

# Molecular epidemiology of ESBL-producing *E. coli* and *K. pneumoniae*: establishing virulence clusters

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**Objective:** To genetically characterize clusters of virulence factors (VFs) among extended spectrum  $\beta$ -lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* and assess whether these clusters are associated with genetic determinants or clinical outcomes.

**Methods:** One hundred forty-eight *E. coli* and 82 *K. pneumoniae* clinical isolates were obtained from 213 patients in Paris, France. Isolates underwent ESBL characterization, MultiLocus Sequence Typing (MLST) typing and phylogenetic group identification. Detection of ten *E. coli* and seven *K. pneumoniae* VF-encoding genes were assessed, from which a *k*-medians partition algorithm with Jaccard similarity measure was used to construct clusters.

**Results:** CTX-M was the predominant ESBL and susceptibility to trimethoprim-sulfamethoxazole (32%), ciprofloxacin (22%) and aminoglycosides (32%) was low. In *E. coli*, there were five identified clusters, with significantly different distributions of ESBL-sequence type ( $P<0.001$ ), ST131 ( $P<0.001$ ) and phylogenetic group ( $P=0.001$ ) between clusters. "Siderophore exclusive", "siderophore exclusive with *iroN*" and "adhesin *sfa/papGIII*-rich" clusters had higher 12-month mortality rates compared to others (49% vs 22%, respectively,  $P=0.02$ ). In *K. pneumoniae*, three different clusters, with significantly different distributions of aminoglycoside-sensitivity ( $P<0.004$ ), MLST-type ( $P<0.001$ ) and relaxase plasmids ( $P=0.001$ ) were described.

**Conclusion:** Distinct clusters of *E. coli* and *K. pneumoniae* VFs are observed within ESBL-producing isolates and are strongly associated with several genetic determinants. Their association with overall morbidity and mortality requires further evidence.

**Keywords:** ESBL, virulence, mortality, *E. coli*, *K. pneumoniae*

## Introduction

Extended spectrum  $\beta$ -lactamase (ESBL)-producing Enterobacteriaceae first emerged during the 1980s and with their extensive transmission have now become pandemic.<sup>1</sup> Coupled with their limited treatment options and difficulty in patient management, these infections have led to increasing morbidity and mortality and represent a major public health concern.<sup>2-4</sup> Since 2000, Enterobacteriaceae producing CTX-M-ESBL have been predominant, yet the reasons for such wide dissemination, including in the community, remain speculative.<sup>1</sup>

Genetic characteristics of strains and plasmids carrying ESBL genes could contribute to dissemination of ESBL-producing Enterobacteriaceae. First, plasmids do not generally contain genes necessary for bacterial vitality. Nonetheless, they sometimes contain genes conferring selective advantages able to resist host responses (ie, detoxification, antibiotic resistance, virulence) and could account for some of the circulating

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strains observed to date.<sup>5</sup> Second, the intrinsic virulence of ESBL-producing strains, much like any bacteria, is principally determined by the presence of virulence genes encoding for virulence factors (VFs).<sup>6</sup> The relationship between VF and ESBL-producing Enterobacteriaceae remain unclear. Finally, certain virulent phylogenetic groups of *Escherichia coli* are described, such as B2 and D. Their capacity to spread has already been described, as observed with B2 O25b:H4-ST131 CTX-M-15 producing *E. coli*.<sup>7</sup>

Only few studies to date have attempted to understand the type of VFs factors shared within strains and whether clusters of VF are associated with other genetic or even clinical characteristics. We aimed to genetically characterize two Enterobacteriaceae most frequently established with carrying ESBL genes, *E. coli* and *K. pneumoniae*.<sup>8</sup> We then used a statistical technique to establish linkage between VF within the two species. Finally, we assessed the association of cluster groups with genetic determinants and clinical outcomes.

## Materials and methods

### Study design

Data were obtained from a cross-sectional study conducted at Saint-Antoine Hospital, Assistance Publique-Hôpitaux de Paris, Paris, France from April 2012 to April 2013.<sup>8</sup> Biological samples testing positive for an ESBL-producing *E. coli* or *K. pneumoniae*, with the exception of rectal swabs, from patients attending in- or outpatient clinics processed at the Department of Microbiology were included. Information was given to each patient for the use of stored samples and personal data related to non-interventional research. All data have been treated anonymously. In accordance with French Public Health law, ethical approval was not required for this study.

