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Clonal distribution of multidrug-resistant *Enterobacter cloacae*

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A multilocus sequence typing (MLST) scheme including 7 housekeeping genes was used to evaluate whether the current spread of multidrug-resistant *Enterobacter cloacae* isolates worldwide might be associated to specific successful clones. Fifty *E. cloacae* clinical isolates of worldwide origin, with various β-lactamase content, and recovered at different periods of time were studied. Forty-four sequence types were identified, highlighting a high clonal diversity with 3 main lineages. This study revealed that a precise identification of the isolates by sequencing of the chromosomal *ampC* gene of *E. cloacae* would provide a significant added value to improve the reliability of the MLST scheme.

1. Introduction

Although *Enterobacter cloacae* is an enterobacterial species that possesses several naturally occurring resistance mechanisms, acquired multidrug resistance is increasingly observed. Resistance to β-lactams in that species may be related to overexpression of chromosomal *ampC* β-lactamase genes, acquisition of plasmid-mediated extended-spectrum β-lactamase (ESBL), or carbapenemase genes, those latter genes encoding KPC type; OXA-48 type; or metallo-β-lactamases of the VIM-, IMP-, and NDM-1 types (Girlich et al., 2014; Ikonomidis et al., 2007; Novak et al., 2014; Pasanen et al., 2014; Pestourie et al., 2014; Poirel et al., 2014). Identifying clonal spread of multidrug-resistant isolates recovered from geographically distant locations remains difficult, since data are being obtained mainly by using the pulsed-field gel electrophoresis technique, which provides heterogeneous and noncomparable data. By contrast, multilocus sequence typing (MLST) provides interlaboratory comparison of epidemiological data. We have used here a recently described MLST scheme including 7 housekeeping genes (Miyoshi-Akiyama et al., 2013) in order to perform an epidemiological comparison of 50 multidrug-resistant *E. cloacae*. This study focuses on multidrug-resistant isolates and, in particular, those producing the NDM-1 or OXA-48 carbapenemases and the ESBL CTX-M-15. The aim was to evaluate i) whether some specific sequence types (STs) with a peculiar β-lactamase content might be disseminated among various countries and ii) whether ESBL- and carbapenemase-producing isolates might correspond to the same strain backgrounds as those of the ESBL-positive but carbapenemase-negative isolates.

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2. Methods

2.1. Clinical isolates

Fifty *E. cloacae* clinical isolates of worldwide origin and recovered at different periods of time (from 1994 to 2013) were included. They were randomly selected from our international collection, including isolates from India, Lebanon, France, Vietnam, Morocco, Turkey, and Algeria. They have been characterized for their β-lactamase content at the molecular level. The isolates were either wild-type strains (n = 3), AmpC overproducers (n = 10), ESBL producers of the CTX-M-15 type (n = 16), and carbapenemase producers (n = 24) (Table 1). Isolates were identified by the API20E biochemical test (bioMérieux, Marcy l'Etoile, France).

2.2. MLST and phylogenetic analysis

MLST primers targeted 7 housekeeping genes (*dnaA*, *fusA*, *gyrB*, *leuS*, *pyrG*, *rplB*, and *rpoB*) with PCR conditions, as recommended (Miyoshi-Akiyama et al., 2013). Purified PCR products were sequenced using an ABI3130 apparatus (Applied Biosystems, Life Technologies SAS, Saint Aubin, France). Concatenated sequences of the 7 DNA fragments (3501 nucleotides in total) were obtained from the Web site (http://pubmlst.org/ecloacae/) and compared using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method. A phylogenic tree was inferred by bootstrap phylogenetic inference using Molecular Evolutionary Genetics Analysis Version 6.0. software (MEGA6) (Tamura et al., 2013). These sequences were compared with 3 concatenated reference sequences (ST2, ST3, and ST9), corresponding to the 3 main clades of ST for *E. cloacae* isolates, as reported by Miyoshi-Akiyama et al. (2013). Searches of the GenBank databases were carried out using the NCBI BLASTn option (www.ncbi.nlm.nih.

Table 1
E. cloacae isolates used in this study.

