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Expansion of a subset within C2 subclade of *Escherichia coli* sequence type 131 (ST131) is driving the increasing rates of Aminoglycoside resistance

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Summary:

ST131 clone is not a uniform population, since subclade C2 drive both the higher virulence and resistance among this clone.

A subset of strains with virotypes E and F among C2 subclade have shown different patterns of resistance phenotype and resistance/virulence genes repertoire from the other C2 strains.

There is a local trend within the C2 subclade in which the generated subset have potential advantages over other ST131 population based on resistance/virulence genes content.

Abstract:

Backgrounds: sequence type 131 (ST131) of *E. coli* is a pandemic clone which drives the increasing rates of antibiotic resistance. While the pervasiveness of ST131 clade C, especially subclades C2 and C1-M27 has been demonstrated in numerous global surveys, no report about the ST131 clades and its virotypes has been published from Iran, so far.

Methods: A collection of 73 consecutive ST131 isolates from extraintestinal specimens were investigated for determination of virotypes, antibiotic susceptibility patterns, resistance/ virulence determinants and clades subsets.

Results: Most of isolates belonged to subclade C2 (33/73 [45.2%]) with the highest virulence factor (VF) scores and resistance rates, followed by C1-M27 [18, (24.6%)], C1-non-M27 [14, (19.1%)] and A [8, (10.9%)]. The distinctive profiles of subclade C2 virulence genes were revealed by "principle coordinates analysis" (PcoA) test. The distribution of *hlyA* virulence gene among subclade C2 was not uniform, so that positive strains [21 (63.6%)] showed significantly higher rates of resistance ($bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, *aac(6')-Ib-cr*, *aac(6')-Ib* , *aac(3)-IIa*) and virulence (*hra*, *tia/hek*, *K5*, *cnf*, *papGII*, *papC*) markers, and gentamicin/tobramycin resistance. Virotype C as the most common virotype [34, (46.5%)] was predominant among subclade C1 population, while virotypes E and F [21, (28.7%)] were detected among subclade C2, with the highest VF scores and aminoglycoside resistance rates.

Conclusions: Appearance of virotypes E and F among subclade C2 strains with higher rates of aminoglycoside resistance/virulence genes content shows the shifting dynamics of this pandemic clone in response to antibiotic selection pressure by establishing subsets with higher survival potential.

Keywords: ST131, Iran, virotype, Antimicrobial resistance, Virulence genes, PcoA, Whole-genome sequencing

Background

Sequence type 131 (ST131), the currently emerged clone of *Escherichia coli* which is disseminated worldwide causes severe hospital-acquired and community-onset infections [1,2]. The pervasiveness of ST131 has been reported by many global surveys and increasing prevalence of fluoroquinolone and cephalosporin resistance in *E. coli* population is attributed to this clone [3].

ST131 strains are closely related and appear to have had a common ancestor, so they are often referred to as a clone, or clonal group [4]. However, whole-genome sequencing analysis of ST131 strains has revealed that this clone is not uniform and three different clades, including clades A, B and C are characterized among this clone [5]. Generally, the clades A and B which are minor parts of ST131 population, are susceptible to fluoroquinolone and cephalosporin [6]. In contrast, clade C (also known as *H*30) represents the largest clade and comprises two sub-clades: C1 (or *H*30R) and C2 (*H*30Rx), both of which are resistant to fluoroquinolone [5]. Furthermore, phylogenetic tree analysis and carriage of a unique prophage-like region, have divided subclade C1 to two subsets, named C1-M27 and C1-non-M27 (C1-nM27) [7]. Apart from the extensive antibiotic resistant phenotypes which are identified among these strains, ST131 is also considered as a highly virulent clone due to the higher capability of causing extraintestinal infections as compared to other clones. [6]. This feature is attributed to diverse putative virulence genes harboured by these strains [8].

Despite the highly conserved sequences which are identified in the core genome of ST131, the accessory genome of this clone is much variable and results in differences in virulence genes content and plasmid repertoire [9]. Considering the virulence genes content, the ST131 clone can be categorized into 12 virotypes which are named from A to F [6]. While the

virotype C is reported as the most common virotype among ST131 clone, the other virotypes have not equal distribution among ST131 population reported from different continents [10].