### Clinical data

A patient was defined as “infected” if his attending physician decided to treat with antibiotics or as “colonization” otherwise. Portal of entry was classified as lung, urinary tract (UTI), digestive tract or unknown according to the attending physician. Acquisition of ESBL-producing Enterobacteriaceae was characterized as follows: hospital-acquired, if the first positive sample was detected >48 hours after admission; health care-associated, if the first positive sample was detected ≤48 hours after admission and the patient underwent hospitalization within 3 months prior or community-acquired, if the first positive sample was detected ≤48 hours

of admission without any recent hospitalization. Data on transfer to the intensive care unit (ICU) and all-cause deaths within 12 months after entry into care were obtained from electronic patient medical records. Only infected patients had available data on ICU transfer.

## Bacterial and genetic characterization of isolated strains

### Species identification

After routine culture at 37°C in Trypticase soy and Drigalski agar, species were identified using the API20E system (BioMérieux, Marcy l’Etoile, France) or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

### Antibiotic resistance

Antibiotic susceptibility patterns were determined by standard diffusion on Mueller–Hinton agar (Bio-Rad Laboratories Inc., Hercules, CA, USA), and results were interpreted according to the Comité de l’Antibiogramme de la Société Française de Microbiologie (CA-SFM)/EUCAST.<sup>31</sup>

### ESBL detection and sequencing

ESBL production was detected using the double-disk test for synergy between clavulanic acid and extended-spectrum cephalosporins (ceftazidime and cefotaxime) on Muller–Hinton agar. For strains with a positive result, ESBL characterization was performed on stored samples using specific PCR amplification and sequencing after bacterial DNA extraction using an InstaGeneMatrix® kit (Bio-Rad).<sup>9,10</sup> For *E. coli* strains, phylogenetic group was characterized using a quadruplex PCR method.<sup>11</sup> A *pabB* and *trpA* allele-specific PCR was used to detect, among B2 strains, those belonging to the pandemic O25b:H4-ST131 clone.<sup>12</sup>

### VFs

PCR was used on stored samples to detect the presence of genes encoding VF in the core genome or in plasmids, including adhesins (*papC*, *papG*, including *papG* alleles, *sfa/foc* and *ibeA*), toxins (*hlyC* and *cnfI*) and iron capture systems (*fyuA*, *iroN* and *iucC*).<sup>13</sup> Sequences were analyzed using the program SeqScape® (Thermo Fisher Scientific, Waltham, MA, USA).<sup>15</sup> Seven VF genes, including the regulator of mucoid phenotype A (*rmpA*), fimbrial adhesion (*mrkD*), iron capture systems (*entB*, *kfu*, *ybtS* and *iutA*) and factor associated with allantoin metabolism (*allS*) as well as capsular serotypes K1 and K2 were determined.<sup>16</sup>

## MultiLocus Sequence Typing (MLST) typing

MLST typing was performed on stored samples using the international *K. pneumoniae* MLST typing scheme.<sup>14</sup>

## Relaxases

Plasmid characterization was performed on stored samples by transferring ESBL genes to azide or rifampin-resistant *E. coli* J53 as a recipient and selecting transconjugants on Drigalski agar supplemented with sodium azide (100 mg/L) or rifampin (256 mg/L) and cefotaxime (2 mg/L) for CTX-M-producing strains or ticarcillin (125 mg/mL) for TEM- and SHV-producing strains. If resistance genes were unable to be transferred by conjugation, plasmids carrying the ESBL gene were extracted with DNA Plasmid Miniprep Kit® (Qiagen NV, Venlo, the Netherlands) and transferred by electroporation in *E. coli* DH10B cells. Plasmids were characterized using PCR-based replicon typing, and multireplicons were studied using the Kado method.<sup>17,18</sup>

## Statistical analysis

Unless otherwise stated, all comparisons were performed using a Pearson's chi-squared test or Fisher's exact test for categorical variables and Kruskal–Wallis rank test for continuous variables. In comparing bacterial characteristics, individual isolates were considered without accounting for patient correlation for those with more than one isolate. In comparing patient-level characteristics, only the last patient visit was considered for those with multiple isolates.

In order to define clusters of VF, we employed a *k*-medians clustering algorithm with the Jaccard similarity measure (specified for binomially distributed data).<sup>19</sup> Since VFs are different between bacterial species, the algorithm was run separately for *E. coli* and *K. pneumoniae*. The optimal number of clusters was tested using a range of *k* = {2, 3, ..., 10}, while the final choice of *k* was based on maximizing the Caliński and Harabasz pseudo-*F* index and evaluating meaningful distributions of VFs (ie, avoiding excess clusters with sparse prevalence of specific VFs). After obtaining the optimal *k*, cluster groups were assigned to each observation based on the similarity measure above.

A *P*-value of <0.05 was considered significant. All analysis was conducted using STATA statistical package (v13.0, College Station, TX, USA).