Isolate	ß-lactamase content	Year and country of isolation	ST	*dnaA*	*fusA*	*gyr*	*leuS*	*pyrG*	*rplB*	*rpoB*	lineage
5434	wild type	2011 France	237	4	6	4	6	37	36	25	1
7725	wild type	2011 France	238	58	40	81	9	79	37	38	3
7746	wild type	2011 France	247	58	61	99	102	97	6	55	7
KAU	overexpressed AmpC	2012 France	108	68	8	75	63	65	34	35	1
VIL	overexpressed AmpC	2012 France	50	4	4	4	6	37	4	25	1
BRE	overexpressed AmpC	2012 France	239	58	37	4	6	4	4	25	1
COUP	overexpressed AmpC + TEM-121	2012 France	248	92	60	100	99	9	42	56	-[a]
BALD	overexpressed AmpC + TEM-2	2012 France	240	8	9	6	9	9	4	8	3
CON	overexpressed AmpC	2012 France	241	4	4	37	6	81	4	25	1
AZA	overexpressed AmpC	2012 France	249	86	25	96	100	100	14	57	8
KHA	CTX-M-15	2008 France	198	68	8	75	63	65	34	6	1
FOU	CTX-M-15	2008 France	78	8	9	6	9	9	6	6	3
3	CTX-M-15 (ArmA)	2008 India	199	46	21	20	96	45	29	54	2
CAU	CTX-M-15 + TEM-1	2008 France	168	59	40	81	9	79	37	38	3
NAI	CTX-M-15 + SHV-12	2008 France	196	8	33	86	9	9	6	8	3
DAS	CTX-M-15 + TEM-1	2008 France	201	53	35	20	44	45	38	8	2
KER	CTX-M-15	2009 France	202	53	35	20	44	45	29	32	2
HOF	CTX-M-15	2009 France	195	53	35	20	44	45	4	53	2
RIV	CTX-M-15	2009 France	251	85	63	101	103	96	6	6	8
CON	CTX-M-15	2009 France	114[b]	53	35	20	44	45	4	6	2
ZER	CTX-M-15 + TEM-1	2010 France	114	53	35	20	44	45	4	6	2
BAL	CTX-M-15	2010 France	114	53	35	20	44	45	4	6	2
BAR	CTX-M-15 + TEM-1	2010 France	207	53	35	20	45	45	4	6	2
SAM	CTX-M-15 + TEM-1	2011 France	114	53	35	20	44	45	4	6	2
CAR	CTX-M-15	2011 France	236	59	64	81	9	79	37	6	8
BRA	CTX-M-15 + TEM-1 + SHV-28	2011 France	200	74	20	20	65	45	4	32	2
NOR	NMC-A	1994 France	250	84	62	95	98	94	43	52	6
KAR	VIM-1 + SHV-70	2011 France	229	87	16	25	97	22	9	15	6
KOW3	VIM-4 + CTX-M-15 + TEM-1 + SHV-31	2011 Kuwait	203	4	4	20	6	92	30	6	1
TWA	IMP-8	2011 Taiwan	194	11	6	4	13	39	4	9	1
TAW	IMP-8 + SHV-12	2011 Taiwan	204	4	4	4	6	95	4	6	1
RAZ	NDM-1	2012 Vietnam	193	49	20	7	44	90	24	32	2
PAY	NDM-1	2012 France	230	49	20	74	44	90	24	32	2
SEN	NDM-1	2012 France	205	4	6	4	61	39	4	25	1
BOQ	NDM-1	2012 France	231	46	20	20	96	45	29	54	2
ABA	NDM-1 + CTX-M-15 + TEM-1 + OXA-1	2013 France	200	74	20	20	65	45	4	32	2
GAT	NDM-1 + CTX-M-15 + TEM-1 + OXA-1	2013 France	206	67	20	20	44	45	4	32	5
IR38 C	NDM-1 + CTX-M-15	2012 India	235	49	21	19	44	94	12	32	4
LIB	NDM-1 + CTX-M-15	2012 Lebanon	32	3	24	3	35	3	16	17	3
MAR17	OXA-48 + TEM-1 + CTX-M-9	2009 Morocco	197	67	21	74	95	45	35	6	2
MAR18	OXA-48 + TEM-1 + SHV-12 + CTX-M-9	2009 Morocco	197	67	21	74	95	45	35	6	2
MAR19	OXA-48 + TEM-1 + CTX-M-15	2009 Morocco	192	46	20	74	44	45	24	42	2
BOU	OXA-48 + TEM-1 + CTX-M-15 + OXA-1	2010 Morocco	245	91	59	19	44	99	4	32	2
TUR	OXA-48 + SHV-5	2010 Turkey	120	46	20	20	44	45	29	6	2
MAR20	OXA-48 + TEM-1 + SHV-12	2011 Morocco	190	9	4	15	6	37	4	9	1
501	OXA-48 + TEM-1 + CTX-M-15 + OXA-1	2011 Morocco	244	90	20	19	44	45	4	6	2
BEU	OXA-48 + TEM-1 + SHV-12 + CTX-M-15 + DHA-1 + OXA-1	2011 France	182	49	20	19	44	90	24	32	2
ESS	OXA-48 + CTX-M-15	2011 Algeria	25	24	14	43	52	27	18	21	6
AZZ	OXA-48 + TEM-1 + CTX-M-15 + OXA-1	2011 Morocco	246	67	59	19	44	99	4	32	2
DOV	OXA-48 + TEM-1 + CTX-M-15 + OXA-1	2011 Morocco	114	53	35	20	44	45	4	6	2