The detection of ST131 and its clades/subclades is important for epidemiological studies. While this being recognized as a pandemic clonal group that threatens public health, ST131 has received less attention in Iran than have other antimicrobial resistant pathogens. The increasing rates of resistance against cephalosporins and fluoroquinolones among *E. coli* isolates from extaintestinal infections and mostly in ST131 population have been reported in recent years from Iran [11,12]. So, in our current study, we aimed to identify the clades and virotypes of ST131 population cultured from extraintestinal specimens during a 19-months surveillance study, and determine the differences of antibiotic susceptibility patterns, virulence and resistance markers between ST131 clades.

Methods:

Strains

In this 19-months cross-sectional study (from March 2015 to September 2016), 338 *E. coli* isolates were cultured from patients with extraintestinal infections admitted to Kosar university hospital of Semnan, Iran. The clinical samples were collected as part of standard care for admitted patients. Isolates were cultured from different specimens including urine, blood, wound and respiratory samples. The genomic DNA of isolates was extracted based on the Cetyl trimethylammonium bromide (CTAB) method [13]. Based on the *gyrB*/*mdh* single nucleotide polymorphism (SNP) multiplex PCR [14], 73 non-duplicate phylogroup B2 ST131 isolates were identified among this bacterial collection. So, the overall prevalence of detected ST131 strains was 21.5%. The O25b/O16 subgroups were determined as described earlier [15]. Allele specific primers for allele 30 of *fimH* corresponding with the main

fluoroquinolone resistance associated subset within this clone were used to identification of *H*30 subclone [16].

Patient Consent Statement

Isolates were taken as part of routine hospital procedure; therefore, patient consent was not required. This study was approved by Ethical committee of Semnan University of Medical Sciences with the ethics code **IR.SEMUMS.REC.1398.219.**

Clades determination

For determination of ST131 clades, the multiplex PCR using seven pairs of primers was used as described by Matsumura et al [17]. Clades and subclades were identified based on the expected amplicons. Amplification was performed using the Ready to use Master Mix (Tempase 2X master mix, Amplicon, Denmark) and recommended concentrations of primers [17].

Virulence factors and virotype determination:

The presence of 35 putative virulence markers was assessed by multiplex PCR [18-21]. Urinary pathogenic *E. coli* (UPEC) isolates were those strains which harboured ≥ 3 of virulence genes including, *yfcV*, *fyuA*, *vat* and *chuA*. The virulence factor (VF) score was the total number of virulence genes detected, adjusted for multiple detection of the *pap* operon [22]. The virotype of the ST131 isolates was established according to the scheme described by Dahbi *et al* [14].

Antimicrobial susceptibility testing

The standard disk diffusion method on Mueller-Hinton agar was used to determine the antibiotic susceptibility patterns of 73 ST131 strains and results were interpreted according to the Clinical and Laboratory Standard Institute (CLSI) guidelines [23]. The number of

antibiotics to which the strain was resistant was considered as resistance score. Isolates with resistance to at least one representative of three or more antimicrobial classes were defined as multidrug resistant (MDR) [24]. ESBL production was assayed using phenotypic combined disk test according to the recommendations of CLSI [23].

Detection of resistance encoding genes

The presence of resistance genes, including ESBLs (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA-1}$ and *bla*$_{CTX-M-15)}$ [25, 26] and plasmid mediated quinolone resistance (PMQR) (*qnrA*, *qnrB*, *qnrS* and *aac(6')-Ib-cr*) was investigated by multiplex PCR according to previously published methods [27]. Furthermore, isolates harbouring the 16S *rRNA* methylase genes (*armA*, *rmtB*, *rmtC*) and aminoglycoside resistance determinants (*aac(3)-IIa*, *aac(6')-Ib*) were detected by single PCR [28,29].

Statistical analysis

In order to compare the proportions and scores, the Fisher's exact test and the Mann-Whitney U-test were used, respectively. The principal coordinate analysis, a multidimensional scaling method analogous to principal component analysis, was used to collapse the molecular data set for simplified between group comparisons [18]. Groups were compared on each of the first three coordinates, which captured most of the variance within the dataset using a two-tailed t-test. *P* values of <0.05 were considered statistically significant.

