Molecular identification of aminoglycoside-modifying enzymes in clinical isolates of *Escherichia coli* resistant to Amoxicillin-Clavulanate isolated in Spain.

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ABSTRACT

The activity of eight aminoglycosides (amikacin, apramycin, arbekacin, gentamicin, kanamycin, neomycin, netilmicin and tobramycin) against a collection of 257 amoxicillin-clavulanate (AMC) resistant *Escherichia coli* was determined by microdilution. We investigated the aminoglycoside resistance rates, the prevalence of aminoglycoside-modifying enzymes (AMEs) genes, the relationship between AME-gene detection and resistance phenotypes to aminoglycosides, and the association with the production of mechanisms of amoxicillin-clavulanate resistance in *E. coli* isolates in Spain.

Aminoglycoside-resistant isolates were screened for the presence of genes encoding common AMEs (*aac(3)-Ia*, *aac(3)-IIa*, *aac(3)-IVa*, *aac(6')-Ib*, *ant(2")-Ia*, *ant(4´)-IIa*, and *aph(3')-Ia*) or 16S rRNA methylases (*armA*, *rmtB*, *rmtC*, and *npmA*). One hundred and five isolates (40.8%) were resistant to at least one of the aminoglycosides tested. Amikacin, apramycin and arbekacin showed better activity with MIC_{90} values of 2 mg/L (arbekacin) and 8 mg/L (amikacin and apramycin). Kanamycin presented the highest MIC_{90} (128 mg/L). The most common AME gene was *aac(6')-Ib* (36 strains, 34.3%), followed by *aph(3')-Ia* (31 strains, 29.5%), *ant(2")-Ia* (29 strains, 27.6%) and *aac(3)-IIa* (22 strains, 20.9%). *aac(3)-Ia*, *aac(3)-IVa*, *ant(4´)-IIa* and four methylases were not detected. The *ant(2")-Ia* gene was usually associated with OXA-1 21/30 (70%); while 23/25 (92%) strains producing CTX-M-15 had the *aac(6´)-Ib* gene. In nosocomial isolates, the most prevalent AME gene was *aac(6´)-Ib* (18/41; 44%) while *ant-(2")-Ia* and *aph(3´)-Ia* genes (20/64; 31.25%) were more frequent in strains of community origin. In 64.6% isolates the phenotypic profile correlates with the presence of commonly encountered AMEs.

Keywords: Aminoglycoside; Aminoglycoside-modifying enzyme; Amoxicillin-Clavulanate; resistance; *Escherichia coli*.

1. Introduction

Clinical use of aminoglycosides declined after the introduction of expanded-spectrum beta-lactams and fluoroquinolones and this correlated with decreased interest in the study of microbiological aspects of these drugs, including the analysis of mechanisms of resistance. The increasing problem of multiresistance in gram-negative bacteria and the introduction of new aminoglycoside analogs (i.e., plazomycin) [1,2] warrant new studies aimed to understand aminoglycoside resistance

Amoxicillin–clavulanate (AMC) is one of the most utilized antimicrobial agents in many countries, including Spain and is a therapeutic option for infection caused by *E. coli* susceptible to this combination [3]. Unfortunately, AMC-resistant *E. coli* are increasingly recognized at the hospital and community levels in Spain [4]. Other drugs, i.e. aminoglycosides, should be considered as alternative therapeutic options against infections caused by AMC-resistant isolates. In these circumstances, an analysis of resistance to aminoglycosides in AMC-resistant *E. coli* is relevant because clinical and epidemiological reasons.

According to the European Antimicrobial Resistance Surveillance-Network-EARS-Net (http://www.ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/database/Pages/database.aspx) in the last decade aminoglycoside-resistant *E. coli* isolates from blood have increased from 6.8% (2001) to 15.6% (2012) in Spain.

Different mechanisms are known to play a role in the development of aminoglycoside resistance in *E. coli* and other gram-negative bacteria, but the production of aminoglycoside-modifying enzymes (AMEs) is the most important. Other mechanisms conferring

aminoglycoside resistance include active efflux of the antimicrobial [5] and reduced intake into the bacterial cell [6].

AMEs are categorized into three classes depending on their modifying activities: acetyltransferases (AACs), phosphotransferases (APHs) and nucleotidyltransferases (ANTs) [7,8]. Recently, a new type of mechanism of increasing importance, methylation of the 16S rRNA has been reported, and it is carried out by methyltransferases [9,10]. Four 16S rRNA methylase genes have been identified in *Enterobacteriaceae* of human origin in different geographical locations: *armA*, *rmtB*, *rmtC*, and *npmA*. *armA* and *rmtB* being the most widespread [9]. High-level resistance (MIC ≥128 ug/ml) to all aminoglycosides except apramycin and neomycin has been described as the phenotype conferred by *armA*, *rmtB*, *rmtC* genes; additionally, resistance to apramycin and neomycin appears to be typical for *npmA*-containing isolates [10]. The AMEs are often found on mobile elements such a plasmids or transposable elements, combined with genes encoding resistance to other antibiotics including AMC, which increases the likelihood of their selection and dissemination [7]. It is known that a single AME can inactivate several aminoglycosides. Phenotypic analysis of aminoglycoside-resistance in clinical laboratories aimed to detect the presence of AMEs [8] is difficult because more than one enzyme can be present and usually a limited number of substrates are tested. In these circumstances, molecular techniques are needed to confirm initial findings [11].

Although aminoglycosides have never completely vanished from clinic use, the ever-increasing resistance to all other common antibiotics has once again focused clinical interests in aminoglycosides and in particular their use in serious gram-negative infections. There is

little information on the production of AMEs in *Enterobacteriaceae* strains resistant to other antimicrobial groups [12,13,14].

The aim of this study was to evaluate the *in vitro* activity of clinically relevant aminoglycosides and the prevalence of commom AMEs genes in *E. coli* resistant to AMC isolated from seven hospitals in Spain.

2. Material and methods

2.1. Bacterial isolates

A total of 257 *E. coli* isolates of clinical origin resistant to AMC obtained between January 2010 and May 2010 in a prospective Spanish multicenter study (seven university hospitals of six Spanish Autonomous Communities) were examined. One hundred and ten of them (43%) produced nosocomial-acquired infections, and 147 (57%) putatively produced community-acquired infections [4,15].

The mechanisms of AMC resistance in these isolates, described in a previous study [4], were: production of OXA-1 (26.1%), hyperproduction of penicillinase (22.9%), production of plasmidic-AmpC (19.5%), hyperproduction of chromosomic AmpC (18.6%), and production of inhibitor-resistant TEM (IRT) (17.5%). In addition, 37 isolates (14.4%) produced extended-spectrum β-lactamases (ESBLs).

2.2. Antimicrobial susceptibility testing

The aminoglycosides used in this study were amikacin (Ak) (Sigma-Aldrich, Spain), apramycin (Ap) (Discovery-Fine Chemicals, UK), arbekacin (Ab) (Discovery-Fine Chemicals), gentamicin (Gm) (Sigma-Aldrich), kanamycin (K) (Sigma-Aldrich), neomycin (Nm) (Discovery-Fine Chemicals), netilmicin (Nt) (Discovery-Fine Chemicals) and tobramycin (To) (Sigma-Aldrich).

MICs of these eight aminoglycosides for the 257 *E. coli* isolates were determined by broth microdilution according to CLSI guidelines [16]. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains.

The results were interpreted using clinical breakpoints as defined by EUCAST [17]. EUCAST epidemiological cut-off values (ECOFFs) for kanamycin and neomycin and EFSA [18] epidemiologic breakpoints for apramycin were used. In addition, when available, breakpoints defined by the CLSI were also used for comparison of clinical categories of isolates possessing AME genes. No breakpoints for arbekacin have been established by EUCAST or CLSI and for this antibiotic; a simple description of MIC values is presented.