[a] Isolates of ST 25, 229, 249, and 250 have been identified as *E. asburiae*; isolate of ST248 has been identified as *E. aerogenes* and should, therefore, be excluded from this MLST scheme if strictly restricted for *E. cloacae* isolates.
[b] *E. cloacae* with ST114 are indicated with a gray shadow.

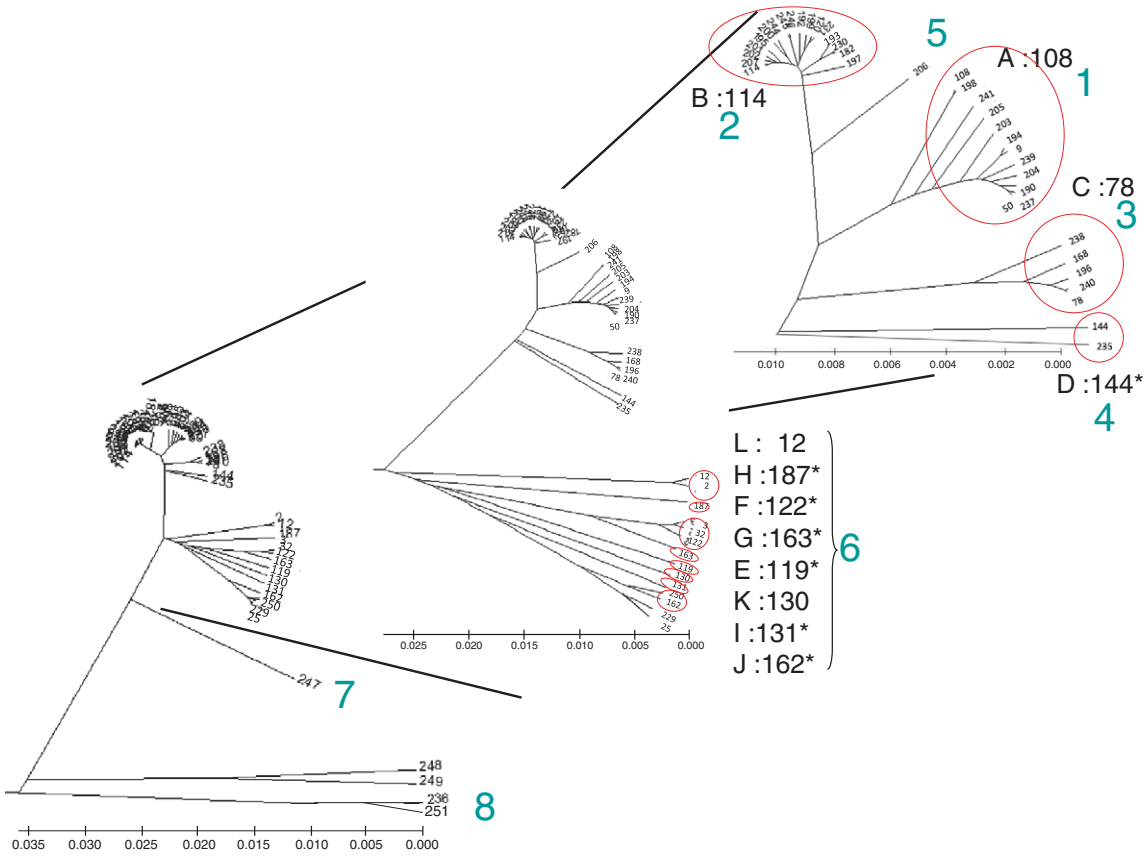

Fig. 1. Unrooted tree of concatenated sequences from combinations of 7 MLST loci. Phylogenetic analysis of 44 distinct ST from fifty *E. cloacae* isolates, 3 reference ST (ST2, ST3, and ST9), from Miyoshi-Akiyama et al. (2013) and STs from the study of Izdebski et al. (2015) as representatives of each cluster (letters a to L) previously reported. The eight lineages identified in this study are indicated with numbers (Girlich et al., 2014; Grundmann et al., 2001; Ikonomidis et al., 2007; Izdebski et al., 2015; Miyoshi-Akiyama et al., 2013; Novak et al., 2014; Pasanen et al., 2014; Pestourie et al., 2014). The STs not identified in our study were marked with an asterisk. The tree was inferred by bootstrap phylogenetic inference using MEGA6 software.

gov), and precise identification of the species was carried out using the *rpoB* sequence on the bioinformatics database leBIBI (umr5558-sud-str1.univ-lyon1.fr/lebibi/lebibi.cgi). The clonal diversity index and confidence intervals (CIs) were calculated according to Grundmann et al. (2001).

2.3. Chromosomal ampC and phylogenetic analysis

Considering that *E. cloacae* is a complex of several species and subspecies, the *ampC* gene encoding the intrinsic AmpC β-lactamase of the species *E. cloacae* was amplified and sequenced with specific primers, namely, ECA (5′-CCCTTTGCTGCGCCCTGC-3′) and ECB (5′-TGCCGCCTCAACGCGTGC-3′). Since amplifications failed for isolates belonging to ST248 and ST249, PCR with primers specific for the intrinsic *ampC* genes of other *Enterobacter* species were also attempted. The *ampC* gene of *Enterobacter aerogenes* was amplified with primers EaerA (5′-CAATCAGACCATCACGCCGT-3′) and EaerB (5′-AGCATCACGATACCGAGATCC-3′) and that of *Enterobacter asburiae* with primers EasbA (5′-GGAACGTACCGTTACGCCGC-3′) and EasbB (5′-GCCGGTTTTATGGACCCAGG-3′). Sequences of the *ampC* genes (790-bp long) were compared using the UPGMA method. A phylogenic tree was inferred by bootstrap phylogenetic inference using MEGA6 (Tamura et al., 2013).