## Results

### Description of bacterial genetic characteristics

In total, 148 *E. coli* and 82 *K. pneumoniae* ESBL-producing strains with available genetic data were included. Isolates

were obtained in 198 patients: 5 were infected with the same species (2 with 2 *E. coli* and 3 with 2 *K. pneumoniae*) and 11 were infected with *E. coli* and *K. pneumoniae*; all of which were collected during different episodes. Genetic characteristics are detailed in Table 1. Isolates were sensitive to trimethoprim–sulfamethoxazole, ciprofloxacin and aminoglycosides in 32%, 22% and 32%, respectively. Among all strains, CTX-M-15 (60%) was the most common ESBL enzyme and was highly prevalent in *K. pneumoniae* (90%). Of the 148 *E. coli* strains, 51 (34%) were ST131. Of the 82 *K. pneumoniae* strains, MLST classes were distributed as follows: ST29 (n=19), ST323 (n=16), ST147 (n=11), ST405 (n=5) and other (n=31). One strain of *K. pneumoniae* had a K2 capsular serotype harboring *rmpA*, defined as

**Table 1** Bacterial determinants of ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae*

n (%)	<i>E. coli</i> (n=148)	<i>K. pneumoniae</i> (n=82)	P-value <sup>a</sup>
ESBL sequence			<0.001
CTX-M-15	64 (43)	74 (90)	
CTX-M-14	35 (24)	2 (2)	
CTX-M-1	22 (15)	0 (0)	
CTX-M-27	11 (8)	0 (0)	
CTX-M others	12 (8)	1 (1)	
SHV-12	2 (1)	0 (0)	
TEM-15	2 (1)	4 (5)	
TEM-20	0 (0)	1 (1)	
Trimethoprim–sulfamethoxazole			
Sensitive	62 (42)	11 (13)	<0.001
Ciprofloxacin			
Sensitive	43 (29)	8 (10)	0.001
Aminoglycoside phenotype			
Sensitive	72 (49)	3 (4)	<0.001
AAC(3)-II	43 (29)	55 (67)	
AAC(6)	13 (9)	22 (27)	
Other <sup>c</sup>	18 (12)	2 (2)	
Relaxase	(n=74)	(n=56)	<0.001
F	28 (38)	8 (14)	
FIK	1 (1)	23 (41)	
II	21 (28)	2 (4)	
Untypable	6 (8)	17 (30)	
Multireplicon <sup>b</sup>	11 (15)	2 (4)	
A/C	2 (3)	1 (2)	
B/O	2 (3)	0 (0)	
C II	1 (1)	0 (0)	
Hia Hiy	1 (1)	0 (0)	
L/M	0 (0)	1 (2)	
R	0 (0)	2 (4)	
XI	1 (1)	0 (0)	

**Notes:** <sup>a</sup>Significance between bacterial species determined using Kruskal–Wallis test for continuous variables and Pearson chi-squared test or Fisher's exact test for categorical variables. <sup>b</sup>Two Relaxases were detected. <sup>c</sup>Includes the following phenotypes: APH(3') (n=14), KTG (n=5) and AAC2 (n=1).

**Abbreviation:** ESBL, extended spectrum β-lactamase.

“hypervirulent”, and was multiresistant (ST86 CTX-M-3 producing K2).<sup>20</sup>

One hundred thirty plasmids were characterized in electroporants or transconjugants from *E. coli* (n=74) and *K. pneumoniae* (n=56), with the most common being IncF plasmids (28%). Of note, 12/21 *E. coli* carrying IncI1 plasmids were CTX-M-1 producers. Eleven *E. coli* had multi-replicons (n=10 Inc F/I1 and n=1 Inc I1/A/C).

## Identification of VFs' clusters within bacterial species

The distribution of *E. coli* and *K. pneumoniae* VF is reported in Table 2. For *E. coli* VFs, five clusters were identified (pseudo-*F* index =45.7). The first cluster without any VF genes (defined as “no VF”) was observed in 19 strains (13%). The second cluster included strains with almost only siderophores *fyuA* and *iucC* (defined as “siderophore exclusive”) (n=62, 42%), while the third one included these VF genes with *iroN* siderophore gene (n=29, 20%) (defined as “siderophore exclusive with *iroN*”). Finally, two other clusters were identified with abundant adhesin VF genes: one cluster with a higher proportion of *sfa/foc* and *papGIII* VF genes (n=14, 3%) (defined as “adhesin *sfa/papGIII*-rich”) and the other with all *papC* and a higher proportion of *papGII* VF genes (n=24, 16%) (defined as “adhesin *papC/papGII*-rich”). Between these clusters, the “adhesin *sfa/papGIII*-rich” cluster was also found to have a higher prevalence of the toxin VF genes *cnfI* and *hly*, whereas the “adhesin *papC/papGII*-rich” cluster had a higher proportion of *iucC* VF genes (Table 2).