The *aac(6´)-Ib-cr* variant is known to confer low level ciprofloxacin and norfloxacin resistance. In isolates with aac*(6´)-Ib* gene, quinolones susceptibility testing was also performed and the corresponding results were interpreted using EUCAST breakpoints

2.3 Molecular characterization of mechanisms of resistance to aminoglycosides

All isolates demonstrating resistance to at least one of the tested aminoglycosides were screened for the presence of aminoglycoside-modifying enzyme genes and methyltransferases by PCR.

As control 25 isolates susceptible to the aminoglycosides were also used in the PCR analysis. Specific sequence primers were chosen within the nucleotide sequence of the published regions of the various genes. Sets of primers for the following genes were included in the PCR assay: *aac(3)-Ia, aac(3)-IIa, aac(3)-IVa*, *aac(6')-Ib, ant(2'')-Ia, ant(4´)-IIa, aph(3')-Ia*, *armA*, *rmtB*, *rmtC* and *npmA*. The primers for AMEs and methyltransferases genes and expected amplicon sizes shown in the supplementary Table S1.

Genomic DNA was extracted using the InstaGene matrix kit (Bio-Rad, Madrid, Spain) according to the manufacturer's instructions, and 2 µl was added to a reaction mixture

containing 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM deoxynucleoside triphosphate, 0.5 µM of each primer, and 1 U of Taq DNA Polymerase (Bioline; ECOGEN, Spain). The amplification conditions were 94°C for 5 min, and then 30 cycles of 94°C for 30 s, 55°C for 30 s (60ºC for *aac(6´)-Ib* gene) and 72°C for 1 min, and a final elongation at 72°C for 10 min. PCR products were analyzed on 1.5% (w/v) agarose gels stained with ethidium bromide.

2.4 DNA sequencing

The amplified products were purified with a QIAquick PCR purification kit (Qiagen Inc., Izasa, Barcelona, Spain). DNA sequences on both strands were determined by using an external resource (Macrogen Inc., Amsterdam, The Netherlands). The BLAST program was used to compare the nucleotide and protein sequences to those available on the internet at the National Center for Biotechnology Information website www.ncbi.nlm.nih.gov.

We sequenced amplicons representative of all AME genes obtained, and found 100% homology with the GenBank sequences. The presence of the *aac(6´)-Ib-cr* variant conferring additional resistance to ciprofloxacin [19] was inferred from the sequence of the corresponding amplicons.

2.5 Statistical analysis

Proportions of isolates with resistance using breakpoints by EUCAST and CLSI were compared using Fisher´s exact test. A *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Antimicrobial susceptibility testing

One hundred and fifty two isolates out of 257 (59.2%) AMC-resistant *E. coli* isolates were aminoglycoside-susceptible, whereas, the remaining 105 isolates (40.8%) were resistant to at least one of the indicated aminoglycosides using the clinical and epidemiological breakpoints defined by EUCAST. The percentage of resistance to the different aminoglycosides is presented in the Table 1.

The highest resistance rates were observed for kanamycin with 35.4% (EUCAST cut-off values) and 32.3% (CLSI breakpoints) of resistant isolates respectively. The percentage resistance to netilmicin obtained using the EUCAST breakpoint (17.1%) was higher than obtained using the CLSI breakpoint (3.5%), significantly ($P<0.0001$). The MIC_{90} value of arbekacin was 2-times lower than that of amikacin and apramycin, and 4-times lower than that of gentamicin, tobramycin and neomycin.

Fifteen different resistance phenotypes were observed among the 105 isolates resistant to aminoglycosides (Supplementary Table S2). The most frequently encountered resistance phenotypes were Gm,K,To (n=29; 27.6%), followed by K,Nm (n=21; 20%), K,Nt,To (n=16; 15.2%) and Gm,Nt,To (n=9; 8.6%). Thirty-nine percent of the isolates (100/257) were resistant to two or more of the tested aminoglycosides. Five isolates were resistant to only one of the considered aminoglycoside: three to gentamicin (MIC, 32 for two isolates and 16 mg/L in on isolate), one to tobramycin (MIC, 8 mg/L) and one to kanamycin (MIC, 64 mg/L). Only seven isolates (6.6%) were resistant to five aminoglycosides, while 55 isolates (52.4%) were resistant to three aminoglycosides and 24 isolates (22.8%) presented resistance to two aminoglycosides.

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222 *3.2. Molecular characterization of mechanisms of resistance to aminoglycosides*.

223 The prevalence of AME genes in *E. coli* resistant to aminoglycoside and the correlation

224 between expected and observed phenotypes are shown in Table 2**.**

225

226 The most common AME gene was *aac(6´)-Ib* (36 strains, 34.3%), followed by *aph(3´)-Ia* (31

227 strains, 29.5%), *ant(2")-Ia* (29 strains, 27.6%), and *aac(3)-IIa* (23 strains, 22%). All the

228 studied isolates were negative for *aac(3)-Ia*, *aac(3)-IVa*, *ant(4")-IIa* and methylases (*armA*,

229 *rmtB*, *rmtC* and *npmA*)

230

231 Eighty-one (77.1%) isolates contained only one of the evaluated AME genes and 16 (15.2%)

232 isolates harboured two of them. The combination of *aac(6´)-Ib* and *aac(3)-IIa* was the most

233 common one (8 isolates, 7.6%), followed by *aac(3)-IIa* plus *aph(3´)-Ia* (4 isolates, 3.8%).

234 Two isolates harboured three genes (*aac(6´)-Ib*, *aac(3)-IIa* and *aph(3´)-Ia*). In six (5.6%)

235 isolates, none of the investigated genes were identified, three of these isolates were resistant

236 to gentamicin (MIC, 32 mg/L), one was resistant to kanamycin (MIC, 128 mg/L), one was

237 resistant to both gentamicin and tobramycin (MIC, 32 mg/L and 8 mg/L, respectively) and the

238 remaining isolate was resistant to both kanamycin and neomycin (Table 2).

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240 The *aac(6')-Ib* gene was detected in 36 isolates (34.3%). All five (1.9%) isolates resistant to

241 amikacin contained the *aac(6´)-Ib* gene. Although it has been described that the *aac(6´)-Ib*

242 gene confers resistance to amikacin, kanamycin, tobramycin, and netilmicin [7], when

243 applying EUCAST breakpoints only four out of the 25 isolates (16%) containing only the

244 *aac(6´)-Ib* gene, presented this specific pattern, while the remaining 21 isolates were resistant

245 to one or more of gentamicin, tobramycin, netilmicin or kanamycin, but not to amikacin.

Using breakpoints established by CLSI none of isolates carrying the *aac(6')-Ib* gene had the expected resistance phenotype to aminoglycosides; in fact, this gene was detected in one strain susceptible to all tested aminoglycosides (Table 2).

The *aac(6')-Ib-cr* variant was found in 34 of the 36 (94.5%) *aac(6´)-Ib* positive isolates. All the 36 isolates with the *aac(6')-Ib* (independently that this were the –cr variant or not) were resistant to ciprofloxacin, levofloxacin, norfloxacin and nadilixic acid; as the –cr variant is known to confer only low level ciprofloxacin and norfloxacin resistance [19] additional mechanisms of chromosomal quinolone resistance probably co-exist in our isolates.

The second most frequent gene was *aph(3')-Ia*, detected in 31 (29.5%) isolates. Of the twenty one isolates positive only for *aph(3')-Ia* gene, 20 isolates had specific pattern resistance to kanamycin and neomycin, and only one isolate was also resistant to gentamicin (Table 2). All isolates with alone *aph(3´)-Ia* had a MIC $\geq$256 mg/L for kanamycin.