Results:

Clades determination, O25b/O16 subgroups and virulence genes content:

Multiplex PCR for clades determination revealed the C2 subclade as the dominant subset [33/73 (45.2%)], followed by C1-M27 [18, (24.6%)], C1-non-M27 (C1-nM27) [14, (19.1%)] and A [8, (10.9%)]. Strains of clade A were identified as O16 subgroup and fim*H*30 negative,

while the remaining 65 isolates belonged to O25b subgroup and harboured the fim*H*30 allele. Seven virulence factors, including *sfa focDE*, *colV*, *ibeA*, *papGIII*, *cdtB* and *neuCK* were not detected among study isolates. The 29 virulence markers were detected at least once, with the lowest rate of 1.3% (*papA*, *papEF*, *hlyF*) to the highest rate of 100% (*usp*, *yfcV*, *fyuA*, *chuA*). Except for two C1 subsets (C1-M27, C1-nM27), the other two clades were considerably different in VF content, with the lowest VF score of clade A (median: 14) to subclade C2 with the highest VF score (median: 19).

Among the C1 strains, capsular type *kpsMT II* was significantly detected among C1-nM27, while *papC*, *papGII*, *hlyA*, *cnf1* and *tia/hek* were negatively associated with both of the C1 subsets. Seven virulence markers (*iss*, *hra*, *cnf1*, *hlyA*, *iroN*, *papGII*, *papC* and *tia/hek*) were significantly associated with subclade C2. Furthermore, positive association was found between the carriage of *hlyA* and *papC*, *papGII*, *K5*, *hra*, *tia/hek* and *cnf1* gene within this population.

Principal coordinate analysis based on the 29 virulence determinants revealed that virulence profiles of subclade C2 were distinctive and differentiated them from other strains. Plotted coordinate 1-coordinate 2 plane showed that the variance of 58.77% was captured and subclade C2 isolates were clustered in the lower left quadrant, clearly separated them from other strains (Figure 1). The differences of aggregate virulence profile were explored using univariate analysis. Subclade C2 strains showed higher aggregate virulence score (median) than other clades. All isolates fulfilled molecular criteria for UPEC. Table 1 shows the prevalence of virulence genes among different clades. As part of the other research project, studied isolates were subjected to Whole Genome Sequencing (WGS). The PCR results of virulence factors confirmed based on WGS results obtained from the analyzing of assembled draft genomes using VFDB [30] and VirulenceFinder 2.0 virulence gene databases [31] (data not shown).

Figure 1 position

Table 1 position.

Clades and resistance profiles/resistance determinants:

Clade A was significantly associated with susceptibility to fluoroquinolone. The two C1 sub-clades, showed different patterns of antibiotic susceptibility profile. The C1-M27 was significantly associated with susceptibility to ampicillin/sulbactam, amoxicillin/clavulanate, gentamicin and tobramycin, and all were phenotypically ESBL/MDR, however, no such an association was found for the C1-nM27 subclade. The resistance rates were increased from clade A with median of 3 to subclade C2 (median: 5). Subclade C2 showed significantly higher rates of resistance against gentamicin, amikacin, tobramycin, amoxicillin-clavulanate, ampicillin-sulbactam, nitrofurantoin, and fluoroquinolone. The most frequently detected resistance marker was $bla_{\text{CTX-M-15}}$ which identified among all [45, 61.6%] except subclade C1-M27 strains. Of the resistance markers studied, $bla_{\text{CTX-M-15}}$, *aac(3)-IIa*, *aac(6')-Ib*, *aac(6')-Ib-cr* and $bla_{\text{OXA-1}}$ were associated with subclade C2, while this clade was conspicuous for the low prevalence of bla_{TEM} (P = 0.01) (Table 2). The PCR results of resistance genes confirmed by analyzing of assembled draft genomes obtained from WGS of isolates using "ResFinder antimicrobial resistance gene database" [32] (Data not shown).

Table 2 position

Closer examination of the C2 subclade showed that *hlyA* gene wasn't uniformly distributed among this clade and strains carrying this virulence marker were significantly positive for *aac(6')-Ib* [21 (100%), P = 0.003], *aac(6')-Ib-cr* [20 (95.2%), P = 0.01], $bla_{\text{OXA-1}}$ [21 (100%), P = 0.01] and *aac(3)-IIa* [19 (90.5%), P = 0.001] (Figure 2). The carriage of *hlyA* among C2 subclade was coincided with resistance phenotype to tobramycin [21 (100%), P = 0.04] and gentamicin [21 (100%), P < 0.001]. Totally, the $bla_{\text{CTX-M-15}}$ positive isolates exhibited a

significantly higher prevalence of resistance to aztreonam [45 (100%), $P < 0.001$], ceftazidime [39 (86.7%), $P = 0.02$], cefepime [34 (75.6%), $P < 0.001$] ampicillin/sulbactam [27 (60%), $P = 0.001$], amoxicillin/clavulanate [33 (73.3%), $P = 0.006$], amikacin [8 (17.8%), $P = 0.02$], tobramycin [32 (71.1%), $P < 0.001$] and gentamicin [29 (64.4%), $P < 0.001$].