Three distinct clusters were identified for *K. pneumoniae* (pseudo-*F* index =154.9) (Table 2). The first cluster (n=40, 49%) was defined by the absence of VF except for *mrkD* and *entB* (defined as “KP common VF”). Two other clusters contained strains that also included mostly *ybtS* (n=27, 33%) (defined as “KP common VF with *ybtS*”) or exclusively *kfu* VF genes (n=15, 18%) (defined as “KP common with *kfu*”).

## Association of bacterial genetic characteristics with VF clusters

Clusters of *E. coli* VF were strongly associated with other genetic determinants and antimicrobial resistance (Table 3). Significant differences in phylogenetic groups were noted ( $P=0.001$ ); however, there was considerable diversity between clusters for all other phylogenetic groups. Distribution of ESBL types was also significantly different between clusters ( $P<0.001$ ), with the largest differences in CTX-M-15 prevalence. ST131 was significantly more prevalent in the “siderophore exclusive” and “adhesin *papC/papGII*-rich” clusters compared to all others ( $P<0.001$ ). More specifically, ST131 compared to non-ST131 isolates had a higher prevalence of the siderophore VFs *fyuA* (100% vs 57%, respectively,  $P<0.001$ ) and *iucC* (94% vs 56%, respectively,  $P<0.001$ ), reflecting the “siderophore exclusive” cluster, as well as the adhesin VF *papGII* (25% vs 7%, respectively,  $P=0.002$ ), reflecting the “adhesin *papC/papGII*-rich” cluster. There were significant differences between clusters in the proportion of strains with ciprofloxacin ( $P=0.001$ ) and

**Table 2** Defining clusters of VFs within bacterial species

<i>Escherichia coli</i>							<i>Klebsiella pneumoniae</i>				
VFs	Total (n=148)	Cluster grouping					VF	Total (n=82)	Cluster grouping		
		No VF (n=19)	Siderophore exclusive (n=62)	Siderophore exclusive with <i>iroN</i> (n=29)	Adhesin <i>sfa/papGIII</i> -rich (n=14)	Adhesin <i>papC/papGII</i> -rich (n=24)			KP common VF (n=40)	KP common VF with <i>ybtS</i> (n=27)	KP common VF with <i>kfu</i> (n=15)
<i>fyuA</i>	106 (72)	0 (0)	50 (80)	24 (83)	10 (71)	22 (92)	<i>ybtS</i>	36 (44)	0 (0)	27 (100)	9 (60)
<i>hly</i>	22 (15)	0 (0)	0 (0)	0 (0)	11 (79)	11 (46)	<i>mrkD</i>	81 (99)	39 (98)	27 (100)	15 (100)
<i>sfa/foc</i>	11 (7)	0 (0)	0 (0)	1 (3)	10 (71)	0 (0)	<i>entB</i>	82 (100)	40 (100)	27 (100)	15 (100)
<i>papC</i>	37 (25)	0 (0)	0 (0)	2 (7)	11 (79)	24 (100)	<i>rmpA</i>	1 (1)	0 (0)	1 (4)	0 (0)
<i>iucC</i>	102 (69)	0 (0)	54 (87)	23 (79)	5 (36)	20 (83)	<i>kfu</i>	15 (18)	0 (0)	0 (0)	15 (100)
<i>papGIII</i>	11 (7)	0 (0)	1 (2)	0 (0)	9 (64)	1 (4)	<i>allS</i>	0 (0)	0 (0)	0 (0)	0 (0)
<i>papGII</i>	20 (14)	0 (0)	0 (0)	1 (3)	1 (7)	18 (75)	<i>iutA</i>	2 (2)	0 (0)	2 (7)	0 (0)
<i>cnfI</i>	22 (15)	0 (0)	1 (2)	2 (7)	11 (79)	8 (33)	<i>magA</i>	0 (0)	0 (0)	0 (0)	0 (0)
<i>ibeA</i>	15 (10)	0 (0)	1 (2)	7 (24)	3 (21)	4 (17)					
<i>iroN</i>	42 (28)	0 (0)	0 (0)	29 (100)	11 (79)	2 (8)					

**Note:** Clusters of VFs were determined among bacterial species, *E. coli* and *K. pneumoniae*, using k-medians clustering with the Jaccard similarity measure.

