Inhibitor-Resistant TEM- and OXA-1-Producing Escherichia coli Isolates Resistant to Amoxicillin-Clavulanate Are More Clonal and Possess Lower Virulence Gene Content than Susceptible Clinical Isolates.

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Inhibitor-resistant TEM and OXA-1 producing *Escherichia coli* isolates resistant to amoxicillin-clavulanate are more clonal and possess lower virulence gene content than susceptible clinical isolates

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Abstract

In a previous prospective multicenter study in Spain, we found that OXA-1 and inhibitor resistant TEM (IRT) β-lactamases constitute the most common plasmid-borne mechanisms of genuine amoxicillin-clavulanate (AMC) resistance in *E. coli*. In the present study, we investigated the population structure and virulence traits of clinical AMC-resistant *E. coli* expressing OXA-1 or IRT and compared these traits to those in a control group of clinical AMC-susceptible *E. coli*. All OXA-1- (n=67) and IRT- (n=45) producing isolates were matched by geographical and temporal origin to the AMC-susceptible control set (n=56). We performed multilocus sequence typing and phylogenetic groups characterization for each isolate and then studied the presence of 49 virulence factors (VF) by PCR and sequencing. The most prevalent clone detected was distinct for each group: C/ST88 was most common in OXA-1 producers, B2/ST131 in IRT producers, and B2/ST73 in AMC-susceptible isolates. The median of isolates per sequence type (ST) were 3.72 in OXA-1 producers, 2.04 in IRT-producers, and 1.69 in AMC-susceptible isolates; the proportions of STs represented by one unique isolate were 19.4%, 31.1%, and 48.2%, respectively. The sum of all VFs detected, calculated as a virulence score, was significantly higher in AMC-susceptible isolates compared with OXA-1and IRT producers (mean, 12.5 *versus*. 8.3 and 8.2 respectively). Our findings suggest that IRT and OXA-1 producing *E. coli* isolates resistant to AMC have a different and less diverse population structure than AMC-susceptible clinical *E. coli* isolates. The AMC-susceptible population also contains more VF than AMC-resistant isolates.

Introduction

Escherichia coli is an important etiologic agent that causes both nosocomial- and community-acquired infections (1). Amoxicillin-clavulanate (AMC) is a widely used antibiotic in many countries, often to treat *E. coli* infections (2,3). According to the European Antimicrobial Resistance Surveillance Network, the percentage of *E. coli* blood isolates in Spain that are non-susceptible to AMC has increased from 9.3% in 2003 to 25.3% in 2012 (EARS-Net [http://ecdc.europa.eu/en/activities/surveillance/EARS-Net/database/Pages/database.aspx]). This increase in resistance coincides with a dramatic increase in community consumption of AMC (4).

AMC resistance in *E. coli* results from a complex epidemiological background involving clonal and non-clonal spread of several resistance mechanisms (5). Production of OXA-1 and inhibitor-resistant TEM (IRT) β-lactamases are the most common plasmid-borne mechanisms of AMC resistance in *E. coli* that do not affect other broad spectrum β-lactam antibiotics (5). Clinical data about patients infected by AMC- resistant *E. coli* have also been provided by our group (6). However, there is very little information available about the population structure and virulence-associated determinants of OXA-1 and IRT-producing *E. coli* compared with AMC-susceptible clinical isolates.

Most extraintestinal pathogenic *E. coli* (ExPEC) isolates belong to phylogenetic group B2 and, to a lesser extent, to group D. ExPEC possess high numbers of virulence-associated factors (VFs), including toxins, adhesins, polysaccharide capsules, siderophores, and invasins that may enable them to evade host defenses and invade host tissues. *E. coli* isolates of phylogroups A and B1 are mainly found as part of the intestinal commensal population and usually possess a lower number of VFs (7,8). In *E. coli* several studies have evaluated the linkage between virulence and resistance to antimicrobials such as quinolones, trimethroprim/sulfamethoxazole, or cephalosporins. Most of these studies have shown that antibiotic susceptible *E. coli* isolates are usually more virulent than resistant ones (9-11). However, in the last few years, B2 *E. coli* clones possessing a high number of

VFs and resistance to several antimicrobials have emerged (i.e. ST131) (12-14). According to recent data (15), eight phylo-groups are now recognized: seven (A, B1, B2, C, D, E, F) belong to *E. coli sensu stricto*, whereas the eighth is the *Escherichia* cryptic clade I.