Considering the *bla*$_{\text{OXA-1}}$, there was a strong association between the carriage of this element and resistance phenotype to amikacin, gentamicin, tobramycin, aztreonam, amoxicillin/clavulanate, ampicillin/sulbactam and susceptibility to fluoroquinolone.

Virotyping of clades and resistance markers:

All except 13 strains were divided in to virotypes A to F. These 13 strains showed unknown arrangements of virulence genes and their virulence patterns are shown in table 3.

Table 3 position

Virotype C was the most common virotype, represented by 34 (46.5%) isolates and predominantly associated with subclade C1 (27/32), including C1-M27 [16 strains within virotype C, (47%)] and C1-nM27 [11 strains within virotype C, (32.3%)] subsets. Most of the virotype C strains identified as virotype C2 [25 (73.5%)], followe by virotype C1 [7 (20.5%)] and virotype C3 [2 (6%)]. In contrast, virotypes E (18 strains) and F (3 strains) belonged to subclade C2 (Table 4).

Table 4 position

The associations among all 29 detected virulence genes were identified using cluster analysis. The isolates were divided according to 100% similarity of virulence genes content. The largest cluster included 20 strains with a unique set of 15 virulence genes (*sat*, *chuA*, *fyuA*, *yfcV*, *iutA*, *k5*, *iucD*, *F10papA*, *iha*, *tsh*, *ompT*, *usp* , *PAI*, *traT*, *iss*) that corresponded to virotype C. These isolates harbored mostly *bla*$_{\text{CTX-M-15}}$ (6/20; 30.%), or were *bla*$_{\text{OXA-1}}$

negative, and mainly represented the C1 subclade (19/20; [95%], including 14 C1-M27 and five C1-nM27 strains).

The second largest cluster, which included 14 subclade C2 strains, contained *bla*$_{\text{OXA-1}}$ (100%), *bla*$_{\text{CTX-M-15}}$ (100%), *aac(3)-IIa* (92.8%), *aac(6')-Ib* (100%),and *aac(6')-Ib-cr* (92.8%) positive strains with a set of 21 virulence markers (*papGII*, *papC*, *F10papA*, *iucD*, *sat*, *cnf1*, *hlyA*, *chuA*, *fyuA*, *yfcV*, *iutA*, *iha*, *hra*, *tsh*, *tia/hek*, *usp*, *K5*, *ompT*, *iss*, *traT*, and *PAI*) that corresponded to virotype E. As expected, these 14 virotype E strains were significantly associated with resistance phenotypes to gentamicin ($P < 0.001$) and tobramycin ($P < 0.001$).

The highest VF score was detected among virotype E (median: 19), followed by virotypes F (median: 17), virotype A (median: 17) and unknown virotype (median: 16).

Discussion:

As far as we know, this is the first study in Iran to investigate and compare the prevalence and genotypes of ST131 sub-clades. Here, we found that the C2 subclade of ST131 was responsible for most of the ST131 infections. All except clade B were detected among study population. Interestingly, virotype and clade patterns had consistency, in which the most common virotypes including virotypes C and E had comprised the subclades C1 and C2 strains, respectively. A subpopulation among subclade C2 lineage was detected which showed higher carriage rates of resistance/virulence markers and were particularly resistant to aminoglycosides, confirming the importance of emerged subsets within this clone.