**Abbreviation:** VFs, virulence factors.

**Table 3** Genetic and patient characteristics between clusters of *Escherichia coli*

	Total	Cluster group					P-value <sup>a</sup>
		No VF	Siderophore exclusive	Siderophore exclusive with <i>iroN</i>	Adhesin <i>sfa/papGIII</i> -rich	Adhesin <i>papC/papGIII</i> -rich	
Genetic characteristics n (%)	(n=148)	(n=19)	(n=62)	(n=29)	(n=14)	(n=24)	
Aminoglycoside							0.07
Sensitive	72 (49)	9 (47)	30 (48)	17 (59)	9 (64)	7 (29)	
AAC(3)-II	43 (29)	6 (32)	16 (26)	6 (21)	3 (21)	12 (50)	
AAC(6')	13 (9)	1 (5)	10 (16)	0 (0)	1 (7)	1 (4)	
APH(3')	12 (8)	1 (5)	4 (6)	5 (17)	1 (7)	1 (4)	
Ciprofloxacin							0.001
Sensitive	43 (29)	6 (32)	12 (19)	9 (31)	11 (79)	5 (21)	
Cotrimoxazole							0.03
Sensitive	62 (42)	2 (11)	28 (45)	13 (45)	8 (57)	11 (46)	<0.001
ESBL sequence							
CTX-M-I	22 (15)	4 (21)	3 (5)	9 (31)	6 (43)	0 (0)	
CTX-M-I4	35 (24)	4 (21)	15 (24)	10 (34)	3 (21)	3 (13)	
CTX-M-I5	64 (43)	8 (42)	30 (48)	8 (28)	2 (14)	16 (67)	
CTX-M-27	11 (7)	1 (5)	10 (16)	0 (0)	0 (0)	0 (0)	
Sequence type 131	51 (34)	0 (0)	28 (45)	8 (28)	1 (7)	14 (58)	<0.001
Phylogenetic group							0.001
A	21 (14)	3 (16)	11 (18)	3 (10)	1 (7)	3 (13)	
B1	12 (8)	6 (32)	4 (6)	1 (3)	0 (0)	1 (4)	
B2	74 (50)	0 (0)	34 (55)	12 (41)	11 (79)	17 (71)	
C	12 (8)	3 (16)	3 (5)	6 (21)	0 (0)	0 (0)	
D	16 (11)	5 (26)	5 (8)	3 (10)	1 (7)	2 (8)	
E	7 (5)	1 (5)	3 (5)	2 (7)	0 (0)	1 (4)	
F	6 (4)	1 (5)	2 (3)	2 (7)	1 (7)	0 (0)	
Relaxase (N=74)							0.15
F	28 (38)	3 (25)	15 (58)	5 (29)	2 (18)	3 (38)	
II	21 (28)	6 (50)	6 (23)	4 (24)	3 (27)	2 (25)	
Multireplicon <sup>b</sup>	11 (15)	2 (8)	1 (4)	5 (29)	2 (18)	1 (13)	
Other	8 (10)	0 (0)	4 (16)	0 (0)	3 (27)	1 (13)	
Non-typed	6 (8)	1 (8)	0 (0)	3 (18)	1 (9)	1 (13)	
Patient characteristics	(n=145)	(n=18)	(n=60)	(n=29)	(n=14)	(n=24)	
Age in years <sup>c</sup>	73 (56–86)	74 (61–85)	75 (56–89)	80 (64–86)	69 (36–84)	67 (56–84)	0.4
% Male	39	33	33	45	50	42	0.7
Any comorbidity	83 (57)	10 (56)	32 (53)	17 (59)	8 (57)	16 (67)	0.9
At risk exposure <3 months	100 (69)	11 (61)	44 (73)	22 (76)	7 (50)	16 (67)	0.4
Hospitalization (N=143)	81 (57)	8 (47)	37 (62)	19 (68)	5 (36)	12 (50)	0.2
Trip abroad (N=143)	11 (8)	1 (6)	4 (7)	3 (11)	0 (0)	3 (13)	0.7
Antibiotics (N=143)	71 (50)	8 (47)	29 (48)	17 (61)	6 (43)	11 (46)	0.8
Episode							0.2
Colonized	74 (51)	9 (50)	36 (60)	12 (41)	7 (50)	10 (42)	
Palliative care assoc.	3 (2)	2 (11)	1 (2)	0 (0)	0 (0)	0 (0)	
Treatment assoc.	68 (47)	7 (39)	23 (38)	17 (59)	7 (50)	14 (58)	
Acquisition							0.8
Nosocomial	69 (48)	8 (44)	28 (47)	16 (55)	5 (36)	12 (50)	
Community-acquired	76 (52)	10 (56)	32 (53)	13 (45)	9 (64)	12 (50)	
Associated bacteremia	18 (12)	3 (17)	4 (7)	6 (21)	0 (0)	5 (21)	0.09
Suspected infection location (N=65)							0.01
Urine	39 (60)	4 (67)	20 (91)	6 (38)	3 (43)	6 (43)	
Digestive tract	20 (31)	1 (17)	2 (9)	7 (44)	4 (57)	6 (43)	
Lungs	3 (5)	0 (0)	0 (0)	2 (13)	0 (0)	1 (7)	
Skin and soft tissue	3 (5)	1 (17)	0 (0)	1 (6)	0 (0)	1 (7)	
Length of stay <sup>c</sup> (N=144)	13 (3–30)	9 (1–32)	10 (2–24)	22 (9–36)	11 (2–20)	16 (10–28)	0.13
Transferred to ICU (N=65)	11 (17)	2 (29)	1 (5)	3 (18)	3 (43)	2 (15)	0.14
Death after 12 months (N=92)	39 (42)	2 (18)	21 (51)	7 (41)	6 (55)	3 (25)	0.2