Our hypothesis was that AMC, the antibiotic most consumed by far in Spain and other countries (3,4), could select not only AMC resistance but also certain specific clones carrying AMC resistance (16). To clarify this subject, the population structure of both resistant and susceptible isolates was studied in parallel. The objective of this study was to determine the population structure and virulence traits of clinical AMC-resistant *E. coli* due to the OXA-1 or IRT-production in comparison with a control group of clinical AMC-susceptible *E. coli*.

Material and Methods

Study design and bacterial isolates

As described previously (5), 257 non-duplicated, AMC-resistant *E. coli* were collected from clinical samples at seven Spanish hospitals in six geographic regions between January and March of 2010. Of them, all 112 (43.6%) isolates producing either OXA-1 (n=67) or IRT (n=45) were included in this study. The IRT types were TEM-40 (n=15), TEM-30 (13), TEM-33 (5), TEM-32 (2), TEM-34 (2), TEM-35 (1), TEM-54 (1), TEM-76 (1), TEM-79 (1), and TEM-185 (4). Among the 67 OXA-1-producing isolates, 25 (37.3%) were also CTX-M-15 producers.

Additionally, 56 AMC-susceptible (MIC<4/2 μg/mL) clinical isolates were simultaneously collected at the participant hospitals to constitute the AMC-susceptible control group. These AMC-susceptible isolates were matched by geographical and temporal origin and were susceptible to several other β-lactam antibiotics. Susceptibility to AMC and other antibiotics including ampicillin, cephalosporins, carbapenems, quinolones, aminoglycosides, and cotrimoxazole was confirmed at the central reference laboratory as described previously (5).

In total, 168 *E. coli* isolates were included in this study: 67 producing OXA-1, 45 producing IRTs, and 56 AMC-susceptible. Origin of the all *E. coli* isolates included in this study is detailed in Table 1.

Molecular epidemiology, phylogenetic groups and detection to O25b and O16 serotypes

Multilocus sequence typing (MLST) and phylogenetic groups were determined in all 168 *E. coli* isolates. MLST was performed according to the University College Cork (Ireland) scheme for *E. coli* developed by M. Achtman et al. (http://mlst.ucc.ie/mlst/dbs/Ecoli). The phylogenetic relationships among the different sequence types (STs) obtained were established according to the eBURST program version 3 (http://linux.mlst.net/burst.htm; July 2013, data last accessed).

In addition, serotypes O25b and O16 were identified by allele-specific PCRs as described (17,18).

Phylogenetic groups were determined both by the former and by the recently updated Clermont et al. method (15, 19).

Virulence factors

In all 168 *E. coli* isolates, the presence of 49 virulence-associated genes, including 19 adhesins, four siderophores, 11 toxins, six capsule synthesis-associated genes and nine miscellaneous VF genes were determined by multiplex PCRs using primers described previously (20-22). Virulence scores were calculated for each isolate as the sum of all VFs detected; *pap*, *sfa/foc*, and *clbB/N* were counted only once regardless of the number of elements or subunits identified.

Statistical analysis

Differences in the prevalence of phylogroups and sequence types between the different groups were assessed by Fisher's exact test. Associations were determined by the calculation of the

odds ratio (OR) with 95% confidence intervals (CIs). The null hypothesis was rejected for values of $P<0.05$. Statistical analysis was performed using GraphPad Prism, version 3.02, software (GraphPad Software, Inc., San Diego, CA). Virulence scores were compared with the Mann-Whitney *U* test.