In our survey, an increasing trend of resistance rate was detected from C1-M27 to C1-nM27 and C2 strains. Considering the susceptibility patterns, significant differences were observed between subclades, particularly between C1-M27 and C2, despite their phylogenetic relatedness. These differences were remarkable in the proportion of resistance to ampicillin/sulbactam, amoxicillin/clavulanate, gentamicin, and tobramycin which were very

low among C1-M27 strains compared with the other isolates. Furthermore, C1-M27 was conspicuously negative for studied resistance markers. This finding indicates that we need to investigate factors other than antibiotic selection pressure to explain the emergence of C1-M27 as the second most prevalent subclade. In a four-years study on isolated *E. coli* strains from blood cultures in Norway, resistance rate against gentamicin was found much higher than cephalosporin among O25b-ST131 isolates [33]. In fact, appearance of a subpopulation with remarkable resistance to aminoglycosides among ST131 which is notorious for fluoroquinolone resistance and producing ESBL, represents an evolving epidemiology of this clone and its subclades to a pandrug resistant population [34].

Here, we identified seven new virotypes including 13 strains with different genetic arrangements, suggesting an endemic distribution of virulence markers probably acquired by mobile genetic elements. Among the known virotypes, virotype C is considered to be the most widely distributed virotype among ST131, occurring in all ST131 clades [35]. Here, virotype C was predominant among subclade C1, specifically C1-M27 strains, as also reported recently from South-West England and Europe [35,36]. In contrast to several European studies that reported the virotype A as a dominant virotype among subclade C2 [10], virotypes E and F were found as the most common virotypes, representative of > 60% of the C2 strains (21 out of 33 strains). Further analysis of subclade C2 revealed a heterogeneous population based on the carriage of *hlyA* virulence marker, as positive strains had remarkably higher rates of some virulence (*hra*, *tia/hek*, *cnf*, *papC*, *papGII*, *K5*) and all except bla_{TEM} resistance genes, and consequently higher resistance phenotype to tobramycin and gentamicin. Interestingly, almost all of this C2 subclade (18 *hlyA*+/ 21 *hlyA*+) was identified as virotype E. In a recently published data from Southeast Asia, a subpopulation among C2 subclade is reported which has been named Southeast Asia-C2 (SEA-C2) lineage [37]. While virotypes of studied strains were not determined in the aforementioned study, the

main features attributed to this subset were the higher carriage rates of some virulence genes mainly *hly*ABCD operon, *cnf1*, and *tia/hek*, and harbouring a conserved plasmid which carried *aac(3)-IIa* , *aac(6')-Ib*, *aac(6')-Ib-cr* , $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, and *tetA*. The strains were very closely related by genome sequence analysis. These findings in conjunction with our data suggest that virotypes E and F are the most prevalent virotypes among C2 subclade originated from Asia, constituting a distinct subset among C2 population.

Our study has some limitations, including small sample size which was collected from a single center, lack of knowledge regarding whether the study isolates were part of a nosocomial outbreak, and most importantly, lack of data regarding phylogenic clustering of strains based on a roboust technique such as whole-genome sequencing.

In conclusion, our study is notable for examining a 19-months collected ST131 strains in a geographical region from which no data has been previously published. We have found that study ST131 strains aren't a uniform population, and subclade C2 like in other regions is driving both the higher virulence and resistance among this high-risk clone. More focus on this clade identified a subset of strains that showed virotypes E and F patterns and their resistance phenotypes and resistance/virulence genes repertoire were different from the other C2 subclade strains. So, our data show a local trend within the C2 subclade in which the generated subset has a major advantage over other ST131 population in the context of resistance/virulence genes content and clonality. So, ongoing monitoring of the dynamics of ST131 local transmission is required to understand the reasons for new virotypes emergence within this region.

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Potential conflicts of interest

The authors declare that they have no competing interests.

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Table 1. Prevalence of virulence markers among ST131 clades.

Virulence factors	A n=8 **(10.9%)**	C1-M27 n=18 **(24.6%)**	C1-nM27 n=14 **(19.1%)**	C2 n=33 **(45.2%)**	Total **n=73**
	n(%)				
	Source of isolates				
	Sputum: 1 UC: 7	Sputum: 2 UC: 16	Sputum: 4 UC: 8 Wound: 2	UC: 31 Wound: 2	
Adherence					
papA	1(12.5)	0	0	0	1 (1.3)
p-value					
papC	2 (25)	0	0	27 (81.8)	29 (39.7)
p-value		<0.001	< 0.001	**<0.001**	
papGII	2 (25)	0	0	28 (78.8)	**30 (41.09)**
p-value		<0.001	0.001	**<0.001**	
papEF	0	0	0	1 (3)	1 (1.3)
F10papA	8 (100)	16 (88.9)	14 (100)	33 (100)	73 (100)
afaDrBC	4 (50)	0	2 (14.2)	3 (9.1)	9 (12.3)
p-value	**0.006**	0.05			
afaFM955459	2 (25)	0	2 (14.2)	3 (9.1)	7 (9.5)
iha	7 (87.5)	16 (88.9)	13 (92.9)	32 (97)	68(93.1)
hra	0	0	3 (21.4)	29 (87.9)	32 (43.8)
p-value [a]	0.008	<0.001		**<0.001**	
Iron uptake					
iucdD	5 (62.5)	16 (88.9)	14 (100)	29 (87.9)	63 (87.6)
p-value					