**Notes:** Characteristics of 148 *E. coli* isolates from 145 patients were compared between five clusters of VFs. <sup>a</sup>Significance was determined using Kruskal–Wallis test for continuous variables and Pearson chi-squared test or Fisher's exact test for categorical variables. <sup>b</sup>2 Relaxases has been detected. <sup>c</sup>Median (IQR).

**Abbreviations:** ESBL, extended spectrum  $\beta$ -lactamase; ICU, intensive care unit; VFs, virulence factors.



trimethoprim–sulfamethoxazole-resistance ( $P=0.03$ ), while no difference was observed with aminoglycoside resistance phenotype.

Clusters of *K. pneumoniae* VF also appeared strongly associated with bacterial characteristics (Table 4). There were no significant differences in ESBL types between clusters ( $P=0.9$ ). Nevertheless, significant differences in the distribution of MLST types were observed ( $P<0.001$ ). ST147 and ST323 were more frequently observed in the “KP common VF” cluster, ST405 in the “KP common VF with *kfi*” cluster (with proportion harboring *kfi* VF at 100% compared to 10% for all other MLST types,  $P<0.001$ ) and ST29 in the “KP common VF with *ybtS*” cluster (with proportion harboring *ybtS* VF at 95% vs 22% for all other MLST types,  $P<0.001$ ). Distribution of relaxase-encoding plasmids was also significantly different between clusters ( $P=0.001$ ). No significant differences were observed in the proportion of isolates resistant to ciprofloxacin ( $P=0.9$ ) and trimethoprim/sulfamethoxazole ( $P=0.5$ ), but isolates from the “KP common VF” cluster appeared to have a higher proportion of the AAC(6′) aminoglycoside-resistant phenotype compared to others ( $P=0.008$ ).

## Patient characteristics and clinical outcomes between VF clusters

A comparison of the five *E. coli* clusters found no significant differences in patient level characteristics or measured clinical outcomes (Table 3). Nevertheless, a significant difference in the distribution of suspected source of infection at admission was noted between clusters ( $P=0.01$ ), with more frequent urine and digestive sources for clusters with more prevalent and varied VF (“siderophore exclusive with *iroN*” and “adhesin *papC/papGII*-rich” clusters). In addition, “siderophore exclusive”, “siderophore exclusive with *iroN*” and “adhesin *sfa/papGIII*-rich” clusters had the highest 12-month mortality rate compared to others ( $P=0.02$ ), while only the “siderophore exclusive with *iroN*” cluster having a high proportion with bacteremia (21%).

A comparison of the three *K. pneumoniae* clusters identified significant differences in proportion with recent travel ( $P=0.02$ ) (Table 4). Furthermore, the “KP common VF with *ybtS*” had the largest proportion of patients recently hospitalized (91%), whereas the “KP common VF” and “KP common VF with *kfi*” had the lowest (56%) ( $P=0.02$ ). No other significant differences in other patient characteristics or clinical outcomes were found (Table 4).

## Discussion

With the widespread dissemination of ESBL-producing Enterobacteriaceae, data on the genetic characteristics of strains are needed. In this large collection, we confirm the preponderance of CTX-M-producing microorganisms, of which CTX-M15 was the most common. CTX-M enzymes were particularly frequent in *K. pneumoniae*, as described by others.<sup>1</sup> We confirmed the high proportion of ST131 *E. coli* strains, first described in 2008 and now widespread.<sup>7</sup>

A wide body of literature has indicated that many VFs are observed in complex combinations among both *E. coli* and *K. pneumoniae* species, while the significance of these groupings vis-à-vis genetic characteristics or their clinical ramifications remains fairly understudied.<sup>6,21</sup> Here, we used a simple form of network analysis in which clusters showing the closest similarity between pairwise combinations of VFs are identified. Other studies, limited to animal populations, have used similar clustering techniques to describe groups of VF.<sup>22,23</sup>

