Impact of the use of beta-lactam antimicrobials on the emergence of *Escherichia coli* resistant to cephalosporins under standard pig rearing conditions

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Running title: Emergence of cephalosporin resistant *E. coli*

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Keywords: Ceftiofur, amoxicillin, betalactams, *E. coli*, pigs

Abstract

The aim of this study was to evaluate if the treatments with ceftiofur and amoxicillin are risk factors for the emergence of cephalosporin resistant (CR) *E. coli* in a pig farm during the rearing period. 100 seven-day-old piglets were divided into two groups, control (n=50) and parenterally treated with ceftiofur (n=50). During the fattening period, both groups were subdivided in two. A second treatment with amoxicillin was administered in-feed to two of the groups; group 1 (untreated, n=20), group 2 (treated with amoxicillin, n=26), group 3 (treated with ceftiofur, n=20) and group 4 (treated with ceftiofur and amoxicillin, n=26). During treatment with ceftiofur faecal samples were collected before treatment (day 0) and at days 2, 7, 14, 21 and 42 post-treatment, whereas with amoxicillin, the sampling was extended 73 days post-treatment. CR *E. coli* were selected on MacConkey agar with ceftriaxone (1mg/L). PFGE, minimal inhibitory concentration to 14 antimicrobials, presence of cephalosporin resistance genes and replicon typing of plasmids were analyzed. Both treatments generated an increase in the prevalence of CR *E. coli*, which was statistically significant in the treated group. Resistance diminished after treatment. A total of 47 CR *E. coli* were recovered during the study period, 15 contained $bla_{\text{CTX-M-1}}$, 10 $bla_{\text{CTX-M-14}}$, four $bla_{\text{CTX-M-9}}$, two $bla_{\text{CTX-M-15}}$ and five $bla_{\text{SHV-12}}$. By the finishing time, all animals were negative for CR *E. coli*. The treatment with ceftiofur and amoxicillin was associated to the emergence of CR *E. coli*, however did not pose enough selective pressure to select for long-term resistant organisms.

Introduction

The major issues of veterinary practitioners when treating a large population of animals are to maximize the likelihood of a favourable clinical outcome at the population level, and to minimize the emergence and development of antimicrobial resistance that could compromise future treatments. When new antimicrobials are introduced on the market, resistant bacteria for which the active substance used to show an excellent activity could quickly emerge. This process should be considered a natural phenomenon, since the antimicrobial would be the factor that determines which strain of each bacterium will be the most frequent under the new selective pressure. It must be highlighted that antimicrobial resistance genes could be present in bacteria before the availability of these drugs (1). Thus, antimicrobial agents are probably only changing the population dynamics; susceptible versus resistant bacteria. It is a known fact that antimicrobial resistance could pose a serious risk for human and animal health; and in general, emergence of resistance reduces the effectiveness of these drugs.

During the last decade, resistance to extended spectrum beta-lactams, especially third- and fourth-generation cephalosporins and penems has raised the concern of the scientific community. The World Health Organization has defined third- and fourth-generation cephalosporins as being "critically important" for use in humans (http://www.who.int/ foodborne_disease/resistance/cia/en/index.html), since the increased presence of resistance to these antimicrobials could seriously compromise the treatment of some life threatening infections, including bacteraemia and meningitis.

A third-generation cephalosporin, ceftiofur, and a fourth-generation cephalosporin, cefquinome, have been developed strictly for veterinary use (2). Ceftiofur is widely used in many different food animals to treat respiratory diseases. Cefquinome can also be used for the treatment of mastitis metritis agalaxia syndrome in sows, exudative epidermitis, and meningitis (3). The systemic use of cephalosporins in food animals that could potentially select for resistance organisms is worrisome due to the role that food producing animals may play in the spread of extended spectrum cephalosporins into the community.

