VIRULENCE FACTORS AND ANTIMICROBIAL RESISTANCE OF ESCHERICHIA COLI ST131 IN COMMUNITY-ONSET HEALTHCARE-ASSOCIATED INFECTIONS IN SICILY, ITALY

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Abstract

*Escherichia coli* ST131 is an emerging resistant agent recently called “superbug” in England. This strain is responsible of community-acquired urinary tract infections and nowadays showing increasing resistance to antibiotics like fluoroquinolones and cephalosporins. Survey of virulent bacterial clone is relevant to control its spreading in community.

We aim to assess the circulation of resistant clones *Escherichia coli* ST131 outside of the hospital to prompt control of outbreak in our geographical area.

We selected 105 *E. coli* resistant isolates from community-acquired urinary infections and performed a multiplex PCR to evaluate if they belonged to the ST131 type. We investigated their set of virulence factors; in particular, *kpsMII, papA, sfaS, focG, iutA, papC, hlyD* and *afa* genes, and finally, we evaluated beta lactamases genes and quinolone resistance determinants. *E. coli* ST 131 clone was present in 66.6% of our isolates and showed positivity to a wide range of resistance genes, in particular *blaCTX-M-15* among beta lactamases and plasmid-related quinolone resistance genes (*qnrA, qnrS* and *aac (6’)-Ib-cr*). Moreover, 81% of the strains showed positivity to at least one of the virulence factor genes.

Our results suggested a high presence of *E. coli* ST131 in community. We suggest antibiotic stewardship for outpatient clinicians and facilities to contain the spread of “superbug” agents.

**Keywords:** *Escherichia coli*, urinary tract infections, antibiotics, fluoroquinolones, cephalosporins
Introduction

Antibiotic resistance stands nowadays as one of the most alarming health problems not only inside hospitals, but also in community [1-2]. This ancient bacterium [3] is recently become attractive because resistant to three or more different drug classes at the same time are considerate "Multi Drug Resistant" (MDR) bacteria [4-5]. Escherichia coli is the primary causative agent for community-acquired urinary tract infections (UTI), whose outcome can be complicated by increasing of antimicrobial resistance. E. coli shows resistance mainly against fluoroquinolones, beta-lactams and third-generation cephalosporins. E. coli ST131 is one of the most virulent bacterial clones: it usually carries Extended Spectrum Beta-Lactamases (ESBL), like the ones belonging to blaCTX-M family, and it is now spreading in community environment [5-6], causing extra-intestinal infections. Many Virulence Factors (VF{s}) can help E. coli to induce infections in hosts: they usually allow pathogen to invade host tissues, to trigger an inflammatory response, and to escape immunity response [7-8]; they may include adhesins (P and S fimbriae, F1C), toxins (haemolysins), group II and III capsules, siderophores (aerobactin system) and invasins [9-11]. Antimicrobial resistance, once confined inside hospital ramparts, has now spread in community too, thus becoming an increasing threat in public health [8,10-12]. Fluoroquinolone resistance in E. coli strains is due qnrA, qnrB, qnrS and aac (6')-Ib-cr genes particularly linked to ST131 E. coli clone.

The increasing use of these antimicrobial molecules in therapies against urinary tract infections could have contributed to ST131 broad diffusion [5,8]. Cefalosporin resistance, while, is mediated by Extended Spectrum Beta-Lactamases (ESBL) production, involving blaCTX-m, blaOXA, blatem and blashv genes, which confer resistance to penicillin, broad spectrum cephalosporins and monobactams [13]. blaCTX-M appears to be now the most common ESBL in Enterobacteriaceae like E. coli; there are a lot of genetic variants forming blaCTX-M family. Among these variants, while blaCTX-M -15 seems to be common in human pathogens, thanks to the diffusion of ST131 pandemic clone [14], blaCTX-M -1 can be found more often in various animal microbiota, mainly birds, poultry, and both wild and breeding mammals [9-15].

The object of this study was to describe E. coli clinical isolates from adults patients with UTI in a community in Sicily, Mediterranean region of Southern Italy.

Materials and methods

Strains. 105 E. coli resistance to Norfloxacin, Levofloxacin and Ciprofloxacin, were collected from three different clinical laboratories located in Palermo, Sicily, between April 2014 and December 2015. All E. coli strains were isolated from urinary samples.

Strain identification and antibiotic susceptibility were done by the Vitek automated system as previously reported [16-19].

DNA extraction. Bacterial strains, stored at -80°C in Brain Heart Infusion Broth (BD) with 10% glycerol were inoculated on Columbia Sheep Blood Agar and incubated at 37°C for 18h. A single colony was suspended in 200 µl sterile bi-distilled water and subjected to DNA extraction protocol according to High Pure PCR Template Preparation Kit (Roche) procedure instructions [20].