*E. coli* clusters ranged from absence of all screened VF to increasing complexity of concomitant VF. The strains with the least number of VF originated from the “no VF”, “siderophore exclusive” and “siderophore exclusive with *iroN*” clusters. These strains were mostly implicated in UTI, particularly the “siderophore exclusive” cluster. This is surprising given that bacteria from these three clusters harbored few adhesins, which are generally involved in UTIs. Given that our study population had a high risk of mortality, the strong presence of *E. coli* as a UTI might be more linked with opportunistic conditions rather than the VFs themselves.<sup>24</sup>

In terms of clusters with increasing VF complexity, the “adhesin *papC/papGII*-rich” and “siderophore exclusive” clusters appears to bring together strains that are from the B2 phylogenetic group and ST131. “Siderophore exclusive” cluster frequently included CTX-M-15 producers or harboring fluoroquinolone resistance, and whose ESBL-producing genes are carried by the IncF plasmid. This cluster closely corresponds to the successful O25b:H4-ST131 CTX-M-15 clone.<sup>7</sup> The “adhesin *sfa/papGIII*-rich” cluster contained strains with Inc11 plasmids carrying CTX-M-1-producing genes. These strains have been observed in both humans and poultry, but in contrary to the O25b:H4-ST131 CTX-M-15 clone, were sensitive to fluoroquinolones.<sup>25–27</sup>

Overall mortality appeared to be higher in certain VF clusters, namely “siderophore exclusive”, “siderophore exclusive with *iroN*” and “adhesin *sfa/papGIII*-rich”, but no relationship was observed in ICU transfer rates. It should

**Table 4** Genetic and patient characteristics between clusters of *Klebsiella pneumoniae*

	Total	Cluster group			P-value <sup>a</sup>
		KP common VF	KP common VF with <i>ybtS</i>	KP common VF with <i>kfu</i>	
Genetic characteristics n (%)	(n=82)	(n=40)	(n=27)	(n=15)	
ESBL sequence					0.9
CTX-M-15	74 (90)	36 (90)	24 (89)	14 (93)	
CTX-M-14	2 (2)	1 (3)	1 (4)	0 (0)	
TEM-15	4 (5)	2 (5)	1 (4)	1 (7)	
Ciprofloxacin					0.9
Sensitive	8 (10)	4 (10)	2 (7)	2 (13)	
Trimethoprim-sulfamethoxazole					0.5
Sensitive	11 (13)	7 (18)	2 (7)	2 (13)	
Aminoglycoside phenotype					0.004
Sensitive	3 (4)	1 (3)	0 (0)	2 (13)	
AAC(3)-II	55 (67)	22 (55)	23 (85)	10 (67)	
AAC(6')	22 (27)	17 (43)	3 (11)	2 (13)	
APH(3')	2 (2)	0 (0)	1 (4)	1 (7)	
MLST					<0.001
ST29	19 (23)	1 (3)	17 (63)	1 (7)	
ST323	16 (20)	14 (35)	2 (7)	0 (0)	
ST147	11 (13)	10 (25)	0 (0)	1 (7)	
ST405	5 (6)	0 (0)	0 (0)	5 (33)	
Others	31 (38)	15 (37)	8 (30)	8 (53)	
Relaxase (N=56)					0.001
FIK	23 (41)	15 (56)	3 (16)	5 (50)	
Untypable	17 (30)	9 (33)	6 (32)	2 (20)	
F	8 (14)	1 (4)	7 (37)	0 (0)	
Others	8 (16)	2 (7)	3 (15)	3 (30)	
Patient characteristics	(n=69)	(n=32)	(n=22)	(n=15)	
Age in years <sup>b</sup>	64 (56–80)	63 (54–84)	70 (59–76)	63 (58–88)	0.9
% Male	68	69	68	67	0.9
Any comorbidity	52 (75)	23 (72)	18 (82)	11 (73)	0.8
At risk exposure <3 months	60 (87)	27 (84)	20 (91)	13 (87)	0.9
Hospitalization	48 (70)	18 (56)	20 (91)	10 (67)	0.02
Trip abroad	6 (9)	0 (0)	3 (14)	3 (20)	0.02
Antibiotics	48 (70)	24 (75)	14 (64)	10 (67)	0.7
Episode					0.4
Colonized	42 (61)	21 (66)	13 (59)	8 (53)	
Infection	27 (39)	11 (34)	9 (41)	7 (47)	
Acquisition					0.17
Nosocomial	56 (81)	26 (81)	20 (91)	10 (67)	
Community-acquired	13 (19)	6 (19)	2 (9)	5 (33)	
Associated bacteremia	8 (12)	3 (9)	5 (23)	0 (0)	0.11
Suspected location of infection					0.2
Urine	9 (36)	3 (33)	1 (11)	5 (71)	
Lungs	8 (32)	2 (22)	4 (44)	2 (29)	
Digestive tract	5 (20)	3 (33)	2 (22)	0 (0)	
Skin and soft tissue	3 (12)	1 (11)	2 (22)	0 (0)	
Length of stay <sup>b</sup>	29 (19–63)	30 (20–86)	42 (17–82)	24 (19–47)	0.5
Transferred to intensive care (N=26)	6 (23)	2 (18)	2 (22)	2 (33)	0.8
Death after 12 months (N=55)	24 (44)	9 (39)	8 (44)	7 (50)	0.8