The *ant(2'')-Ia* gene was found in 29 (27.6%) isolates. All of them showed the expected resistance phenotype (gentamicin, tobramycin and kanamycin) when using the EUCAST breakpoints, while 21 isolates had the expected phenotype by CLSI (Table 2).

Finally, the *aac(3)-IIa* gene was present in 23 strains (22%). Eight out of the 9 isolates with only *acc(3)-IIa* had a phenotypic resistance pattern consistent with that described for this gene (resistance to gentamicin, tobramycin and netilmicin). It is remarkable that MICs of gentamicin (64-128 mg/L) were higher than those of tobramycin (8-64 mg/L).

As a control for our results, PCR analysis revealed the absence of AME genes in 25 isolates susceptible to the evaluated aminoglycosides.

Although in this study none of the isolates showed high-level resistance (MIC> 128 mg/L) to tested aminoglycosides, ten strains with MIC of amikacin ≥ 16 mg/L were screened for *armA*, *rmtB*, *rmtC* and *npmA* 16S rRNA methylase genes by PCR and all of them were negative for these genes.

Phenotypic detection of resistance did not correlate well with the PCR results of genes investigated in this study. Using EUCAST criteria sixty-four (64.6%) isolates were concordant between resistance phenotypes observed and PCR results (100% concordance for strains with *ant(2")-Ia* gene). By CLSI, we observed agreement between the resistance phenotypes and the AME genotype in 46 (46.4%) isolates.

3.3 Correlation between AMEs genes detected and mechanisms of resistance to amoxicillin-clavulanate.

The percentages of strains resistant to aminoglycosides considering the mechanisms of resistance to amoxicillin-clavulanate are presented in Table 3. Interestingly, isolates producing OXA-1 (alone or combined with an ESBLs) were more often resistant to an aminoglycoside than isolates with others AMC-resistant mechanism, in contrast isolates hyperproduced chromosomal AmpC or producers inhibitor-resistant TEM (IRTs) were significantly more susceptible to aminoglycosides.

The different combinations of AME genes obtained and the mechanism of resistance to AMC [4] are shown in Table 4. Among the 105 aminoglycoside-resistant *E. coli* characterized in

this study, 59 isolates (56.2%) produced OXA-1, 19 isolates (18.1%) produced a plasmidic AmpC, 16 isolates (15.2%) overproduced a TEM-1, 11 isolates (10.5%) hyperproduced chromosomal AmpC and 10 isolates (9.5%) were inhibitor-resistant TEM (IRTs) producers. In addition, a total of 27 isolates (25.7%) were ESBLs-producers (25 isolates CTX-M-15 and two isolates CTX-M-14 producers), all but one of them had an AMC resistance mechanism mainly OXA-1 (24 isolates, 88.8%).

As shown in the table 4, the OXA-1 gene alone (the most prevalent mechanism of resistance to AMC [4]) was usually associated with *ant(2'')-Ia* (21 isolates, 70%), all isolates with *ant(2'')-Ia* belong to the clone ST88 phylogroup A ($p<0.0001$) [20]. On the other hand, 18 strains (17.1%) producing both OXA-1 and ESBL (CTX-M-15) contained *aac(6´)-Ib-cr* alone or in combination with another AME gene. In nine strains producing only an IRTs, five and four strains with only *aph(3´)-Ia* and *aac(3)-IIa* have been found respectively. The *aph(3´)-Ia* gene was present in 8 out of 13 (61.5%) strains producing plasmidic-AmpC and in 4 out of 6 (66%) hyperproducing chromosomal-AmpC.

The *ant-(2'')-Ia* and *aph(3´)-Ia* genes were more frequent in strains of community origin (20/64; 31.25%) than the *aac(6´)-Ib* (18/64, 28.16%) or *aac(3)-IIa* (14/64; 21.8%) genes. In contrast, in strains of nosocomial origin the most frequent AME gene was *aac(6´)-Ib*, which was present in 18/41 (44%) isolates, while 26,8%, 22% and 19.5% of these isolates contained the *aph(3´)-Ia, ant-(2'')-Ia* and *aph(3´)-Ia* genes, respectively.

4. Discussion

The overall incidence of aminoglycoside resistance found in our study was higher than the incidence that has been presented in other reports, which may reflect our bias in considering bacteria with a defined resistance phenotype, rather than unselected isolates [21,22,23].

In a recent study in Spain the percentages of resistance obtained in 330 aminoglycoside-resistant enterobacteria, (80% *E. coli*) were 26.3% to kanamycin, 18% to gentamicin, 17% to tobramycin, 3.6% to netilmicin and 1.5% to amikacin [21]. In 20 European University hospitals participating in the European SENTRY Antimicrobial Surveillance Programme the percentages of resistance to gentamicin, tobramycin and amikacin in *E. coli* obtained from three Spanish hospitals were 9.2%, 4.4% and 0.7% respectively [23]. However, in ESBL-producing *E. coli*, the resistance rates were higher than those obtained in our study; resistance to tobramycin, gentamicin and amikacin in a study in Norway were 94%, 73% and 6% respectively [12].

In this study the most common AME gene was *aac(6´)-Ib* followed by *aph(3´)-Ia*, *ant(2'')-Ia* and *aac(3)-IIa*. In a previous study in a single centre in Barcelona (Spain), the most frequent genes were *aph(3´)-Ia* (13.9%), *aac(3)-IIa* (12.4%), *aac(6´)-Ib* (4.2%) and *ant(2'')-Ia* (3.6%) [21]. In *Enterobacteriaceae* isolated from blood cultures in a hospital in Athens, the *aac(6´)-Ib* gene alone or combined with *aac(3)-I* was the most prevalent mechanism of aminoglycoside resistance [22]. In ESBL-producing *E. coli* isolates the prevalence of *aac(3)-IIa* and *aac(6´)-Ib* genes was more elevated than were detected in this study; 77.6% and 47.8% respectively [12]. In 50 carbapenem-resistant *K. pneumoniae* strains, *aac(6´)-Ib* gene was the most prevalent AME detected in 98% of strains [14]. In other study, the most prevalent resistance gene in Europe [24] was *aac(3)-II*.

In our study, only five (1.9%) strains were amikacin-resistant, and all of them produced the *aac(6´)-Ib* gen. However, low MIC values of amikacin despite the possesion of *aac(6')-Ib-cr* have been described [25]. The explanation could be either an impaired gene expression or that amikacin is a poor substrate for this enzyme [26]. Kim *et al*. [27] have also found a very high percentage of amikacin-susceptible *Enterobacter cloacae* isolates harbouring the *aac(6´)-Ib* gene (84.5% and 55.2% according to the CLSI and EUCAST breakpoints, respectively) with only two isolates containing mutations associated with the loss of amikacin resistance. Recently Almaghrabi *et al*. [14] did not find correlations between AMEs and amikacin MICs or resistance in carbapenem-resistant *K. pneumoniae* strains.

The second most frequent gene in this study was *aph(3')-Ia* that confers resistance to kanamycin and neomycin and is widely distributed, mainly among Gram-negatives within wide host range plasmids and transposons [7]. As kanamycin is not prescribed in Spain, the persistence of this gene may be related to its genetic linkage with other resistance genes. In *aac(3)-IIa* positive isolates, for which resistance to gentamicin, tobramycin and netilmicin is expected, it is remarkable that the MICs of gentamicin (128-64 mg/L) were higher compared with tobramycin (64-8 mg/L). It has been described that the encoded enzyme has a stronger affinity for gentamicin than for tobramycin or netilmicin [11]. All of the 23 isolates positive for *aac(3)-IIa* had MIC values for netilmicin above to 4 mg/L, that it is the clinical breakpoints by EUCAST, however only six isolates (26.1%) were defined as resistant by CLSI (MIC $\geq$ 32 mg/L); thus showing that the CLSI breakpoints for netilmicin does not reliably indicate the presence of this gene.