ST131 type assignment. To evaluate the circulation of ST 131 group, we performed a Multiplex PCR for mdh and gyrB genes, according to James R. Johnson model, which examines certain SNPs associated to these two genes. ST131 positivity is showed by two amplicons, whose length is respectively 275 bp and 132 bp [10]. Reactions were conducted in 25 µl PCR tubes, each one containing Green GoTaq® Flexi Buffer 1 X, MgCl2 4 mM, each dNTPs with a final concentration of 0.8 mM, each primer with a final concentration of 0.75 mM and 1.25 U of GoTaq® Flexi DNA Polymerase (Promega). PCR reactions require an initial denaturation at 94 °C for 5 minutes, then 28 cycles with 30" 94°C denaturation, 30" at 65 °C annealing, 30" at 68 °C elongation and a final 3 minutes extension step at 72 °C.
Plasmid-mediated quinolone resistance (PMQR) aac, qnrA, qnrB and qnrS genes were investigated according to Chi Hye Park model [15]. Each 25 µl PCR tube contained Green GoTaq® Flexi Buffers 1X, 1,5 mM MgCl2, 20 mM of each primer, 2,5 mM of each dNTP, 1 U of GoTaq® Flexi DNA Polymerase (Promega) and 1,5 µl of DNA. PCR reactions were performed with an initial denaturation for 4' at 95°C, followed by 35 cycles with 45'' denaturation at 94°C, 45'' annealing at 55°C, 45'' polymerization at 72°C and a final extension step at 72°C for 10'. Similar conditions applied to qnrA, qnrB and qnrS amplification, only with different annealing temperatures (53°C for qnrA and 57°C for qnrB and qnrS).

Cephalosporin resistance. Beta-lactamases such as OXA, TEM, SHV (Multi TSO) and blaCTX-M-like ESBL were investigated with Multiplex PCR reactions, according to Dallenne model [18]. Multi TSO PCR was conducted in 25 µl tubes, each containing Green GoTaq® Flexi Buffers 1X, 1,5 mM of MgCl2, 2,5 mM of each dNTPs, 1 U of GoTaq® Flexi DNA Polymerase (Promega) and 0,4 pmol/µL of each primer. Reactions were conducted with an initial 94°C denaturation for 10', followed by 24 cycles with 30'' denaturation at 94°C, per 30'' annealing at 61°C and 1' extension at 68°C, with a final extension step at 72°C for 10'. Three bacterial strains were used as positive control: E. coli RS218 strain, E. coli V27 strain and E. coli 2H16 strain.

Results

In the present study, we analyzed 105 quinolone-resistant E. coli isolated from UTI. We found that E. coli ST131 strains were predominant (66.6% ST131 positive; 33.3% non-ST131) among our samples.

The genes aac(6')-Ib-cr, qnrA, qnrB and qnrS correlated to quinolone resistance were found in only 56.2% of our isolates. In particular, aac(6')-Ib-cr and qnrA genes both appeared in 27 of 105 isolates; while 5 of 105 isolates were positive to qnrS. No strain showed positivity to qnrB gene.

About to relation between PQMR genes and ST131 strains, while aac and qnrA were equally present in both ST131 and non-ST131 strains, qnrS presence seems to be higher in non-ST131 strains (Figure 1).

We furthermore analysed for cephalosporin resistance determinants, and specifically for blaOXA blaSHV, blaTEM genes. 67.6% of our E. coli isolates were positive to at least one of these genes, 71 strains out of 105. Their association looked quite infrequent, with only 4 strains showing blaOXA and blaSHV together, and 3 strains showing blaOXA and blaTEM together. No strain showed an association between blaSHV and blaTEM.

Cephalosporin resistance genes have been then related to ST131 and non-ST131 types. Interestingly, blaOXA and blaTEM seem to be more common in ST131 strains, while on the other hand blaSHV seems to be significantly related to non-ST131 strains. In fact, among the 35 positive strains to blaOXA, 28 belong to ST131 and 7 to non-ST131 type; blaSHV showed positivity on 7
non-ST131 strains and 2 ST131, and bla\textsubscript{TEM} appeared positive in 16 ST131 strains and 11 non-ST131 (Figure 2).

We therefore investigated for presence of virulence factors (VFs) in our isolates by using two different multiplex PCRs, each one searching for 4 VFs genes. First multiplex PCR was used to research hly\textit{D}, afa, iut\textit{A} and pap\textit{C} genes (Figure 3\textit{a}), 86.7% of our bacterial strains carries at least one of these VFs genes. Three strains were positive for hly\textit{D} and afa; 10 strains for iut\textit{A} and pap\textit{C} together, 5 strains for afa and iut\textit{A} genes but no strain showed association between hly\textit{D} and iut\textit{A} genes. The most common association appeared to be hly\textit{D} + iut\textit{A} + pap\textit{C}, that we found in 17 out of 105 strains (16.2%). The Multiplex PCR was used to investigate about kps\textit{MII}, pap\textit{A}, sfa\textit{S}, and foc\textit{G} genes presence (Figure 3\textit{b}), 81% of our isolates showed positivity to at least one of them; the most common association was the one between kps\textit{MII} e foc\textit{G}, which we found in 30 strains (28.6%); in addition, all strains carrying pap\textit{A} genes were positive to foc\textit{G} gene too.

hly\textit{D} and pap\textit{A} genes were present only in ST131 strains, while iut\textit{A} appeared to be more common on non-ST131. The remaining VFs genes appeared to be equally distributed on ST131 and non-ST131 isolates.