**Notes:** Characteristics of 82 *K. pneumoniae* isolates from 69 patients were compared between three clusters of VFs. <sup>a</sup>Significance was determined using Kruskal–Wallis test for continuous variables and Pearson chi-squared test or Fisher's exact test for categorical variables. <sup>b</sup>Median (IQR).

**Abbreviations:** ESBL, extended spectrum  $\beta$ -lactamase; VFs, virulence factors.

be mentioned, however, that patients in our study population had multiple co-morbidities and were for the most part immunosuppressed, which likely contributed to a higher risk of overall mortality in general. Other research has in fact shown that host factors and portal of entry likely outweigh bacterial determinants in predicting the severity of *E. coli* bacteremia.<sup>8,24</sup> In another study of *E. coli* bacteremia among pregnant women, lower virulence scores were actually associated with more severe episodes.<sup>28</sup> The link between these groups of VF and mortality will need to be addressed in larger studies.

There are certain noteworthy aspects of the *K. pneumoniae* strains observed in our study with three specific clones. CTX-M-15-producing genes carried on IncFIIk plasmid and ST147 resistant to quinolones clones of *K. pneumoniae* have been previously described.<sup>29,30</sup> These two clones have been isolated in different services at our hospital. Additionally, we isolated 19 ST29 strains, for the most part resistant to quinolones or trimethoprim/sulfamethoxazole, phenotype AAC(3)-II and with *entB*, *mrkD* and *ybtS*, from patients in the gastrointestinal surgery department. Plasmids from this ST belonged to the IncF group and carried CTX-M-15 genes. There are few data in the literature to date on ST29. Rep and ERIC PCR confirmed the clonality of these strains, thus suggesting cross-transmission.

Furthermore, it is worth noting that strains observed in our study population produced for the most part CTX-M ESBL. Four *E. coli* and five *K. pneumoniae* isolates were non-CTX-M producing and their presence was only observed in adhesin *papC/papGII*-rich VF clusters of *E. coli* and in all VF clusters of *K. pneumoniae*. As expected, excluding non-CTX-M-producing isolates did not change our overall analysis (data not shown). Nevertheless, we stress that these findings stem from *E. coli* and *K. pneumoniae* with predominately CTX-M ESBL production.

Some limitations of our study need to be stated. First, there are a multitude of methods able to determine clusters (ie, hierarchical clustering, principal component analysis, latent class analysis, etc). We opted for a more simplistic method based on the few parameters (ten VF at most), binomial distribution of these parameters and relatively few numbers of patients. We cannot infer how robust these clusters would be in other settings. Second, this study was conducted at a single hospital center and the isolates obtained herein might not be representative of other settings. These results should be interpreted while keeping in mind the diverse range of departments included. Third, strains from both infection and colonization were considered and

since there was substantial variability between these two groups, additional heterogeneity could have affected cluster composition. Fourth, some individuals had more than one infection with the same species but at different time points. The repeated data in these patients would not alter the cluster analysis, but could bias statistical comparisons. Given the few patients in whom this was observed (n=5), any effect from this bias would likely be minimal. Finally, *K. pneumoniae* isolates were not overly abundant and their identified VFs were limited in spectrum. The cluster composition, as a result, frequently led to a single VF. Larger collections of isolates would be needed to obtain more meaningful results.

## Conclusion

Distinct clusters of *E. coli* and *K. pneumoniae* VF were observed within ESBL-producing isolates. These clusters were linked to ESBL-producing plasmids and phylogenetic groups for *E. coli*, ST and relaxase types for *K. pneumoniae*, and resistance to specific antibiotic agents for both species.

Further evidence in healthier populations would be needed to sufficiently address whether VF are associated with clinical outcomes or if they can be used to predict severe morbidity and mortality during treatment. Analyzing genetic factors using a clustering approach could be beneficial for future studies.

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