We observed frequent disagreement between the resistance phenotypes and the AME genotype in 35 (35.3%) or 53 (53.3%) isolates, according to the EUCAST and CLSI

breakpoints respectively. It is possible that these differences are related to some extend to the fact that adequate breakpoints are still to be defined for some compounds and that EUCAST epidemiological breakpoints for some compounds are really different of the CLSI clinical breakpoints. In *E. coli*, Davis *et al*. [28] observed genotypic-phenotypic discrepancies to aminoglycosides in 24.7% of the strains and characterized mutations or deletions in inactive AMEs genes in isolates where the gene was present but the expected corresponding phenotype resistance was absent.

CTX-M-15 plasmid-mediated dissemination of *aac(6´-Ib-cr* among *Enterobacteriaceae* isolates has been observed in multiple European countries [29,30]. We have also found that 23 out of 25 strains producing CTX-M-15 had the *aac(6´)-Ib-cr* gene of which 69.5% belonged to clonal complex ST131 and 30.5% were ST23 or ST10 [20].On the other hand, it is also remarkable that in five out 11 isolates hyper-producing chromosomal AmpC harboured the *acc(6´)-Ib* gene, in contrast with results of Lindemann *et al*. [12] who did not find *aac(6´)-Ib* in any of isolates with AmpC hyperproduction.

In conclusion, the most notable finding of this study was that strains exhibited a remarkable AME diversity. Overall, we identified nine AME patterns, which correlated with different levels of aminoglycoside resistance. The *aac(6')-Ib* enzyme was most common gene detected and it was associated with strains producing CTX-M-15 while the *ant(2'')-Ia* gene was usually associated with OXA-1. For 35.4% isolates the aminoglycoside resistance phenotype was an inadequate predictor of the AME genotype, suggesting unsuitable setting of breakpoints for aminoglycosides or the contribution of multiple concurrent resistance mechanisms. A full understanding of AMEs and other molecular mechanisms of diminished susceptibility to currently available aminoglycosides will allow clinicians to incorporate these

agents most rationally into treatment regimens against amoxicillin-clavulanate resistant *E. coli* infections

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Declarations

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Table Legends

Table 1. *In vitro* activity of eight aminoglycosides against 257 AMC-resistant *E. coli*.

Table 2. Prevalence of aminoglycoside-modifying enzymes (AME) genes and correlation between antibiograms and AME genes in 105 AMC-resistant *E. coli*.

Table 3. Number of isolates resistant to aminoglycoside considering the mechanism of resistance to amoxicillin-clavulanate in 257 *E. coli*

Table 4. Correlation between aminoglycosides-modifying enzymes (AMEs) genes obtained and mechanism of resistance to amoxicillin-clavulanate in 105 *E. coli*.

Supplementary Table

Table S1. Primers used in detection of aminoglycoside-modifying enzymes (AME) and methyltransferases genes and expected amplicon sizes.

Table S2- Phenotypic profiles to aminoglycosides observed among the 105 AMC-resistant *E. coli*.

Reviewers' comments

Reviewer #3:

Major

I agree with Author's comment that these results are of epidemiological importance, and have also clinical implications. Better opinion for clinical use is "aminoglycosides should be considered as alternative or combined therapeutic options against infections caused by AMC-resistant isolates."(Line 81-82).

The sentence has been modified as suggested by the Reviewer.

Number of isolates is incorrect. (Total numbers of isolates has to be 25 isolates) (Table2. Aac(6')-Ib).

We have corrected the mistake: In the column “Observed phenotype resistance (no. of isolates) for *aac(6´)-Ib*, the value for Gm is “1”, not “19”. Then the final number for this enzyme is: 3+17+2+1+1+1=25.

There are several calculation and formatting errors. Please check the numbers again in Table1 and also in the manuscript.

Ex. Table1. 24.7→24.8, 23.6→23.8, 20→20.0, 2.85→2.9, 0.95→1.0, 1.9→2.0 etc.

Line98-99 59.2→59.1, 40.8→40.9

The numbers have been modified as suggested.

Minor

gen. → gene. (Line 342)

breakpoints → breakpoints. (Line158)

Table2. N° →No. , n°→no.

All the indicated changes have been made.

We thank Reviewer#3 for his comments and observations.

Molecular identification of aminoglycoside-modifying enzymes in clinical isolates of *Escherichia coli* resistant to Amoxicillin-Clavulanate isolated in Spain.

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Running title: Identification of AME-genes in AMC-resistant *E. coli*.

ABSTRACT

The activity of eight aminoglycosides (amikacin, apramycin, arbekacin, gentamicin, kanamycin, neomycin, netilmicin and tobramycin) against a collection of 257 amoxicillin-clavulanate (AMC) resistant *Escherichia coli* was determined by microdilution. We investigated the aminoglycoside resistance rates, the prevalence of aminoglycoside-modifying enzymes (AMEs) genes, the relationship between AME-gene detection and resistance phenotypes to aminoglycosides, and the association with the production of mechanisms of amoxicillin-clavulanate resistance in *E. coli* isolates in Spain.

Aminoglycoside-resistant isolates were screened for the presence of genes encoding common AMEs (*aac(3)-Ia*, *aac(3)-IIa*, *aac(3)-IVa*, *aac(6')-Ib*, *ant(2")-Ia*, *ant(4´)-IIa*, and *aph(3')-Ia*) or 16S rRNA methylases (*armA*, *rmtB*, *rmtC*, and *npmA*). One hundred and five isolates (40.9%) were resistant to at least one of the aminoglycosides tested. Amikacin, apramycin and arbekacin showed better activity with MIC_{90} values of 2 mg/L (arbekacin) and 8 mg/L (amikacin and apramycin). Kanamycin presented the highest MIC_{90} (128 mg/L). The most common AME gene was *aac(6')-Ib* (36 strains, 34.3%), followed by *aph(3')-Ia* (31 strains, 29.5%), *ant(2")-Ia* (29 strains, 27.6%) and *aac(3)-IIa* (23 strains, 22.0%). *aac(3)-Ia*, *aac(3)-IVa*, *ant(4´)-IIa* and four methylases were not detected. The *ant(2")-Ia* gene was usually associated with OXA-1 21/30 (70%); while 23/25 (92%) strains producing CTX-M-15 had the *aac(6´)-Ib* gene. In nosocomial isolates, the most prevalent AME gene was *aac(6´)-Ib* (18/41; 44%) while *ant-(2")-Ia* and *aph(3´)-Ia* genes (20/64; 31.25%) were more frequent in strains of community origin. In 64.6% isolates the phenotypic profile correlates with the presence of commonly encountered AMEs.

Keywords: Aminoglycoside; Aminoglycoside-modifying enzyme; Amoxicillin-Clavulanate; resistance; *Escherichia coli*.

1. Introduction

Clinical use of aminoglycosides declined after the introduction of expanded-spectrum beta-lactams and fluoroquinolones and this correlated with decreased interest in the study of microbiological aspects of these drugs, including the analysis of mechanisms of resistance. The increasing problem of multiresistance in gram-negative bacteria and the introduction of new aminoglycoside analogs (i.e., plazomycin) [1,2] warrant new studies aimed to understand aminoglycoside resistance

Amoxicillin–clavulanate (AMC) is one of the most utilized antimicrobial agents in many countries, including Spain and is a therapeutic option for infection caused by *E. coli* susceptible to this combination [3]. Unfortunately, AMC-resistant *E. coli* are increasingly recognized at the hospital and community levels in Spain [4]. Other drugs, i.e. aminoglycosides, should be considered as alternative or combined therapeutic options against infections caused by AMC-resistant isolates. In these circumstances, an analysis of resistance to aminoglycosides in AMC-resistant *E. coli* is relevant because clinical and epidemiological reasons.