Previous studies have demonstrated statistically significant association between the use of ceftiofur and reduced susceptibility to third generation cephalosporins in *Escherichia coli* (4, 5). However, they did not find association between ceftiofur usage and presence of ESBL genes ($bla_{\text{CTX-M}}$) and more importantly, none of these studies have examined other drug-use practice that can cross- or co-select for cephalosporin resistance. To our understanding, there is a lack of comprehensive studies performed under standard pig rearing conditions, analysing the presence and factors that can contribute to both, emergence and increase in occurrence of CR *E. coli* in pig farms.

For this reason, this study intends to evaluate if the treatments with two different beta-lactams, ceftiofur and amoxicillin, are risk factors associated to the emergence of CR *E. coli* during two stages (preweaning-growing and finishing) of the rearing period, and assess if there is enough selective pressure to maintain resistant strains during the life-time of the animals.

Materials and Methods

Study design

This study was conducted on a conventional commercial pig farm in the northeast of Spain. During the six months previous to the study the site remained depopulated, cleaned and disinfected with standard operation procedures under field conditions. Sixty eight sows were housed in the climate control house, and faecal samples were collected to examine the presence of CR *E. coli*. After farrowing, a total of 100 seven-day-old piglets from 10 different sows were spatially divided into two groups: untreated control (n=50) and parenterally treated (n=50) with ceftiofur (5 mg/Kg of body weight in one shot) following the summary of product characteristics of a commercial presentation (Naxcel®, Zoetis SLU). Three animals from the control group died of non infectious causes during the course of the study. Faecal samples were taken manually from the rectum of piglets in six occasions; before treatment (day 0) and at days 2, 7, 14, 21 and 42 post-treatment (Table 1).

During the fattening period (day 70), each of the previous groups was subdivided into two (Table 2). A treatment with amoxicillin (Maymoxi®, Laboratorios Maymó) was administered in feed for 14 days to two of the new four groups (10 mg/kg of body weight/day). At that point in time, there were a total of four groups: untreated control group, or animals that did not receive any treatment with beta-lactams (n=20); group 2, animals orally treated with amoxicillin during finishing (n=26); group 3, animals parenterally treated with ceftiofur during preweaning (n=20) and group 4, animals treated with ceftiofur and amoxicillin (n=26). The four groups remained spatially separated until their departure to the abattoir. Faecal samples were taken from all

115 animals before administration of amoxicillin (day 0) and on days 2, 7, 14, 21 and 45 and
116 73 post-treatment. A final sampling was performed at slaughter time. During the course
117 of the study, farm biosecurity was extreme. Animals of different groups were spatially
118 separated in designated pens to avoid contact. Overboots were used and replaced at the
119 entrance of each pen. Sampling was always initiated from the control group to the
120 treated group to minimize transmission of resistant bacteria from pen to pen.

121

122 The study was performed in a commercial farm where the treatments, housing and
123 husbandry conditions were conformed to the European Union (EU) Guidelines. In
124 particular, the medicinal product used in this study (Naxcel®) is EU registered
125 (EU/2/05/053/001), and it was used according to veterinary rules without any additional
126 requirement. Thus, it was not necessary to comply with ethical standards and approvals
127 to carry out this experimental work, since it did not require any invasive procedures
128 (only collection of faecal samples), or management other than the field standards
129 protocols set by the company.

130

131 *E. coli* isolation and identification

132 Faecal samples were transported to the laboratory at 4ºC on the same day of sampling.
133 Samples were homogenised and 1 g of faeces was dissolved in 10 ml of MacConkey
134 broth supplemented with ceftriaxone (1 mg/ml). After overnight enrichment at 37 ^{o}C,
135 10µl were plated onto MacConkey agar with ceftriaxone (1 mg/ml). Three colonies for
136 each plate were stored and one was confirmed as *E. coli* by Vitek-2 (Biomerieux) and
137 further characterized.

Pulsed field gel electrophoresis and phylotyping

To assess the clonality of the strains and the epidemiological relatedness, all isolates were analyzed for genetic relatedness by PFGE using *XbaI* according to the CDC PulseNet protocol (6). The *Salmonella* Braenderup H9812 strain was used as molecular standard. PFGE profiles were compared using Fingerprinting II Informatixe software (Applied Maths, Sint-Martens-Latem, Belgium). Isolates were considered to have a unique pattern when at least one band difference was detected. The analysis of the bands generated was performed using the Dice coefficient and unweighted pair group method with arithmetic averages (optimization of 1.5% and position tolerance 1.5%).

