Clin. Microbiol. 42, 4769-4775.
- 392 Vinué, L., Sáenz, Y., Martínez, S., Somalo, S., Moreno, M.A., Torres, C., Zarazaga, M.,
- 393 2009. High prevalence and diversity of Extended-Spectrum Beta-Lactamases in faecal
- 394 *Escherichia coli* isolates of healthy humans. Clin. Microbiol. Infect. (in press)
- 395

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	А	STR-TET-NAL	aadA, tet(B)		
AV-13.1A	+	CTX-M-14a	20	\mathbf{B}_1	STR-TET-NAL-CIP-SXT-CHL	aadA, tet(B), sull	S83L+D87G	S80I
AV-8.1A	+	CTX-M-14a	ND	D	STR-TET-NAL-CIP-SXT-CHL-GEN-TOB	aadA, tet(A), sul1, sul2, cmlA, aac(3)-II, bla _{TEM-1b}	S83L+D87N	S80I
AV-11-1A	+	СТХ-М-32	18	B_1	STR-TET-NAL-CIP-SXT	aadA, tet(B), sul2, sul3,	S83L+D87N	S80I
AV-12-1B	+	СТХ-М-32	19	\mathbf{B}_1	STR-TET-NAL-CIP-SXT	aadA, tet(A), tet(B), sul2	S83L+D87N	S80I
AV-1.1A	+	TEM-52	8	\mathbf{B}_1	STR-TET-NAL-CIP-SXT-CHL	aadA, tet(A), sull	S83L+D87N	S80I
AV-2.1A	+	TEM-52	9	А	STR-TET-NAL	aadA, tet(A)		
AV-3.1A	+	TEM-52	3	А	STR-TET-NAL	aadA, tet(A)		
AV-6.1A	+	TEM-52	2	\mathbf{B}_1	TET-NAL-CIP-SXT-CHL	tet(A), sul1, sul2, cmlA	S83L+D87N	S80I
AV-12.1A	+	TEM-52	1	B ₁	TET-NAL-CIP-SXT	tet(A), tet(B), sul2	S83L+D87N	S80I
AV-13.1B	+	TEM-52	6	А	STR-TET-NAL-CIP	aadA, tet(B)	S83L+D87N	S80I
AV-14.1A	+	TEM-52	1	B ₁	STR-TET-NAL-CIP-SXT	aadA, tet(A), sul2, sul3	S83L+D87N	S80I
AV-15.1A	+	TEM-52	15	D	STR-TET-NAL-CIP-SXT	aadA, tet(B), sul2, bla _{TEM-1b}	S83L+D87N	S80I
AV-18.1A	+	TEM-52	15	D	STR-TET-NAL-CIP-SXT	aadA, tet(B), sul2, bla _{TEM-1b}	S83L+D87N	S80I
AV-16.1A	+	TEM-52	1	\mathbf{B}_1	STR-TET-NAL-CIP	aadA, tet(A)	S83L+D87N	S80I
AV-19.1A	+	TEM-52	12	А	TET	tet(B)		
AV-20.1A	+	TEM-52	12	А	TET	tet(A)		
AV-21.1A	+	TEM-52	6	А	TET-NAL-SXT	tet(A), tet(B), sul1, sul2		

AV-23.1A	+	TEM-52	13	D	STR-TET-NAL-SXT-GEN	tet(B), sul2, aac(3)-II		
AV-24.1A	+	TEM-52	7	D	STR-TET-NAL-CIP-SXT	aadA, tet(B), sul2	S83L+D87N	S80I
AV-52.1B	+	TEM-52	1	B_1	STR-TET-NAL-CIP-SXT	aadA, tet(B), sul2	S83L+D87N	S80I
AV-36.1A	+	TEM-52	12a	А	STR-TET-NAL-SXT-GEN-TOB-	aadA, tet(B), sul1, sul2, aac(3)-I, aac(3)-IV		
AV-43.1A	+	TEM-52	1	B_1	STR-TET-NAL-CIP-SXT	aadA, tet(A), tet(B), sul2	S83L+D87N	S80I
AV-44.1A	+	TEM-52	ND	А	STR-TET-NAL-CIP-SXT	aadA, tet(A), sul2, sul3	S83L+D87N	S80I
AV-50.1A	+	TEM-52	4	B_1	STR-TET-NAL	aadA, tet(A)		
AV-66.1A	+	TEM-52	14	D	ТЕТ	<i>tet</i> (A)		
AV-69.1A	+	TEM-52	ND	B_1	STR-TET-NAL-SXT	aadA, tet(A), sul1, sul2		
AV-73.1A	+	TEM-52	ND	А	TET-NAL-CIP	<i>tet</i> (B)	S83L+D87Y	S80I
AV-5.1A	+	TEM-52	5	D	STR-TET-NAL-CIP-SXT-CHL	aadA, tet(B), sul2, sul3	S83L+D87N	S80I
AV-65.1B	+	TEM-52	10	B ₁	STR-TET-NAL-SXT	aadA, tet(A), tet(B), sul1, sul2		
AV-48.1A	+	TEM-52	11	B ₁	NAL	bla _{TEM-1b}		
AV-68.1B	-	none	16	B ₁	STR-TET-NAL-SXT	<i>tet</i> (B), <i>sul2</i> , <i>bla</i> _{TEM-1b}		
AV-75.1A	-	none	16	B ₁	STR-TET-NAL-SXT	aadA, tet(B), sul1, sul2, bla _{TEM-1b}		
AV-9.1A	-	none	21	B_1	STR-TET-CHL-GEN-TOB	aadA, tet(B), cmlA, aac(3)-II		

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

determined.

CCEPTED JUSCRI PT

E. coli	ESBL gene	Mutations in <i>ampC</i> promoter region
AV-68.1B	none	-42 (C \rightarrow T), -18 (G \rightarrow A), -1 (C \rightarrow T), +58(C \rightarrow T)
AV-75.1A	none	-42 (C→T), -18 (G→A), -1 (C→T), +58(C→T)
AV-9.1A	none	-18 (G \rightarrow A), -1 (C \rightarrow T), and +58(C \rightarrow 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

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.}

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

-ga.

5 Caption

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

Solo S

10

Figure 1



Phylogenetic group: **■** A **■** B1 **□** D