According to the European Antimicrobial Resistance Surveillance-Network-EARS-Net (http://www.ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/database/Pages/database.aspx) in the last decade aminoglycoside-resistant *E. coli* isolates from blood have increased from 6.8% (2001) to 15.6% (2012) in Spain.

Different mechanisms are known to play a role in the development of aminoglycoside resistance in *E. coli* and other gram-negative bacteria, but the production of aminoglycoside-modifying enzymes (AMEs) is the most important. Other mechanisms conferring

aminoglycoside resistance include active efflux of the antimicrobial [5] and reduced intake into the bacterial cell [6].

AMEs are categorized into three classes depending on their modifying activities: acetyltransferases (AACs), phosphotransferases (APHs) and nucleotidyltransferases (ANTs) [7,8]. Recently, a new type of mechanism of increasing importance, methylation of the 16S rRNA has been reported, and it is carried out by methyltransferases [9,10]. Four 16S rRNA methylase genes have been identified in *Enterobacteriaceae* of human origin in different geographical locations: *armA*, *rmtB*, *rmtC*, and *npmA*. *armA* and *rmtB* being the most widespread [9]. High-level resistance (MIC ≥128 mg/L) to all aminoglycosides except apramycin and neomycin has been described as the phenotype conferred by *armA*, *rmtB*, *rmtC* genes; additionally, resistance to apramycin and neomycin appears to be typical for *npmA*-containing isolates [10]. The AMEs are often found on mobile elements such a plasmids or transposable elements, combined with genes encoding resistance to other antibiotics including AMC, which increases the likelihood of their selection and dissemination [7]. It is known that a single AME can inactivate several aminoglycosides. Phenotypic analysis of aminoglycoside-resistance in clinical laboratories aimed to detect the presence of AMEs [8] is difficult because more than one enzyme can be present and usually a limited number of substrates are tested. In these circumstances, molecular techniques are needed to confirm initial findings [11].

Although aminoglycosides have never completely vanished from clinic use, the ever-increasing resistance to all other common antibiotics has once again focused clinical interests in aminoglycosides and in particular their use in serious gram-negative infections. There is

little information on the production of AMEs in *Enterobacteriaceae* strains resistant to other antimicrobial groups [12,13,14].

The aim of this study was to evaluate the *in vitro* activity of clinically relevant aminoglycosides and the prevalence of commom AMEs genes in *E. coli* resistant to AMC isolated from seven hospitals in Spain.

2. Material and methods

2.1. Bacterial isolates

A total of 257 *E. coli* isolates of clinical origin resistant to AMC obtained between January 2010 and May 2010 in a prospective Spanish multicenter study (seven university hospitals of six Spanish Autonomous Communities) were examined. One hundred and ten of them (43%) produced nosocomial-acquired infections, and 147 (57%) putatively produced community-acquired infections [4,15].

The mechanisms of AMC resistance in these isolates, described in a previous study [4], were: production of OXA-1 (26.1%), hyperproduction of penicillinase (22.9%), production of plasmidic-AmpC (19.5%), hyperproduction of chromosomic AmpC (18.6%), and production of inhibitor-resistant TEM (IRT) (17.5%). In addition, 37 isolates (14.4%) produced extended-spectrum β-lactamases (ESBLs).

2.2. Antimicrobial susceptibility testing

The aminoglycosides used in this study were amikacin (Ak) (Sigma-Aldrich, Spain), apramycin (Ap) (Discovery-Fine Chemicals, UK), arbekacin (Ab) (Discovery-Fine Chemicals), gentamicin (Gm) (Sigma-Aldrich), kanamycin (K) (Sigma-Aldrich), neomycin (Nm) (Discovery-Fine Chemicals), netilmicin (Nt) (Discovery-Fine Chemicals) and tobramycin (To) (Sigma-Aldrich).

MICs of these eight aminoglycosides for the 257 *E. coli* isolates were determined by broth microdilution according to CLSI guidelines [16]. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains.

The results were interpreted using clinical breakpoints as defined by EUCAST [17]. EUCAST epidemiological cut-off values (ECOFFs) for kanamycin and neomycin and EFSA [18] epidemiologic breakpoints for apramycin were used. In addition, when available, breakpoints defined by the CLSI were also used for comparison of clinical categories of isolates possessing AME genes. No breakpoints for arbekacin have been established by EUCAST or CLSI and for this antibiotic; a simple description of MIC values is presented.

The *aac(6´)-Ib-cr* variant is known to confer low level ciprofloxacin and norfloxacin resistance. In isolates with aac*(6´)-Ib* gene, quinolones susceptibility testing was also performed and the corresponding results were interpreted using EUCAST breakpoints.

2.3 Molecular characterization of mechanisms of resistance to aminoglycosides

All isolates demonstrating resistance to at least one of the tested aminoglycosides were screened for the presence of aminoglycoside-modifying enzyme genes and methyltransferases by PCR.

As control 25 isolates susceptible to the aminoglycosides were also used in the PCR analysis. Specific sequence primers were chosen within the nucleotide sequence of the published regions of the various genes. Sets of primers for the following genes were included in the PCR assay: *aac(3)-Ia, aac(3)-IIa, aac(3)-IVa, aac(6')-Ib, ant(2'')-Ia, ant(4´)-IIa, aph(3')-Ia, armA, rmtB, rmtC* and *npmA*. The primers for AMEs and methyltransferases genes and expected amplicon sizes shown in the supplementary Table S1.

Genomic DNA was extracted using the InstaGene matrix kit (Bio-Rad, Madrid, Spain) according to the manufacturer's instructions, and 2 µl was added to a reaction mixture

containing 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM deoxynucleoside triphosphate, 0.5 µM of each primer, and 1 U of Taq DNA Polymerase (Bioline; ECOGEN, Spain). The amplification conditions were 94°C for 5 min, and then 30 cycles of 94°C for 30 s, 55°C for 30 s (60ºC for *aac(6´)-Ib* gene) and 72°C for 1 min, and a final elongation at 72°C for 10 min. PCR products were analyzed on 1.5% (w/v) agarose gels stained with ethidium bromide.

2.4 DNA sequencing

The amplified products were purified with a QIAquick PCR purification kit (Qiagen Inc., Izasa, Barcelona, Spain). DNA sequences on both strands were determined by using an external resource (Macrogen Inc., Amsterdam, The Netherlands). The BLAST program was used to compare the nucleotide and protein sequences to those available on the internet at the National Center for Biotechnology Information website www.ncbi.nlm.nih.gov.

We sequenced amplicons representative of all AME genes obtained, and found 100% homology with the GenBank sequences. The presence of the *aac(6´)-Ib-cr* variant conferring additional resistance to ciprofloxacin [19] was inferred from the sequence of the corresponding amplicons.

2.5 Statistical analysis

Proportions of isolates with resistance using breakpoints by EUCAST and CLSI were compared using Fisher´s exact test. A *P* value of <0.05 was considered statistically significant.

3. Results

3.1. Antimicrobial susceptibility testing

One hundred and fifty two isolates out of 257 (59.1%) AMC-resistant *E. coli* isolates were aminoglycoside-susceptible, whereas, the remaining 105 isolates (40.9 %) were resistant to at least one of the indicated aminoglycosides using the clinical and epidemiological breakpoints defined by EUCAST. The percentage of resistance to the different aminoglycosides is presented in the Table 1.

The highest resistance rates were observed for kanamycin with 35.4% (EUCAST cut-off values) and 32.3% (CLSI breakpoints) of resistant isolates respectively. The percentage resistance to netilmicin obtained using the EUCAST breakpoint (17.1%) was higher than obtained using the CLSI breakpoint (3.5%), significantly ($P<0.0001$). The MIC_{90} value of arbekacin was 2-times lower than that of amikacin and apramycin, and 4-times lower than that of gentamicin, tobramycin and neomycin.