The isolates were discriminated in pyhlogenetic groups (A, B1, B2, C, D and E) according to the method previously described by Clermont *et al*. (7, 8)

Antimicrobial susceptibility testing

Disc diffusion was performed according to CLSI guidelines using the following discs (Oxoid, UK): cefoxitin, 30 mg; cefepime, 30 mg; ceftazidime, 30 mg; cefotaxime, 30 mg; cefotaxime+clavulanic acid, 30+10 mg; and ceftazidime+clavulanic acid, 30+10 mg. The disc combinations of cefotaxime and cefotaxime/clavulanic acid, ceftazidime and ceftazidime/clavulanic acid were used for the identification of ESBLs; cefoxitin was used for the detection of *ampC*-type beta-lactamase (9). Minimum inhibitory concentration (MIC) against ampicillin, ciprofloxacin, nalidixic acid, gentamicin, streptomycin, tetracycline, florfenicol, colistin sulphate, sulphametoxazole, trimethoprim, chloramphenicol, kanamycin, cefotaxime and ceftazidime was determined by microdilution methods (VetMIC GN-mo, National Veterinary Institute,

Uppsala, Sweden). Results were interpreted as epidemiological cut-off values following EUCAST recommendations.

Detection of resistance genes

Resistance to third-generation cephalosporins was analysed by PCR for the presence of the *bla*$_{TEM}$, *bla*$_{CTX}$, *bla*$_{CMY-1}$, *bla*$_{CMY-2}$ and *bla*$_{SHV}$, genes as described previously (10). Detection of plasmid-mediated AmpC beta-lactamase genes was assessed by multiplex PCR (11). Sequence analysis was performed using Vector NTI advance 11 (InforMax, Inc., Bethesda, MD). The amplified nucleotide sequences were compared to previously described sequences obtained from public databases (www.ncbi.nlm.nih.gov, http://www.lahey.org/Studies/).

Mating experiments and plasmid characterization

Filter mating experiments were performed to assess the capacity of the plasmids to conjugate. For this analysis, fourteen strains containing ESBL genes were selected. They comprised representative isolates from five PFGE clusters and nine PFGE types. Mating assays were performed as described elsewhere (12), using the strains as donors and rifampicin-resistant *E. coli* HB101 as recipient. Transconjugants were selected on LB agar plates containing rifampicin (50 mg/L) and ceftriaxone (1mg/L) and were confirmed by PFGE.

Plasmidic DNA was purified from these 14 wild-type isolates and later from transformants using a Qiagen Plasmid Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Plasmids were introduced to electrocompetent plasmid-free *E. coli* cells by electroporation. Transformants were selected in brain heart infusion agar supplemented with ceftriaxone (1mg/L) and PCR for confirmation of the cephalosporin resistant genes was performed. The presence of a unique plasmid in the transformants and their sizes were determined using S1-PFGE (13). Finally, plasmids were classified by PCR-based replicon typing (14). Additionally, susceptibility testing was performed in all transformants to assess transferability of resistance genes unrelated to cephalosporins.

Results

Emergence of cephalosporin resistance during treatment

All 68 sows were negative for CR *E. coli*. However, before administration of ceftiofur, five and seven of the seven-day-old piglets among the control and the treated groups respectively, yielded CR *E. coli* (Table 1). During this first treatment, a total of 12 (4.1%) and 23 (8%) CR *E. coli* were isolated from the control (n=288 samples) and the treated group (n=300 samples), respectively. The difference in the proportion of CR *E. coli* recovered in the two groups was statistically significant (p=0.04). The highest percentage of samples positive for CR *E. coli* was obtained within the treated group (22%), 48 hours post-treatment, showing a statistical tendency (p=0.1) when compared to the corresponding figure (10%) of the control group.