Results

Phylogenetic groups

Phylogenetic groups distribution is depictured in Figure 1. OXA-1 producers mostly belonged to phylogroup C and B2; most IRT-producers belonged to phylogoup B2, and the vast majority of susceptible isolates were of the B2 phylogroup (Table 1).

E. coli isolates producing OXA-1 but not CTX-M-15 ESBL belonged to phylogroup C, but the majority of isolates (78.3%) producing OXA-1 plus CTX-M-15 belonged to phylogroup B2.

Of the 168 *E. coli* isolates, 55 (32.7%) belonged to the newly described phylogroups C (29.1%) or F (3.6%). No phylogroup E isolates were detected.

By the former Clermont method (19), 49 (29.2%) isolates were classified as phylogroup A; however, 45 of them (91.8%) were reclassified as phylogroup C.

MLST results

Eighteen different STs were identified among the 67 OXA-1-producing isolates (mean of isolates per ST: 3.72, range: 1-25). Twenty-two different STs were identified among the 45 IRT-producing isolates (mean of isolates per ST: 2.04, range: 1-8), and the 56 AMC-susceptible isolates were identified as 33 different STs (mean of isolates per ST: 1.69, range: 1-12) (Table 1, Table 2). The proportions of STs represented by one unique isolate were 19.4% (OXA-1 group), 31.1% (IRT group), and 48.2% (AMC-susceptible group) (Table 1).

The most prevalent ST was different for each group. In the OXA-1-producing isolates, ST88 (25 isolates, 37.3%) and ST131 (22, 32.8%) were the most common. In contrast, the IRT-producing isolates were most commonly ST131 (8, 17.8%), ST73 (5, 11.1%), and ST23 (5, 11.1%). Finally,

149 ST73 (12, 21.4%) and ST95 (5, 8.9%) were most common among the AMC-susceptible isolates
150 (Table 2). Four novel STs were identified: STs 3292, 3312, and 3361 in the AMC-susceptible group
151 and STs 2817 and 3312 in the IRT-producing group.

152 To determine whether a specific sequence type was significantly correlated with an AMC
153 resistance mechanism, we conducted further statistical analysis. We found that a number of sequence
154 types were significantly more prevalent in one specific group. ST131 was more prevalent in the
155 OXA-1-producing group (P=0.0001) and in the IRT-producing group (P=0.06), and in than in the
156 AMC-susceptible group (Table 1). ST88 was significantly more prevalent in the OXA-1-producing
157 group than in the IRT-producing and AMC-susceptible groups (P<0.0001) (Table 1). ST73 was
158 significantly more prevalent in AMC-susceptible group than in OXA-1-producing group (P=0.0005)
159 (Table 1). ST131 and ST73 isolates were detected in all seven participating hospitals, and ST88 was
160 detected in five of them.

161 Isolates of ST131, ST73, and ST95 belonged to the phylogroup B2, and isolates of ST88,
162 ST23, and ST10 isolates belonged to the phylogroup C.

163 Most OXA-1- and CTX-M-15- producing isolates belonged to ST131 (18/25, 72%) while
164 isolates only producing OXA-1 without CTX-M-15 belonged mainly to ST88 (25/48, 52.1%).

165 All except four B2/ST131 isolates belonged to O25b serotype, three of the non-O25b isolates
166 (two IRT-producers and one AMC-susceptible) were serotype O16 and the other one was non-O25b
167 non-O16 serotype.

168

169 *Virulence factors*

170 To determine whether there was a relationship between virulence factors and resistance mechanisms,
171 we thoroughly screened the isolates for 49 different VFs and analyzed their virulence relative to the
172 AMC resistance mechanisms present. Data showing the virulence gene content among OXA-1-
173 producing, IRT-producing, and AMC-susceptible isolates are summarized in Table 3. Most of the 49

VFs studied were more frequently detected in the AMC-susceptible isolates than in the OXA-1- or IRT-producing isolates. Nineteen VFs were significantly associated to the AMC-susceptible group, whereas only six were associated to the OXA-1 group (P=0.005; Table 3). Overall, the AMC-susceptible group exhibited a significantly higher virulence score in comparison with the OXA-1 group (mean virulence score 12.5 *versus* 8.3; P<0.0001). In relation to the AMC-susceptible and the group producing IRTs, 12 VFs were significantly associated with the AMC-susceptible group whereas only one was associated to the IRT group (P<0.001; Table 3); AMC-susceptible group also exhibited a significantly higher virulence score in comparison with the IRT group (mean score, 12.5 *versus* 8.2; P<0.0001). Overall, these data suggest that AMC-susceptible isolates may have a high potential of virulence.