Fifteen different resistance phenotypes were observed among the 105 isolates resistant to aminoglycosides (Supplementary Table S2). The most frequently encountered resistance phenotypes were Gm,K,To (n=29; 27.6%), followed by K,Nm (n=21; 20.0%), K,Nt,To (n=16; 15.2%) and Gm,Nt,To (n=9; 8.6%). Thirty-nine percent of the isolates (100/257) were resistant to two or more of the tested aminoglycosides. Five isolates were resistant to only one of the considered aminoglycoside: three to gentamicin (MIC, 32 for two isolates and 16 mg/L in on isolate), one to tobramycin (MIC, 8 mg/L) and one to kanamycin (MIC, 64 mg/L). Only seven isolates (6.6%) were resistant to five aminoglycosides, while 55 isolates (52.4%) were resistant to three aminoglycosides and 24 isolates (22.8%) presented resistance to two aminoglycosides.

3.2. Molecular characterization of mechanisms of resistance to aminoglycosides.

The prevalence of AME genes in *E. coli* resistant to aminoglycoside and the correlation between expected and observed phenotypes are shown in Table 2**.**

The most common AME gene was *aac(6´)-Ib* (36 strains, 34.3%), followed by *aph(3´)-Ia* (31 strains, 29.5%), *ant(2")-Ia* (29 strains, 27.6%), and *aac(3)-IIa* (23 strains, 22.0%). All the studied isolates were negative for *aac(3)-Ia*, *aac(3)-IVa*, *ant(4")-IIa* and methylases (*armA*, *rmtB*, *rmtC* and *npmA*)

Eighty-one (77.1%) isolates contained only one of the evaluated AME genes and 16 (15.2%) isolates harboured two of them. The combination of *aac(6´)-Ib* and *aac(3)-IIa* was the most common one (8 isolates, 7.6%), followed by *aac(3)-IIa* plus *aph(3´)-Ia* (4 isolates, 3.8%). Two isolates harboured three genes (*aac(6´)-Ib*, *aac(3)-IIa* and *aph(3´)-Ia*). In six (5.7%) isolates, none of the investigated genes were identified, three of these isolates were resistant to gentamicin (MIC, 32 mg/L), one was resistant to kanamycin (MIC, 128 mg/L), one was resistant to both gentamicin and tobramycin (MIC, 32 mg/L and 8 mg/L, respectively) and the remaining isolate was resistant to both kanamycin and neomycin (Table 2).

The *aac(6')-Ib* gene was detected in 36 isolates (34.3%). All five (1.9%) isolates resistant to amikacin contained the *aac(6´)-Ib* gene. Although it has been described that the *aac(6´)-Ib* gene confers resistance to amikacin, kanamycin, tobramycin, and netilmicin [7], when applying EUCAST breakpoints only four out of the 25 isolates (16%) containing only the *aac(6´)-Ib* gene, presented this specific pattern, while the remaining 21 isolates were resistant to one or more of gentamicin, tobramycin, netilmicin or kanamycin, but not to amikacin.

Using breakpoints established by CLSI none of isolates carrying the *aac(6')-Ib* gene had the expected resistance phenotype to aminoglycosides; in fact, this gene was detected in one strain susceptible to all tested aminoglycosides (Table 2).

The *aac(6')-Ib-cr* variant was found in 34 of the 36 (94.5%) *aac(6´)-Ib* positive isolates. All the 36 isolates with the *aac(6')-Ib* (independently that this were the –cr variant or not) were resistant to ciprofloxacin, levofloxacin, norfloxacin and nadilixic acid; as the –cr variant is known to confer only low level ciprofloxacin and norfloxacin resistance [19] additional mechanisms of chromosomal quinolone resistance probably co-exist in our isolates.

The second most frequent gene was *aph(3')-Ia*, detected in 31 (29.5%) isolates. Of the twenty one isolates positive only for *aph(3')-Ia* gene, 20 isolates had specific pattern resistance to kanamycin and neomycin, and only one isolate was also resistant to gentamicin (Table 2). All isolates with alone *aph(3´)-Ia* had a MIC $\geq$256 mg/L for kanamycin.

The *ant(2'')-Ia* gene was found in 29 (27.6%) isolates. All of them showed the expected resistance phenotype (gentamicin, tobramycin and kanamycin) when using the EUCAST breakpoints, while 21 isolates had the expected phenotype by CLSI (Table 2).

Finally, the *aac(3)-IIa* gene was present in 23 strains (22%). Eight out of the 9 isolates with only *acc(3)-IIa* had a phenotypic resistance pattern consistent with that described for this gene (resistance to gentamicin, tobramycin and netilmicin). It is remarkable that MICs of gentamicin (64-128 mg/L) were higher than those of tobramycin (8-64 mg/L).

As a control for our results, PCR analysis revealed the absence of AME genes in 25 isolates susceptible to the evaluated aminoglycosides.

Although in this study none of the isolates showed high-level resistance (MIC> 128 mg/L) to tested aminoglycosides, ten strains with MIC of amikacin ≥ 16 mg/L were screened for *armA*, *rmtB*, *rmtC* and *npmA* 16S rRNA methylase genes by PCR and all of them were negative for these genes.

Phenotypic detection of resistance did not correlate well with the PCR results of genes investigated in this study. Using EUCAST criteria sixty-four (64.6%) isolates were concordant between resistance phenotypes observed and PCR results (100% concordance for strains with *ant(2'')-Ia* gene). By CLSI, we observed agreement between the resistance phenotypes and the AME genotype in 46 (46.5%) isolates.

3.3 Correlation between AMEs genes detected and mechanisms of resistance to amoxicillin-clavulanate.

The percentages of strains resistant to aminoglycosides considering the mechanisms of resistance to amoxicillin-clavulanate are presented in Table 3. Interestingly, isolates producing OXA-1 (alone or combined with an ESBLs) were more often resistant to an aminoglycoside than isolates with others AMC-resistant mechanism, in contrast isolates hyperproduced chromosomal AmpC or producers inhibitor-resistant TEM (IRTs) were significantly more susceptible to aminoglycosides.

The different combinations of AME genes obtained and the mechanism of resistance to AMC [4] are shown in Table 4. Among the 105 aminoglycoside-resistant *E. coli* characterized in

this study, 59 isolates (56.2%) produced OXA-1, 19 isolates (18.1%) produced a plasmidic AmpC, 16 isolates (15.2%) overproduced a TEM-1, 11 isolates (10.5%) hyperproduced chromosomal AmpC and 10 isolates (9.5%) were inhibitor-resistant TEM (IRTs) producers. In addition, a total of 27 isolates (25.7%) were ESBLs-producers (25 isolates CTX-M-15 and two isolates CTX-M-14 producers), all but one of them had an AMC resistance mechanism mainly OXA-1 (24 isolates, 88.8%).

As shown in the table 4, the OXA-1 gene alone (the most prevalent mechanism of resistance to AMC [4]) was usually associated with *ant(2'')-Ia* (21 isolates, 70%), all isolates with *ant(2'')-Ia* belong to the clone ST88 phylogroup A ($p<0.0001$) [20]. On the other hand, 18 strains (17.1%) producing both OXA-1 and ESBL (CTX-M-15) contained *aac(6´)-Ib-cr* alone or in combination with another AME gene. In nine strains producing only an IRTs, five and four strains with only *aph(3´)-Ia* and *aac(3)-IIa* have been found respectively. The *aph(3´)-Ia* gene was present in 8 out of 13 (61.5%) strains producing plasmidic-AmpC and in 4 out of 6 (66%) hyperproducing chromosomal-AmpC.