206 A total of 552 faecal swabs were collected during the second part of the study when
207 animals were treated with amoxicillin in-feed (Table 2). Previously to the treatment
208 with amoxicillin, all animals were negative for CR *E. coli*. Two, seven, one and one CR
209 *E. coli* were recovered from group 2 (treated only with amoxicillin) after 2, 7, 14 and 45
210 days post-treatment, respectively. One extra isolate was obtained from group 4 (treated
211 with ceftiofur and amoxicillin) after 21 days post-treatment. No other positive samples
212 were obtained in the rest of the groups during the study period. The highest percentage
213 of samples positive for CR *E. coli* (27%) was obtained after seven days of amoxicillin
214 treatment, within the group treated with amoxicillin and with no previous history of
215 ceftiofur use. Significant differences were observed (Fisher test, p=0.02) between the
216 proportion of CR *E. coli* isolated from animals treated with amoxicillin and the rest of
217 the groups after seven days of treatment. By the finishing time, all animals were
218 negative for CR *E. coli*.

219

220 PFGE and phylogenetic analysis

221 Electrophoresis of *Xba*I-digested genomic DNA from the 47 CR *E. coli* isolates
222 revealed 22 different profiles (Fig. 1). *Xba*I profiles typically had 14 to 21 restriction
223 fragments between 20 and 1135 kb (Fig. 1). Indistinguishable fingerprints were present
224 in isolates from different animals, and also in isolates obtained from the same animal at
225 different sampling times (annex 1), indicating the persistence of clones during the
226 course of the treatment. Interestingly, none of the clones obtained during the treatment
227 with ceftiofur were recovered during treatment with amoxicillin. Additionally, 10 out of
228 12 isolates recovered during amoxicillin treatment presented identical PFGE pattern. A

229 total of 66%, 25%, 4% and 4% belonged to the phylogroups A, B1, C and E,
230 respectively.

231

232 MIC determination

233 All 47 CR *E. coli* isolates (Figure 1) were resistant to ampicillin (WT ≤ 8mg/L) and
234 cefotaxime, (WT ≤ 0.25 mg/L) and all but four (belonging to the ceftiofur study) were
235 resistant to ceftazidime (WT ≤ 0.5 mg/L). Regarding the remaining antimicrobial
236 families tested (tetracyclines, sulphamides, trimethoprim, aminoglycosides, quinolones,
237 phenicols and polymyxins), all isolates but two were multiresistant (15), ranging from
238 resistance to three families of antimicrobials to resistance to six. MIC differences were
239 detected among isolates according to treatment and sow. Higher levels of resistance
240 were found during the ceftiofur treatment against phenicols (both, chloramphenicol
241 (WT ≤ 16 mg/L) and florfenicol (WT ≤ 16 mg/L)) and gentamicin (WT ≤ 2 mg/L) when
242 compared to the amoxicillin treatment, whereas levels of resistance were lower against
243 ciprofloxacin (WT ≤ 0.064 mg/L), nalidixic acid (WT ≤ 16 mg/L), trimethoprim (WT ≤
244 2 mg/L), and kanamycin (WT ≤ 8 mg/L). Litter from sow number 25 had all 10 positive
245 CR isolates but one with the same resistance phenotype (beta-lactams – quinolones -
246 trimetophim), whereas the remaining isolates obtained from the rest of the sows
247 exhibited higher diversity of resistance traits. One isolate was resistant to colistin (WT ≤
248 2 mg/L).

249

250 Detection of genes responsible for ESBL resistance

ESBL genes were detected in 36 of these 47 CR *E. coli* strains, and in most cases were combined with the $bla_{\text{TEM-1}}$ gene. Fifteen strains were confirmed to contained $bla_{\text{CTX-M-1}}$, 10 $bla_{\text{CTX-M-14}}$, four $bla_{\text{CTX-M-9}}$, two $bla_{\text{CTX-M-15}}$ and five $bla_{\text{SHV-12}}$. Four isolates were resistant to cefoxitin and the genotype could not be determined. Seven isolates with MIC 0.5 mg/L and 2 mg/L for cefotaxime and ceftazidime respectively, were negative for all PCRs tested, suggesting low susceptibility to cephalosporins probably by upregulation of the AmpC promoter.