2.4. Nucleotide sequence accession numbers

The *ampC* gene sequences of isolates 7746 of ST247, RIV of ST251, AZA of ST249, NOR of ST250, and ESS of ST25 appear in the GenBank database with accession numbers KM985624 to KM985628.

3. Results and discussion

3.1. MLST and phylogenetic analysis

Forty-four STs were identified among the 50 *E. cloacae* isolates, highlighting a high clonal diversity (Table 1). Out of 44 distinct ST identified, 39 (88.6%) corresponded to novel ones. The clonal diversity index was 93.7% with a CI value of 92.5–94.9%, indicating a high genetic diversity. Phylogenetic analysis revealed little association either to the antibiotic resistance gene content or to the geographic origin of the isolates (Table 1 and Fig. 1). This result is consistent with results from Izdebski et al. (2015), who reported very recently a clonal diversity of 97.5% among expanded-spectrum cephalosporin-resistant *E. cloacae* isolates from Europe and Israel (Izdebski et al., 2015). The discriminatory ability of the different loci, measured as the number of alleles obtained for each gene, varied from 17 (for *rpoB* and *rplB*) to 21 (for *dnaA*) (Table 1). This result differs from that obtained by Miyoshi-Akiyama et al. (2013), reporting the *leuS* and *pyrG* alleles as the most polymorphic ones. The result of the nucleotide diversity analysis within the sample, performed on alleles of each MLST gene, demonstrated significant diversity in accordance with the data from Miyoshi-Akiyama et al. (2013) and Izdebski et al. (2015).

The most frequently identified alleles were *dnaA*-53 (9 isolates), *fusA*-20 (10 isolates), *gyrB*-20 (16 isolates), *leuS*-44 (18 isolates), *pyrG*-45 (19 isolates), *rplB*-4 (21 isolates), and *rpoB*-6 (15 isolates) (Table 1). Even though the combination of all these alleles in a given ST profile has not been reported yet, it is very close to that of the most frequently identified ST of this study (ST114), differing only by a single locus (*fusA*) (Table 1).