The *ant-(2'')-Ia* and *aph(3´)-Ia* genes were more frequent in strains of community origin (20/64; 31.25%) than the *aac(6´)-Ib* (18/64, 28.16%) or *aac(3)-IIa* (14/64; 21.8%) genes. In contrast, in strains of nosocomial origin the most frequent AME gene was *aac(6´)-Ib*, which was present in 18/41 (44%) isolates, while 26,8%, 22% and 19.5% of these isolates contained the *aph(3´)-Ia, ant-(2'')-Ia* and *aph(3´)-Ia* genes, respectively.

4. Discussion

The overall incidence of aminoglycoside resistance found in our study was higher than the incidence that has been presented in other reports, which may reflect our bias in considering bacteria with a defined resistance phenotype, rather than unselected isolates [21,22,23].

In a recent study in Spain the percentages of resistance obtained in 330 aminoglycoside-resistant enterobacteria, (80% *E. coli*) were 26.3% to kanamycin, 18% to gentamicin, 17% to tobramycin, 3.6% to netilmicin and 1.5% to amikacin [21]. In 20 European University hospitals participating in the European SENTRY Antimicrobial Surveillance Programme the percentages of resistance to gentamicin, tobramycin and amikacin in *E. coli* obtained from three Spanish hospitals were 9.2%, 4.4% and 0.7% respectively [23]. However, in ESBL-producing *E. coli*, the resistance rates were higher than those obtained in our study; resistance to tobramycin, gentamicin and amikacin in a study in Norway were 94%, 73% and 6% respectively [12].

In this study the most common AME gene was *aac(6´)-Ib* followed by *aph(3´)-Ia*, *ant(2'')-Ia* and *aac(3)-IIa*. In a previous study in a single centre in Barcelona (Spain), the most frequent genes were *aph(3´)-Ia* (13.9%), *aac(3)-IIa* (12.4%), *aac(6´)-Ib* (4.2%) and *ant(2'')-Ia* (3.6%) [21]. In *Enterobacteriaceae* isolated from blood cultures in a hospital in Athens, the *aac(6´)-Ib* gene alone or combined with *aac(3)-I* was the most prevalent mechanism of aminoglycoside resistance [22]. In ESBL-producing *E. coli* isolates the prevalence of *aac(3)-IIa* and *aac(6´)-Ib* genes was more elevated than were detected in this study; 77.6% and 47.8% respectively [12]. In 50 carbapenem-resistant *K. pneumoniae* strains, *aac(6´)-Ib* gene was the most prevalent AME detected in 98% of strains [14]. In other study, the most prevalent resistance gene in Europe [24] was *aac(3)-II*.

In our study, only five (1.9%) strains were amikacin-resistant, and all of them produced the *aac(6´)-Ib* gene. However, low MIC values of amikacin despite the possesion of *aac(6')-Ib-cr* have been described [25]. The explanation could be either an impaired gene expression or that amikacin is a poor substrate for this enzyme [26]. Kim *et al*. [27] have also found a very high percentage of amikacin-susceptible *Enterobacter cloacae* isolates harbouring the *aac(6´)-Ib* gene (84.5% and 55.2% according to the CLSI and EUCAST breakpoints, respectively) with only two isolates containing mutations associated with the loss of amikacin resistance. Recently Almaghrabi *et al*. [14] did not find correlations between AMEs and amikacin MICs or resistance in carbapenem-resistant *K. pneumoniae* strains.

The second most frequent gene in this study was *aph(3')-Ia* that confers resistance to kanamycin and neomycin and is widely distributed, mainly among Gram-negatives within wide host range plasmids and transposons [7]. As kanamycin is not prescribed in Spain, the persistence of this gene may be related to its genetic linkage with other resistance genes. In *aac(3)-IIa* positive isolates, for which resistance to gentamicin, tobramycin and netilmicin is expected, it is remarkable that the MICs of gentamicin (128-64 mg/L) were higher compared with tobramycin (64-8 mg/L). It has been described that the encoded enzyme has a stronger affinity for gentamicin than for tobramycin or netilmicin [11]. All of the 23 isolates positive for *aac(3)-IIa* had MIC values for netilmicin above to 4 mg/L, that it is the clinical breakpoints by EUCAST, however only six isolates (26.1%) were defined as resistant by CLSI (MIC $\geq$ 32 mg/L); thus showing that the CLSI breakpoints for netilmicin does not reliably indicate the presence of this gene.

We observed frequent disagreement between the resistance phenotypes and the AME genotype in 35 (35.4%) or 53 (53.5%) isolates, according to the EUCAST and CLSI

breakpoints respectively. It is possible that these differences are related to some extend to the fact that adequate breakpoints are still to be defined for some compounds and that EUCAST epidemiological breakpoints for some compounds are really different of the CLSI clinical breakpoints. In *E. coli*, Davis *et al*. [28] observed genotypic-phenotypic discrepancies to aminoglycosides in 24.7% of the strains and characterized mutations or deletions in inactive AMEs genes in isolates where the gene was present but the expected corresponding phenotype resistance was absent.

CTX-M-15 plasmid-mediated dissemination of *aac(6´-Ib-cr* among *Enterobacteriaceae* isolates has been observed in multiple European countries [29,30]. We have also found that 23 out of 25 strains producing CTX-M-15 had the *aac(6´)-Ib-cr* gene of which 69.5% belonged to clonal complex ST131 and 30.5% were ST23 or ST10 [20].On the other hand, it is also remarkable that in five out 11 isolates hyper-producing chromosomal AmpC harboured the *acc(6´)-Ib* gene, in contrast with results of Lindemann *et al*. [12] who did not find *aac(6´)-Ib* in any of isolates with AmpC hyperproduction.

In conclusion, the most notable finding of this study was that strains exhibited a remarkable AME diversity. Overall, we identified nine AME patterns, which correlated with different levels of aminoglycoside resistance. The *aac(6')-Ib* enzyme was most common gene detected and it was associated with strains producing CTX-M-15 while the *ant(2'')-Ia* gene was usually associated with OXA-1. For 35.4% isolates the aminoglycoside resistance phenotype was an inadequate predictor of the AME genotype, suggesting unsuitable setting of breakpoints for aminoglycosides or the contribution of multiple concurrent resistance mechanisms. A full understanding of AMEs and other molecular mechanisms of diminished susceptibility to currently available aminoglycosides will allow clinicians to incorporate these

agents most rationally into treatment regimens against amoxicillin-clavulanate resistant *E. coli* infections

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Declarations

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Table Legends

Table 1. *In vitro* activity of eight aminoglycosides against 257 AMC-resistant *E. coli*.

Table 2. Prevalence of aminoglycoside-modifying enzymes (AME) genes and correlation between antibiograms and AME genes in 105 AMC-resistant *E. coli*.

Table 3. Number of isolates resistant to aminoglycoside considering the mechanism of resistance to amoxicillin-clavulanate in 257 *E. coli*

Table 4. Correlation between aminoglycosides-modifying enzymes (AMEs) genes obtained and mechanism of resistance to amoxicillin-clavulanate in 105 *E. coli*.

Supplementary Table

Table S1. Primers used in detection of aminoglycoside-modifying enzymes (AME) and methyltransferases genes and expected amplicon sizes.

Table S2- Phenotypic profiles to aminoglycosides observed among the 105 AMC-resistant *E. coli*.

Table

Table 1. *In vitro* activity of eight aminoglycosides against 257 AMC-resistant *E. coli*.