Conjugation and transformation

Eight of the 14 selected strains were able to transfer the cephalosporin resistant genes by conjugation. Additionally, 11 out of 14 strains transferred cephalosporin resistant genes to the electrocompetent strain. The 11 transformants together with the three transconjugants resulting from the wild-type strains were subjected to S1-nuclease, and the presence of one unique plasmid was confirmed. Sizes of plasmids varied between aprox. 33.4 Kb and 173.4 Kb (Table 3). IncI1 was the most common replicon followed by IncN. Four of the strains presented two different replicons on the same plasmid, and no replicons were detected in one of the transformants.

The transformants/transconjugants were also resistant to streptomycin (n=10), tetracycline (n=9), sulphamethosaxole (n=8), trimethoprim (n=4), ciprofloxacin (n=2), and kanamycin (n=1).

Discussion

Cephalosporin resistant *E. coli* strains were found in samples from seven-day old piglets prior receiving any medication. Moreover, we could not detect them from the sows despite using an enrichment step for isolation of the specific resistance trait. The high clonality of the isolates demonstrated by PFGE does not plead for a vertical transmission, but rather for multiple acquisitions of strains with limited colonization properties, perhaps from an external origin (personnel working at the farm, food source, presence of rodents or other vectors). Other studies have also detected high diversity of CR strains in newborn piglets (16). Further identification of the possible sources of contamination to prevent the entrance of resistant bacteria into the farm should be carried out. On the other hand, in some cases, PFGE results suggest that some of the clones were shared among piglets of the same pen (like the litters from sows numbers 25 and 11, see annex 1), indicating a common source within the pen. Perhaps the mothers were colonized with CR *E. coli* but the limitation of the bacteriological techniques did not allow their detection. Hence, the farm was cleaned and depopulated during the six months previous to the study; incorrect cleaning and disinfection of the premises may play a role in the persistence of these organisms. Since environmental samples of the barn were not taken prior the study, this option cannot be ruled out. Thus, a further visit to this farm, after one year of finishing this trial and applying a cleaning and disinfection protocol, demonstrated the presence of CR *E. coli* in the environment with a different PFGE profile to the ones isolated from faeces (data not shown).

After 48 hours of the parenteral treatment with ceftiofur, an increase in the prevalence of CR *E. coli* was detected. These levels decreased after the first week of treatment. In the case of in-feed amoxicillin treatment, similar increase was observed after seven days

298 of treatment. In the last visit, prior departure to the abattoir, all the animals were
299 negative for CR *E. coli*. Results from this study are in agreement with other studies
300 performed in calves (17, 18), in which CR *E. coli* emerged for short time while
301 treatment was in course, and diminished shortly after treatment. Perhaps the resistant
302 population could not compete well with the sensitive population after withdrawal of the
303 antibiotic (17). However, during treatment with beta-lactam antimicrobials, animal
304 faeces could become a source of resistant bacteria. Biosecurity measures should be
305 undertaken during treatment, such as faeces removal or isolation of animals under
306 medication to avoid transfer of resistance. Additionally, farmers are at potential risk of
307 contamination during exposure to animals shedding CR bacteria. Studies have
308 demonstrated that ESBL genes and plasmids obtained from *E. coli* of farmers, exhibited
309 genetic similarity to those obtained from *E. coli* isolated from animals belonging to their
310 farms (19).

311

312 It is interesting to notice the emergence of new clones of CR *E. coli* within the group
313 treated only with amoxicillin and without previous history of ceftiofur use. One would
314 expect that the second treatment would re-select the same clones that emerged during
315 the first treatment, but that was not the case. Out of 12 CR *E. coli* isolates obtained
316 during the second treatment, nine belonged to the same clonal group by PFGE and
317 presented the same cephalosporin resistance genes, $bla_{\text{CTX-M-14}}$; whereas the two extra
318 isolates clustered alone and contained $bla_{\text{CTX-M-1}}$ and $bla_{\text{SHV-12}}$. A recent study by
319 Hansen *et al* (16) has described a reduction in the diversity of CR strains during the life
320 cycle, which is at a first glance what our results suggest. However, these conclusions
321 cannot be drawn based on our study, since one unique CR *E. coli* isolate per animal has

been characterized and therefore, some of the resistant isolates from the first application of the drug could have been missed in the second round of sampling.