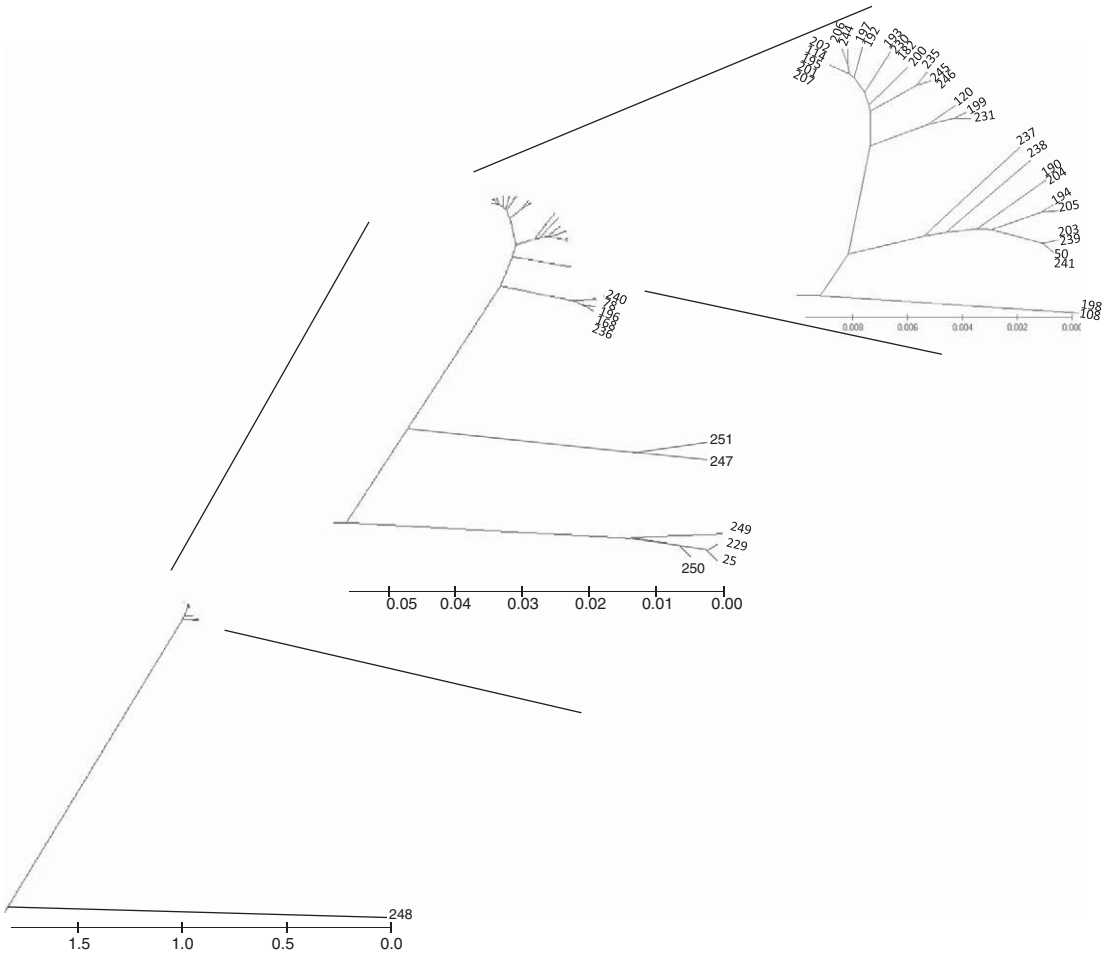

Fig. 2. Unrooted tree of chromosomal *ampC* sequences from the 50 *Enterobacter* isolates. Phylogenetic analysis of the 50 *E. cloacae* isolates used in this study after sequencing of their chromosomal *ampC* gene. The tree was inferred by bootstrap phylogenetic inference using MEGA6 software. The isolate of ST248, identified as an *E. aerogenes* isolate, and ST25, ST229, ST249, and ST250, identified as *E. asburiae* isolates after sequencing of the *ampC* genes, should be excluded from MLST lineages.

The global phylogeny revealed 8 lineages. Three major lineages (namely, 1, 2, and 3) encompassed most *E. cloacae* isolates, represented by ST78 like (group 3), ST108 like (group 1), and ST114 like (group 2) (Fig. 1). Each of these lineages contained concatenated sequences showing >99% nucleotide identity and shared ca. 98% of nucleotide identity with each other. In order to compare with the 3 main clades reported by Miyoshi-Akiyama et al. (2013), concatenated sequences of ST2, ST3, and ST9 (respectively, corresponding to clades 1, 2, and 3) were included in our phylogenetic analysis. Surprisingly, the 8 lineages identified in our study did not correlate with the 3 clades previously reported. For instance, ST2 and ST3 from Miyoshi-Akiyama et al. (2013) were included in the same lineage containing ST32 and showed ca. 94% nucleotide identity with the ST114 lineage (Fig. 1).