				Clinical breakpoints		Resistant (%)		
Antimicrobial agent	Range (mg/L)	MIC_{50} (mg/L)	MIC_{90} (mg/L)	EUCAST	CLSI	EUCAST	CLSI	*P*-value
Gentamicin	0.125-128	0.5	32	>4	≥16	60 (23.3)	55 (21.4)	0.672
Tobramycin	0.125-128	0.5	32	>4	≥16	79 (30.7)	66 (25.7)	0.239
Amikacin	0.25-64	2	8	>16	≥64	5 (1.9)	1 (0.4)	0.216
Kanamycin	1-256	4	128	>8[a]	≥64	91 (35.4)	83 (32.3)	0.514
Neomycin	0.125-128	1	32	>8[a]	NA	30 (11.6)	NA	
Netilmicin	0.125-128	0.25	16	>4	≥32	44 (17.1)	9 (3.5)	**<0.0001**
Apramycin[b]	1-16	4	8	>8[b]	NA	4 (1.5)	NA	
Arbekacin[c]	0.25-32	0.5	2	NA	NA	NA	NA	

[a] For kanamycin and neomycin, we used EUCAST epidemiological cut-off values (ECOFFs).

[b] For apramycin epidemiological breakpoints from the European Food Safety Authority (EFSA) were used.

[c] NA, not available. Breakpoints have not been established by EUCAST or CLSI for arbekacin. Statistically significant values are in bold type. The clinical breakpoints and ECOFFs are done according to the EUCAST guidelines (http://www.escmid.org/sites/).

Table 2. Prevalence of aminoglycoside-modifying enzymes (AME) genes and correlation between antibiograms and AME genes in 105 AMC-resistant *E. coli*.

AME genes	No. of isolates (%)	Expected phenotype resistance	Observed phenotype resistance (no. isolates) EUCAST	Observed phenotype resistance (no. isolates) CLSI
ant(2")-Ia	26 (24.8)	Gm,K,To	as expected (26)	as expected (21) Gm,To (2) K,To (1) K (1) Susceptible (1)
aac(6´)-Ib	25 (23.8)	Ak,K,Nt,To	as expected (4) K,Nt,To (16) Gm,K,To (2) K,To (2) To (1)	K,Nt,To (3) K,To (17) To (2) Gm (1) K (1) Susceptible (1)
aph(3´)-Ia	21 (20.0)	K,Nm	as expected (20) Gm,K,Nm (1)	as expected (20) Gm,K (1)
aac(3)-IIa	9 (8.6)	Gm,Nt,To	as expected (8) Gm,K,Nt,To (1)	as expected (2) Gm,To (2) Gm (5)
aac(6´)-Ib, aac(3)-IIa	8 (7.6)	Ak,Gm,K,Nt,To	as expected (1) Gm,K,Nt,To (7)	as expected (1) Gm,K,Nt,To (2) Gm,K,To (4) Gm,To (1)
aac(3)-IIa, aph(3´)-Ia	4 (3.8)	Gm,K,Nm,Nt,To	as expected (3) Gm,Nt,To (1)	Gm,K,To (2) Gm,K (1) Gm (1)
ant(2")-Ia, aph(3´)-Ia	3 (2.9)	Gm,K,Nm,To	as expected (2) Gm,K,To (1)	as expected (2) K,To (1)
aac(6´)-Ib, aph(3´)-Ia	1 (1.0)	Ak,K,Nm,Nt,To	Gm,K,Nt,To (1)	Gm,K,To (1)
aac(6´)-Ib, aac(3)-IIa, aph(3´)-Ia	2 (2.0)	Ak,G,K,Nm,N,T	Gm,K,Nm,Nt,To (2)	Gm,K,Nt,To (1) Gm,K,To (1)
None	6 (5.7)		Gm (3) K (1)	Gm (4) K (2)

		Gm,To (1)
		K,Nm (1)
Total no. of isolates	105 (100)	

Ak, amikacin; Gm, gentamicin; K, kanamycin; Nm, neomycin; Nt, netilmicin; To, tobramycin.

Table 3. Number of isolates resistant to aminoglycoside considering the mechanism of resistance to amoxicillin-clavulanate in 257 *E. coli*.

	No of isolates with resistance* to (%)								
Mechanisms of resistance to AMC (no. of isolates)	Ak	Gm	K	Nm	Nt	To	Aminoglycoside resistant	Aminoglycoside susceptible	*P*-value
OXA-1 (35)	1	25	30	2	8	29	30 (11.7)	5 (1.9)	**<0.0001**
TEM-1 (50)		6	11	9	4	5	15 (5.8)	35 (13.6)	0.158
p-AmpC (40)		12	8	6	5	9	13 (5.1)	27 (10.5)	0.386
c-AmpC (41)		1	6	4		2	6 (2.3)	35 (13.6)	**0.0009**
IRTs (42)		4	5	5	4	4	9 (3.5)	33 (12.8)	**0.016**
ESBLs (1)		1	1			1	1 (0.4)		
OXA-1/ ESBLs (19)	4	6	18	2	16	18	18 (7.0)	1 (0.4)	**<0.0001**
p-AmpC/ OXA-1 /ESBLs / or both (10)		3	5		3	6	6 (2.3)	4 (1.6)	0.327
c-AmpC/ OXA-1/ ESBLs / or both (7)		2	5		4	5	5 (1.9)	2 (0.8)	0.132
TEM-1/ ESBLs (4)			1	1			1 (0.4)	3 (1.2)	0.648
IRTs/ ESBLs (3)			1	1			1 (0.4)	2 (0.8)	1.000
SHV-1 (5)								5 (1.9)	
Total (257)	5	60	91	30	44	79	105 (40.9)	152 (59.1)	

* EUCAST Breakpoints. Ak, amikacin; Gm, gentamicin; K, kanamycin; Nm, neomycin; Nt, netilmicin; To, tobramycin.

Bolface data indicate statistically significant differences.

OXA-1, production of OXA-1; TEM-1, hyperproduction of TEM-1; p-AmpC, production of plasmidic AmpC; c-AmpC, hyperproduction of chromosomal AmpC; IRTs, production of inhibitor-resistant TEM; SHV-1, hyperproduction of SHV-1; ESBLs, production of extended-spectrum β-lactamases.

Table 4. Correlation between aminoglycosides-modifying enzymes (AMEs) genes obtained and mechanism of resistance to amoxicillin-clavulanate in 105 *E. coli*.

AMEs genes	Mechanisms of resistance to AMC (no. of isolates)												
	OXA-1 (30)	TEM-1 (15)	p-AmpC (13)	c-AmpC (6)	IRTs (9)	ESBLs (1*)	OXA-1/ ESBLs (18)	p-AmpC/ OXA-1 (4)	c-AmpC/ OXA-1 (1)	TEM-1/ ESBLs (1*)	IRTs/ ESBLs (1)	p-AmpC/ OXA-1/ ESBLs (2)	c-AmpC/ OXA-1/ ESBLs (4)
aac(6')-Ib (25)	4		1	1			13	1	1			2	2
ant(2'')-Ia (26)	21	1				1		2					1
aph(3')-Ia (21)	1	8	2	3	5					1	1		
aac(3)-IIa (9)		4	1		4								
aac(6')-Ib, aac(3)-IIa (8)	3						3	1					1
ant(2'')-Ia, aph(3´)-Ia (3)			2	1									
aac(3)-IIa, aph(3´)-Ia (4)	1		3										
aac(6')-Ib, aph(3´)-Ia (1)							1						
aac(6')-Ib, aac(3)-IIa, aph(3´)-Ia (2)			1				1						
None (6)		2	3	1									

OXA-1, production of OXA-1; TEM-1, hyperproduction of TEM-1; p-AmpC, production of plasmidic AmpC; c-AmpC, hyperproduction of chromosomal AmpC; IRTs, production of inhibitor-resistant TEM; ESBLs, production of extended-spectrum β-lactamases (*two isolates CTX-M-14 and the others CTX-M-15 producers).

Supplementary data

Click here to download Supplementary data: Supplementary Table S1 and S2 (22-12-14).doc