It appears that both treatments with beta-lactams have selected for a wide range of cephalosporin resistance genes from different families, and these genes were recovered during both treatments. Previous studies analyzing the presence of cephalosporin resistance genes in pig farms in Spain, described the presence of different *bla* genes with SHV-12 being the most frequent (20), a completely different picture to other European countries where SHV-12 is associated to human infections (21). Results from this study have shown the co-existence of many different resistant genes within one farm. The most frequent CTX-M variants in ESBL producers in animals and food of animal origin are currently CTX-M-1 and CTX-M-14, while CTX-M-15 ESBL-producing *E. coli* have only exceptionally been observed in the veterinary context (22). However, this study has demonstrated the presence of CTX-M-15 genes in healthy pigs harboured in high molecular weight plasmids of aprox. 150 Kb containing two replicons; FIA and FIB. Are we seeing a similar change in the evolution of resistance than we have perceived in the human side (23), where it was a shift in occurrence from CTX-M-14 and CTX-M1 towards CTX-M15?

Specific bacterial strains are more successful at colonizing individual animals. That appears to be the case of one animal (number 29) shedding the same resistant clone during the first 20 days of life. Even though CR *E. coli* were isolated from this same animal at mature age (138 days), PFGE demonstrated the presence of a different clone; however, in all cases the isolates yielded $bla_{\text{CTX-M-1}}$. Transformation experiments and

346 replicon typing revealed the presence of a great variety of plasmids of many different
347 sizes harbouring the same resistant genes, with the most common replicons being IncI1
348 and IncN. However, further studies should be performed at the animal level and at the
349 farm level to assess both, the occurrence and spread of plasmids within the pig bacterial
350 population in a particular farm, and the persistence and transmission of these plasmids
351 from herd to herd.

352

353 Additionally, CR *E. coli* recovered during the course of the study were phenotypically
354 resistant to different families of antimicrobials and half of them were resistant to
355 ciprofloxacin, even though fluoroquinolones were never used to treat these animals.
356 Although fluoroquinolone resistant is mostly conferred via gyrA/parC mutation in the
357 bacterial chromosome, two of the transformants exhibited resistance to
358 fluoroquinolones. Plasmid mediated quinolone resistance has been in some cases
359 associated to the same plasmids as those harbouring cephalosporin resistance genes
360 (24). Furthermore, as demonstrated by the phenotype of the transformants exhibiting
361 resistance to several antimicrobial families, co-selection by plasmids bearing resistance
362 genes for different antimicrobial families probably plays an important role in the
363 maintenance of resistance mechanisms. In depth studies should be performed to avoid
364 the transmission of these resistance genes from farm to fork, since several studies have
365 demonstrated the presence of resistant *E. coli* and in particular CR *E. coli* of pig origin
366 in the abattoir (25-27). Although animals from this study departed to the abattoir free of
367 CR *E. coli*, it should be notice that this study was conducted under control conditions
368 and no extra-medication apart from ceftiofur and amoxicillin was applied during the
369 course of the study. However, conventional farming could also require the
370 administration of macrolides, polymyxins and tetracyclines during the fattening period,

371 which could co-select for CR *E. coli* (28, 29). Nowadays, there are a scarce data linking
372 antimicrobial consumption in veterinary medicine and the generation of antimicrobial
373 resistance bacteria; hence, it seems clear that the use of different families of
374 antimicrobials in the same population could be a risk factor for the development of
375 antimicrobial resistance in several microorganisms under field conditions (30).