Finally, we analysed for ESBL bla\textsubscript{CTX-M} gene. We found out that 49 of our samples (46.7%) carried bla\textsubscript{CTX-M} genes and, among these, 35 belonged to ST131. All positive samples were submitted for sequencing, showing bla\textsubscript{CTX-M}G variant for ST131 strains, and bla\textsubscript{CTX-M}\textit{i} variant for the remaining 14 non-ST131 samples.

**Discussion**

Antimicrobial resistance has become a public health priority worldwide. The antibiotic resistance situation is not uniform in EU, and in general higher resistance frequencies are reported by countries in eastern and southern Europe [23]. Italy is one of the European countries with increasing spread of antimicrobial-resistant microorganisms especially in adult and Pediatric Intensive Care Units [24-27] The clinical management infections due to multidrugs gram negative microorganism is difficult and nowadays under debates [28-30]

Knowledge of a recent Escherichia coli associated outbreak prompted interviewers to ask about baking and spreading of new types of E. coli called “superbug” England [31] prompted us to investigate the prevalence of this strain in our geographic area.

In the present study, we focused attention on 105 E. coli strains isolated from patients with UTI, most of our samples came from elderly people living in nursing homes or rehabilitation centres.

E. coli strains of ST131 were found to constitute the majority (66.6%) of quinolone resistant and ESBL producers. These data are in accordance with previous studies illustrating the emerging of resistant ST131 E. coli clone [31]; and they are quickly diffusing and adapting both to hospital and community environments [32,33].

The acc(6\textsuperscript{b})-Ib-cr and qnr\textit{A} genes, which contribute to quinolone resistance, were found in the same percentage of ST131 and non-ST131 strains, while the gene qnr-S were associated more frequently with non-ST131 strains. The latter gene, is still less diffuse in E. coli, and recently qnr-S has been spreading within E. coli strains in other Mediterranean area like Greece [24]; noticeably, this resistance gene appeared to be frequent in pets and courtyard animals and in Italian studies suggesting the role of food chain in antibiotic resistance transmission to humans [8].

The genes acc(6\textsuperscript{b})-Ib-cr, qnr\textit{A}, qnr\textit{B} and qnr\textit{S} correlated to quinolone resistance were found in only 56.2%, we hypnotize that the strains of E. coli in which we didn’t found any PMQR genes, could carry chromosome-located resistance genes, like efflux pumps, or point mutations in gyr\textit{A} or par\textit{C} genes. We therefore focused on ESBL genes, like. In 67.6% of our samples we found at least one of bla\textit{OXA}, bla\textit{SHV}, bla\textit{TEM} and bla\textit{CTX-M} genes. While usually ST131 type appears to be the greatest ESBL producer, we found that SHV is frequent associate to non-ST131 type, as shown in Figure 3\textit{b}.

Nearly half of our isolates belong to ST131 strains showed bla\textsubscript{CTX-M}G, the most common
ESBL in human *E. coli*, while *blaCTX-M-1*, was found in chicken and pigs, occurred among non-ST131 strains, as confirmed elsewhere [35]. Pets and breeding animals are in fact the main reservoir for MDR genes, which spread by food, water and animal dejections [5, 8, 11, 35].

*FocG* gene present in 81 strains, *iutA* gene present in 88 *E. coli* analysed and *kpsMII* gene positive in 34 of our samples were considered to be the most frequent VFs among our strains suggesting a more predominant role for this gene in extra-intestinal pathogen. Finally the presence of *papC* gene in 34 strains and *papA* in 4 strains can support the hypothesis that resistant bacteria could come from breeding animals. Overall, our bacterial strains didn't own a heavy set of VFs genes; it has been underlined the relationship between quinolone resistance and VFs gene reduction, due to the loss of VFs genes or, conversely, to the fact that less virulent strains are more prone to acquire resistance determinants [4, 5, 35].

A separate consideration deserves so-called "frailty patients" that in the community are children, the elderly or immunocompromised subjects such as HIV positive and cancer patients [36-41].

In this category, the spread of resistant pathogens could trigger outbreaks as observed in Canada. For this reason, nowadays educational programs are being proposed in Italy to contain old and new infectious agents through vaccination especially in extreme ages such as children and the elderly [41-45].

In conclusion, we can assume that antimicrobial stewardship requirements to be comprehensive of the involvement of paediatricians and family physicians to control the antibiotic abuse.

References


Figure 1. Percentage of PQMR genes.

Figure 2. Distribution of ESBL genes \((bla_{oxa}, bla_{SHV}, bla_{TEM})\) among ST131 and non-ST131 isolates.
Figure 3. Virulence Factors found in our isolate; a) First multiplex PCR was used to research hlyD, afa, iutA and papC genes presence; b) The Multiplex PCR was used to investigate about kpsMII, papA, sfaS, and focG genes presence.