The phylogenetic analysis of Izdebski et al. (2015) performed on 195 *E. cloacae* resistant to expanded spectrum cephalosporins and collected across Europe and Israel distinguished 80 STs and contained 12 clusters of very related STs. Comparing with those data, we were able to redefine our lineages accordingly, aiming to make both studies corremating as much as possible. The phylogenetic analysis was, therefore, completed by including the 12 STs representing the 12 different clusters as defined by Izdebski et al. (2015). The first 4 lineages could be well correlated with those (A to D) from Izdebski et al. (2015), but some STs that had not been previously identified could not be related to any of the previously described clusters.

3.2. Chromosomal ampC and phylogenetic analysis

Sequencing of the *ampC* genes showed a lower diversity than that obtained with the 7 housekeeping genes used for the MLST. The phylogenetic tree obtained with the *ampC* sequences showed only 5 main lineages and differed from that obtained with the concatenated sequences (Fig. 2). Noticeably, isolates with the same ST harbored the same *ampC* sequence (ST114, ST197, and ST200). However, the *ampC* genes of ST247 and ST251 isolates, those of ST25, ST229, ST249, and ST250 isolates, and that of the ST248 isolate significantly differed from those of all other isolates (Fig. 2). The *ampC* gene of the ST247 isolate shared the highest identity with that of ST251, with a value of 97.3% and a maximum of 89.4% nucleotide identity with that of other *E. cloacae* isolates.

Since the amplification of the *ampC* gene from a single isolate belonging to ST248 failed, primers EaerA and EaerB specific for the *E. aerogenes ampC* gene were used and gave a positive result. Sequence analysis identified an *ampC* gene sharing 100% nucleotide identity with that of *E. aerogenes* EA1509E (Preston et al., 2000), and no more than 73.4% nucleotide identity with the *ampC* genes identified from *E. cloacae*. As suggested in Fig. 1, this ST248 isolate might, therefore, be excluded from the MLST lineage, considering that this isolate was initially misidentified. Indeed, the closest *rpoB* sequence from that of the ST248 isolate was that of *E. aerogenes* CP002824. This indicates that the fragment length of the *rpoB* gene used in the MSLT scheme might be too short for a precise identification of the species. By contrast, the use of the chromosomal *ampC* gene should be preferred for identification of the species.

In addition, since the amplification of the *ampC* gene from the ST249 isolate failed with primers specific for *E. cloacae*, primers EasbA and EasbB specific for the *E. asburiae ampC* gene were used and gave a positive result. Sequencing identified this *ampC* gene with 93% nucleotide identity compared to that of *E. asburiae*. Moreover, all others isolates grouped

with that of ST249 in Fig. 2, e.g., ST25, ST229, and ST250, harbored chromosomal *ampC* genes with 98–99% nucleotide identity with that of *E. asburiae*. All those isolates should, therefore, be excluded from the MLST scheme of *E. cloacae* species by further modifying the specificity of the typing (i.e., targeting other housekeeping genes, modifying primer locations).

3.3. Clonal distribution of multiresistant E. cloacae isolates

All these observations, although limited to a little number of isolates, suggest that there was no significant relationship between a specific β-lactam resistance profile and the major *E. cloacae* clonal groups (ST78, ST114, and ST108). Nevertheless, *E. cloacae* isolates belonging to ST114 were prevalent. Moreover, even though all $bla_{CTX\text{-}M\text{-}15}$–positive isolates did not all belong to ST114, most of them actually belonged to the clonal complex 114, with ST114 as the main representative ST (Table 1; Fig. 1).

4. Conclusion

We found here an important clonal diversity of multidrug-resistant *E. cloacae* isolates. No obvious link with a specific β-lactamase content or with a specific geographical origin could be evidenced. Nevertheless, the most prevalent ST identified was ST114. Further studies will be required to further evaluate the possible involvement of this clone in the dissemination of the $bla_{CTX\text{-}M\text{-}15}$ gene. Moreover, this study highlights some drawbacks of the current MLST scheme that may erroneously include non *E. cloacae* species and, therefore, bias the analysis. We believe that including the chromosomal *ampC* gene of *E. cloacae* would provide an added value to the reliability of the scheme.

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Transparency declarations

None to declare.

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