376

377 Taken together these results suggest that the use of ceftiofur and amoxicillin at different
378 stages of the rearing cycle are independent risk factors for the selection of CR *E. coli*.
379 Both beta-lactam antimicrobials do select for resistant *E. coli* during the course of the
380 treatment. However, CR *E. coli* appear to be transitory and disappear in the absence of
381 the selective pressure. Both treatments did not select for long-term resistant organisms
382 since we could not detect CR *E. coli* when the animals departed to the abattoir.
383 However, further studies should be designed to identify other risk factors associated to
384 the persistence of resistance determinants to minimize the recirculation of strains and/or
385 plasmids within farms.

386

387 **Funding**

388 This work was supported by project AGL2011-28836 from the Ministerio de Economia
389 y Competitividad of Spain. KCV is a PhD student register with the Universidad
390 Autonoma de Barcelona.

391

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Table 1: Results obtained during the visits after treatment with ceftiofur. Sampling in day 0 was performed prior injecting the animals with ceftiofur.

Sampling days	Age (days)	Positive animals in the control group (N=50*)	Positive animals in the treated group (N=50)
0	6-8	5 (10%)	7 (12%)
2	8-10	5 (10%)	11 (26%)
7	13-15	1 (2%)	0
14	20-22	1 (2%)	5 (8%)
21	27-29	0	0
41	47-49	0	0

*three animals from the control group died after 7 days of treatment decreasing the size of the group to 47 animals.

501 Table 2: Results obtained during the course of the study after treatment with
502 amoxicillin. Sampling in day 0 was performed just before the beginning of the
503 treatment.

		Positive animals in each group			
Sampling days	Age (days)	Group 1 (N=20)	Group 2 (N=26)	Group 3 (N=20)	Group 4 (N=26)
0	70	0	0	0	0
2	72	0	2 (8%)	0	0
7	77	0	7 (27%)	0	0
14	84	0	1 (4%)	0	0
21	115	0	0	0	1 (4%)
45	138	0	1 (4%)	0	0
73	155	0	0	0	0

504 Group 1: untreated with antimicrobials, Group 2: untreated with ceftiofur and treated
505 with amoxicillin, Group 3: treated with ceftiofur and not treated with amoxicillin,
506 Group 4: treated with ceftiofur and with amoxicillin.