Virulence factors in B2 and non-B2 isolates

In order to determine whether there was a relationship between phylogroups and virulence gene content, we next analyzed these data relative to each phylogroup. All isolates, whether AMC-susceptible or AMC-resistant, that belonged to phylogroup B2 exhibited a higher number of VFs and consequently a higher virulence score than non-B2 strains (supplementary Table A). The highest virulence scores were observed in the B2-AMC-susceptible isolates, which possessed a mean VF score of 13.5 in comparison with the non-B2-AMC-susceptible isolates which had a mean VF score of 10.2 (P=0.021). The lowest virulence scores were observed in the non-B2 AMC-resistant isolates, and within this group the OXA-1 and IRT producers possessed a mean virulence score of 7.7 and 7.3, respectively. The virulence gene content of the isolates belonging to phylogenetic groups B2 (53.4%) and non-B2 (46.6%) in relation to the AMC-resistance mechanism is summarized in supplementary Table S1. Overall, we found that, as expected, phylogroup B2 contained the greatest number of virulence factors and the highest virulence score.

Virulence factors in isolates of the most prevalent sequence types

To determine whether there was a relationship between the most prevalent clones detected and the virulence traits, we next performed statistical analysis on these parameters, as shown in Table 3. ST131 isolates exhibited similar virulence score and range of VF (mean: 9.1; range: 5-13) as isolates belonging to ST88 (mean 9.2; range: 5-13). ST73 isolates possessed the highest virulence score (mean: 13.2; range: 8-18). There were more virulence factors statistically associated to B2/non-ST131 isolates than to B2/ST131 isolates (13 *versus* 6), and the virulence score was significantly higher in the former (mean 12.5 and 9.1, respectively; $P<0.001$). In contrast, the analysis comparing B2/ST73 and B2/non-ST73 isolates revealed that the former had a significantly higher virulence score than the latter (mean 13.2 *versus*. 10.7, respectively; $P=0.003$) (Table 3). In relation to ST88 clone, C/ST88 isolates possessed a higher virulence score in comparison with C/non-ST88 isolates (mean of 9.2 *versus* 6.6, respectively; $P=0.004$) (Table 3).

Among ST131 isolates, serotypes O16 and O25b showed similar virulence scores and range of VF [mean score 9.3 (range, 7 to 12) and 9.2 (range 5 to 13), respectively]

Discussion

To our knowledge, this is the first study comparing the population structure and virulence-associated genes of AMC-resistant *E. coli* isolates, either producing IRTs or OXA-1, with AMC-susceptible isolates. Historically, the population of *E. coli*, both environmental and human-associated, has been genetically very diverse (23); however the emergence and dissemination of multiresistant and virulent clones of ExPEC have been described recently (24,25), mainly associated to the successful B2/ST131 clone (25,26). Our data suggest that AMC-resistant *E. coli* isolates have a different and less diverse population structure than AMC-susceptible *E. coli*, mainly due to the OXA-1-producing isolates. The mechanism by which antibiotic consumption leads to the selection of certain clones is poorly understood; a recent study suggests that different clones of *E. coli* vary markedly in their

response to antibiotics despite comparable MICs; these results seem to support the ability of antibiotics to select certain successful clones (27).

Association between antimicrobial resistance and virulence in *E. coli* is a controversial topic (9-11,25,26). We observed an inverse relationship between resistance to AMC due to OXA-1 and IRT production and virulence potential. These results are in agreement with previous studies concluding that *E. coli* isolates resistant to non-fluorinated quinolones, fluoroquinolones, or trimethroprim/sulfamethoxazole were associated with reductions in their virulence traits (9-11). In contrast, *E. coli* multiresistant and virulent clones have been described last years (24-26).