iutA	5 (62.5)	16 (88.9)	13 (92.9)	31 (93.9)	65 (89)
p-value	0.03				
yfcV	8 (100)	18 (100)	9 (100)	33 (100)	73 (100)
fyuA	8 (100)	18 (100)	9 (100)	33 (100)	73 (100)
chuA	8 (100)	18 (100)	9 (100)	33 (100)	73 (100)
iroN	0	0	0	5 (15.2)	5 (6.8)
				0.01	
Invasion					
Tia/hek	2 (25)	0	3 (21.4)	29 (87.9)	34 (46.5)
P value		<0.001	0.04	< **0.001**	
Autotransporter					
sat	5 (62.5)	16 (88.9)	13 (92.9)	32 (97)	66 (90.4)
P value	0.02				
vat	2 (25)	0	0	0	2 (2.7)
P value	0.01				
tsh	7 (87.5)	18 (100)	14 (100)	33 (100)	72 (98.6)
Toxins					
cnF1	0	0	0	21 (61.6)	21 (28.7)
P value		0.001	0.007	<**0.001**	
hlyA	0	0	0	21 (61.6)	21 (28.7)
P value		0.001	0.007	<**0.001**	
usp	8 (100)	18 (100)	9 (100)	33 (100)	73 (100)
hlyF	0	0	0	1 (3)	1 (1.3)
Protection					
kpsMTII	1 (12.5)	0	7 (50)	3 (9.1)	11 (15)
P value		0.05	<**0.001**		
K5	7 (87.5)	17 (94.4)	5 (35.7	28 (84.8)	57 (78)

P value			<0.001		
iss	0	16 (88.9)	13 (92.9)	33 (100)	62 (84.9)
	<0.001			**0.001**	
traT	8 (100)	17 (94.4)	12 (85.7)	31 (93.9)	68 (93.1)
Miscellaneous					
PAI	6 (75)	18 (100)	13 (92.9)	33 (100)	70 (95.8)
P value	0.03				
ompT	7 (87.5)	18 (100)	14 (100)	33 (100)	92 (98.6)
VF score (mean, median)	13.75, 14	14.28, 15	15.14, 15	18.6, 19	

Notes: [a] Comparison between each clades with all other clades combined. Boldface values indicate significant associations. *P*-values in rows shown for differences that were statistically significant ($P<0.05$). Underlining indicates a negative association. UC: urine culture.

Table 2. Antibiotic resistance rates and prevalence of resistance markers among different clades of ST131.

Antibiotics	A n=8	C1-M27 n=18	C1-nM27 n=14	C2 n=33	Total (%)
	n(%)				
Imipenem	1 (12.5)	(0)	1 (7.1)	0	2 (2.7)
Meropenem	0	1 (5.6)	0	0	1 (1.4)
Ertapenem	1 (12.5)	2 (11.1)	2 (14.3)	4 (12.1)	9 (12.3)
Piperacillin-tazobactam	2 (25)	3 (16.7)	2 (14.3)	7 (21.2)	14 (19.2)
Ampicillin-sulbactam	5 (62.5)	2 (11.1)	5 (35.7)	20 (60.6)	32 (43.8)
P value [a]		0.002		**0.01**	
Amoxicillin-clavulanate	6 (75)	5 (27.8)	7 (50)	26 (78.8)	44 (60.3)
P value		0.002		**0.004**	
Trimethoprim/sulfamethoxazole	6 (75)	14 (77.8)	8 (57.1)	23 (69.7)	51 (69.9)
Aztreonam	6 (75)	16 (88.9)	12 (85.7)	31 (93.9)	65 (89)
Cefepime	5 (62.5)	8 (44.4)	7 (50)	22 (66.7)	42 (57.5)
Ceftazidime	6 (75)	12 (66.7)	11 (78.6)	27 (81.8)	56 (76.5)
Cefotaxime	6 (75)	18 (100)	12 (85.7)	32 (97)	68 (93.2)
Amikacin	0	0	0	8 (24.2)	8 (11)
P value				**0.001**	
Gentamicin	2 (25)	1 (5.6)	3 (21.4)	25 (75.8)	31 (42.5)