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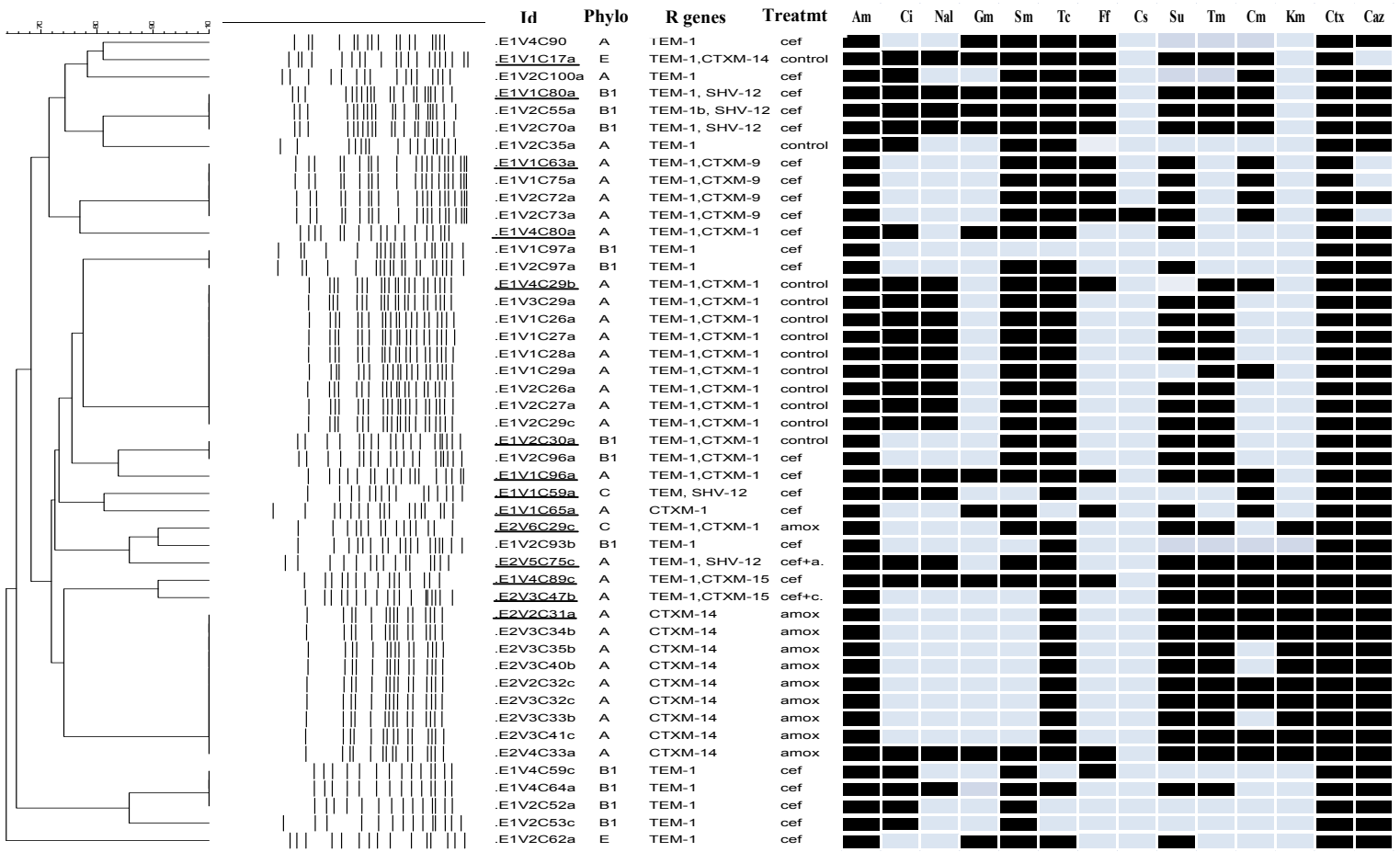
Figure 1. Dendogram showing the genotypic relatedness of the CR *E. coli* isolated during the course of the study, phylogeny, cephalosporin resistance genes, treatment and phenotypic diversity.



Am: ampicillin, Ci:ciprofloxacin, Nal: nalidixic acid, Gm: gentamicin, Sm: streptomycin, Tc: tetracycline, Ff: florfenicol, Cs: colistin, Su: Sulphametoxazole, Tm: trimethoprim, Cm: chloramphenicol, Km: kanamycin, Ctx: cefotaxime, Caz:ceftazidime.

Underlined are those strains selected for transformation and conjugation experiments

526 Table 3: Results of the conjungations and transformations experiments together with

527 plamid replicons, and plasmids sizes obtained.

				Inc Families found					
Wild type	Resistance gene	Conjugation results	Transformation results	I1	N	FIA	FIB	A/C	Molecular Weight (Kb)
E1V1C17a	CTXM-14	TC1b	**TF1a**	+					120
E1V1C80a	SHV-12	**TC2a**		+					138,9
E1V1C63a	CTXM-9	**TC3b**		+					138,9
E1V4C80a	CTXM-1	TC4a	**TF4a**		+				40
E1V4C29b	CTXM-1		**TF5a**		+		+		140
E1V2C30a	CTXM-1		**TF6a**	+					138
E1V1C96a	CTXM-1	TC7a	**TF7a**		+				40
E1V1C59a	SHV-12	TC8c	**TF8**	+	+				180
E1V1C65a	CTXM-1		**TF9a**		+				50
E2V6C29c	CTXM-1	TC10a	**TF10a**					+	180
E2V5C75c	SHV-12		**TF11a**						140
E1V4C89c	CTXM-15		**TF12a**			+	+		150+
E2V3C47b	CTXM-15		**TF13a**			+	+		150
E2V2C31a	CTXM-14	**TC14a**		+					120

528 In bold all transconjugants and transformants used for replicon typing