The pandemic clone B2/ST131, previously associated with multiple mechanisms of antibiotic resistance (25), was predominant in the isolates producing both OXA-1 and IRTs, but it was uncommon in AMC-susceptible isolates. The virulence profile of B2/ST131 isolates observed in this study was similar to that of other B2/ST131 isolates in Spain, producing other resistance mechanisms such as an extended spectrum β-lactamase (12,13). However, the virulence score of B2/ST131 isolates was lower than that other B2 isolates not belonging to ST131 (mean 9.10 *versus.* 12.5 respectively), which were mainly found in the AMC-susceptible group. This finding is in agreement with a previous study suggesting that ST131 isolates could be less virulent than previously supposed, and less virulent than other B2 non-ST131 clones (14).

Although the genetic diversity detected in AMC-susceptible isolates was great, the high prevalence of isolates belonging to the B2/ST73 clone in this AMC-susceptible group (21.4% of all susceptible isolates) is remarkable. The ST73 linage has been recently found as one of the most prevalent STs in uropathogenic isolates in England (16.6% of 300 isolates) (28) and in isolates causing spontaneous bacterial peritonitis and bacteremia in patients with cirrhosis in France (8% of 110 isolates) (29). Most ST73 isolates were antibiotic susceptible, in accordance with previous studies (28,29); however, ST73 has also been associated with the production of ESBLs of the CTX-M- type in Egypt and Japan (30,31). In addition, our study showed that isolates belonging to ST73

exhibited the highest virulence score (mean of 13.2, Table 3), in agreement with another study showing that ST73 was one of the most virulent clones detected in the UK (28). Although several authors have demonstrated that the overall virulence score of an *E. coli* isolate is directly related to its ability to cause invasive infections and lethality (32,33), a single, specific VF may enhance the virulence potential of a defined strain (34,35) beyond the virulence score.

As we described previously (5), 37.3% of the OXA-1-producing isolates belonged to ST88. ST88 has also been described in association with chromosomal mediated AmpC overproduction in a French hospital (36), but so far its virulence profile had not been reported. Our ST88 isolates belonged to the recently proposed phylogroup C and they possessed a high virulence score (mean of 9.2). Most of the virulence-associated traits of isolates belonging to ST88 were adhesins, protectins, and siderophores that may facilitate persistence and survival in adverse circumstances. Phylogroup C has been previously identified in a virulent strain causing an outbreak in a neonatal ward (37). Interestingly, in this study 30% of *E. coli* isolates belonged to phylogroup C, mainly due to the reclassification of prevalent clonal complexes ST10 and ST23 (Table 2) previously classified as phylogroup A by the former Clermont method (19). In this study, carried out in clinical isolates, phylogroup A was very uncommon (2.4%) in contrast with the 18-28% of prevalence described previously in two different collections of human faecal isolates (15).

Concluding remarks

Our findings suggest that IRT- and OXA-1-producing *E. coli* isolates resistant to AMC have a different and less diverse population structure than AMC-susceptible clinical *E. coli* isolates, mainly due to OXA-1 producers. AMC-susceptible isolates had more VFs than AMC-resistant isolates. We

also provide information about the higher numbers of virulence traits in the B2/ST73 clone compared with the B2/ST131 and C/ST88 clones.

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412 Figure 1. Distribution of phylogenetic groups in 67 OXA-1-producers, 45 IRT-producers, and 56
413 susceptible *Escherichia coli* isolates.

414 Table 1. Different population markers indicating genetic and virulence variations between OXA-1-
415 producing-, IRTs-producing, and susceptible *Escherichia coli* isolates.

Table 2. The distribution of sequence type (ST) and phylogenetic group among IRT-producing-, OXA-1-producing-, and susceptible isolates.

Table 3. Distribution of virulence determinants among IRT producing, OXA-1-producing, and AMC susceptible isolates.