P value		<u>≤0.001</u>		**<0.001**	
Tobramycin	1 (12.5)	0	2 (14.3)	30 (90.2)	33 (45.2)
P value		<u>≤0.001</u>	<u>0.01</u>	**<0.001**	
Ciprofloxacin	0	18 (100)	13 (92.9)	33 (100)	64 (87.7)
P value	<u>≤0.001</u>			**0.003**	
Levofloxacin	0	18 (100)	14 (100)	33 (100)	65 (89)
P value	<u>≤0.001</u>			**0.007**	
Nitrofurantoin	0	1 (5.6)	1 (7.1)	8 (24.2)	10 (13.7)
P value				**0.03**	
MDR	6 (75)	18 (100)	10 (71.4)	33 (97.1)	69 (90.8)
			<u>0.02</u>		
Resistance rates (mean, median)	2.63, 3	3.39, 3	3.36, 3.50	4.74, 5	
ESBL	6 (75)	18 (100)	12 (85.7)	33 (97.1)	69 (94.5)
Resistance markers					
bla$_{\text{CTX-M-15}}$	5 (62.5)	0	9 (64.3)	31 (93.9)	45 (61.6)
P value		<u>≤ 0.001</u>		**< 0.001**	
bla$_{TEM-}$	8 (100)	0	9 (64.3)	7 (21.2)	24 (32.8)
P value	**< 0.001**	<u>≤ 0.001</u>	**0.01**	<u>0.01</u>	
bla$_{OXA-1}$	0	0	0	29 (87.9)	29 (39.7)
P value	<u>0.01</u>	<u>≤0.001</u>	<u>≤0.001</u>	**<0.001**	
Aac6Ib-cr	0	0	1 (7.1)	27 (81.8)	28 (38.3)
P value	<u>0.02</u>	<u>≤ 0.001</u>	<u>≤0.01</u>	**0.001**	
qnrS	0	0	4 (28.6)	0	4 (5.4)
P value			**0.001**		
Aac3IIa	6 (75)	0	4 (28.6)	23 (69.7)	33(45.2)
P value		<u>≤0.001</u>		**<0.001**	
Aac6Ib	1 (12.5)	0	1 (7.1)	28 (84.8)	30 (41)

P value	< 0.001	0.005	**<0.001**	

Notes: [a] Comparison between each clade with all other clades combined. Bold values indicate significant associations. *P*-values in rows shown for differences that were statistically significant ($P<0.05$). Underlining indicates a negative association.

Table 3. Unknown virotypes detected among 13 strains with their clades and serotypes detected in o

	Virulence genes [a]												Clades	O25/O16
Unknown Virotypes **N:13**	*afaDraBC*	*afaFM955459*	*iroN*	*sat*	*papGII*	*cnf*	*hlyA*	*kpsMTII*	*k5*	*papC* [b]	*vat* [b]	*iss* [b]		
Pattern 1 N:2	+	+	-	+	+	-	-	-	+	+	+	-	A	O16
Pattern 2 N:2	+	+	-	+	-	-	-	-	+	-	-	+	C2	O25
Pattern 3 N:2	+	-	-	-	-	-	-	-	+	-	-	-	A	O16
Pattern 4 N:3	-	-	+	+	+	+	+	-	+	+	-	+	C2	O25
Pattern 5 N:1	-	-	-	-	-	-	-	+	-	-	-	-	A	O16
Pattern 6 N:1	-	-	-	-	-	-	-	-	+	-	-	-	C1-M27	O25
Pattern 7 N: 2	-	-	-	-	-	-	-	-	-	-	-	+	C1	O25

[a] **:** The virulence genes *cdtB, neuC k, papGIII* and *ibeA* which are used for virotyping, were not detected.

[b] **:** These three genes are not considered for virotyping, and are shown because of the same arrangement among isolates of each pattern.

Table 4. Virotyping of clades and prevalence of resistance genes among virotypes

	Virotype A (n=3)	**Virotype B (n=2)**	**Virotype C (n=34)**	**Virotype E (n=18)**	**Virotype F (n=3)**	**Unknown (n=13)**
			Clades			
Resistance markers	C1-nM27: 2 C2: 1	C2: 2	A: 3 C1-M27: 16 C1-nM27: 11 C2: 4	C2: 18	C2: 3	A: 5 C1-M27: 2 C1-nM27: 1 C2: 5
bla$_{TEM}$	3 (100)	1 (50)	11 (32.4)	1 (5.6)	2 (66.7)	6 (46.2)
P value				0.004		
bla$_{OXA-1}$	1 (33.1)	1 (50)	2 (5.9)	18 (100)	3 (100)	4 (30.8)
P value				**< 0.001**		
bla$_{CTX-M-15}$	1 (33.3)	1 (50)	13 (38.2)	18 (100)	3 (100)	9 (69.2)
P value				**< 0.001**		
Aac6Ib	1 (33.3)	1 (50)	4 (11.8)	18 (100)	2 (66.7)	4 (30.8)
P value				**< 0.001**		
Aac3IIa	0	0	7 (20.6)	17 (94.4)	2 (66.7)	3 (23.1)
P value				**< 0.001**		
Aac6Ib-cr	1 (33.3)	1 (50)	3 (8.8)	17 (94.4)	2 (66.7)	4 (30.8)
P value				**< 0.001**		
qnrS			4 (11.8)			
Virulence score (mean, median)	17.67, 17	14.50, 14.50	14.94, 15	18.83, 19	17	15.17, 16
Resistance score (mean, median)		3.50, 3.50	3.62, 4	4.94, 5		3.17, 3

a: Thirteen strains were not categorized to defined virotypes. Boldface values indicate significant association. Underlining indicates a negative association.

Figure 1. Principal coordinate analysis (PCoA) of virulence gene profiles among 73 ST131 isolates. The PCoA was based on results for all 29 virulence genes studied. Each isolate is plotted based on its values for PCoA coordinates 1 (*x* axis) and 2 (*y* axis), which collectively capture 58.77% of total variance in the data set.

Figure 2. Figure 2. Comparison of virulence/resistance genes content between two subsets (*hly* A^{+} and *hlyA*$^{-}$) of subclade C2 strains. Black and blue stars indicate the association of that gene with *hlyA* $^{-}$ and *hlyA* $^{+}$ strains, respectively.

Figure 1

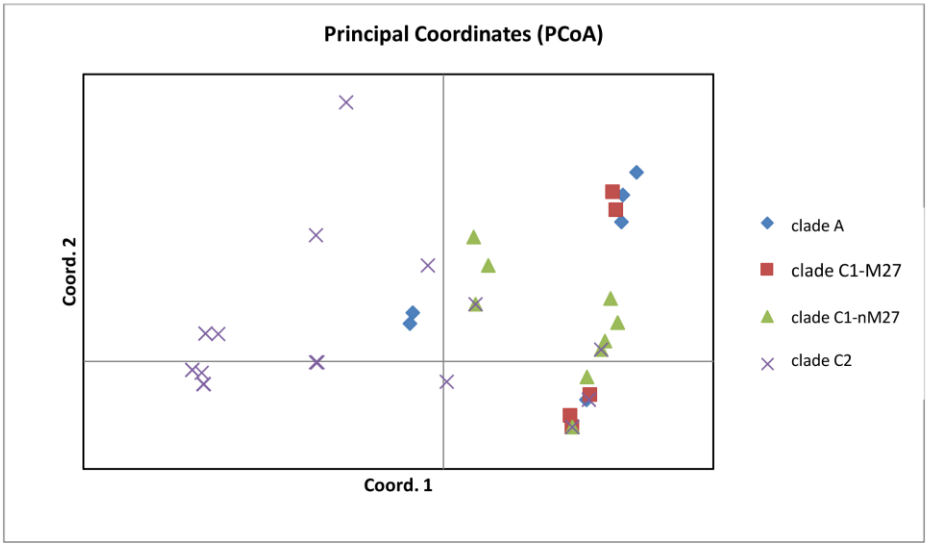

Figure 2