Five GES-producing Enterobacteriaceae isolates that displayed an extended-spectrum \(\beta\)-lactamase (ESBL) phenotype harbored two GES variants: GES-7 ESBL and GES-6 carbapenemase. In all isolates, the two GES alleles were located on the same integron that was inserted into an 80-kb IncM1 self-conjugative plasmid. Whole-genome sequencing suggested in vivo horizontal gene transfer of the plasmid along with clonal diffusion of Enterobacter cloacae. To our knowledge, this is the first description in Europe of clustered Enterobacteriaceae isolates carrying two GES \(\beta\)-lactamases, of which one has extended activity toward carbapenems.

GES-type extended-spectrum \(\beta\)-lactamases (ESBLs) are increasingly reported in Pseudomonas aeruginosa, Acinetobacter baumannii, Escherichia coli, and Klebsiella pneumoniae (1, 2, 3). More than 27 GES variants have now been identified throughout the world (3). One feature of these enzymes is that they may modify their spectrum of hydrolysis by point mutations. GES-2 was the first described example of an ESBL that extended its spectrum of activity against carbapenems through a single point mutation (4). To date, at least 12 variants (GES-2, -4, -5, -6, -13, -14, -15, -16, -18, -20, -21, and -24) possess a substitution at position 170 and are theoretically able to hydrolyze carbapenems. Some variants also have the ability to hydrolyze cefotixin (GES-4, GES-5, GES-6, and GES-11) and/or aztreonam (GES-9 and GES-14) (3). The \(\text{bla}_{\text{GES}}\) genes have been essentially described as gene cassettes associated with class 1 or class 3 integrons on plasmids with different types of replicases (3, 5).

Here, we describe enterobacterial isolates recovered from a Belgian hospital that harbored two variants of \(\text{bla}_{\text{GES}}\) genes that were inserted back to back in the same integron, one coding for an ESBL and the second one coding for a carbapenemase.

In May 2007, an ESBL-producing Enterobacter cloacae isolate (EB-1) was isolated from the stool sample of a two-year-old girl who was hospitalized in the pediatric surgical ward of a Belgian hospital for a follow-up after a second liver transplantation. This patient underwent a first transplantation in Israel in January 2006 and was transferred to Belgium for a second transplantation. During initial hospitalization, all of the stool samples obtained for screening of ESBL carriage remained negative. The child left the hospital in May 2006 and did not travel until rehospitalization in May 2007. One week after the first case, an ESBL-producing Citrobacter youngae isolate (EB-2), displaying a similar resistance pattern, was cultured from the urine sample of a 1-year-old girl who was hospitalized for acute pyelonephritis in the same ward. A third child, a 3-year-old boy transferred from Macedonia for a liver transplant in November 2007, was negative for ESBL carriage at admission. He was found to be colonized with ESBL-producing Enterobacteriaceae in December 2007, first with an E. cloacae isolate (EB-3) and then with another E. cloacae isolate (EB-4) and a K. pneumoniae isolate (EB-5).

Antibiograms performed by disk diffusion and interpreted according to EUCAST guidelines (http://www.eucast.org) revealed that all isolates (EB-1 to EB-5) were resistant to penicillins, cephalothin, and ceftazidime but remained susceptible to cefotaxime, ceftazidime, aztreonam, and imipenem. Moreover, synergy images were demonstrated between clavulanic and third/fourth-generation cephalosporins, suggesting the presence of an ESBL. Etests revealed that the MICs for aztreonam were in the intermediate range and that the MICs of carbapenems were in the susceptible range (imipenem, MICs \(\leq 0.5\) \(\mu\)g/\(\mu\)l; ertapenem, MICs \(\leq 0.047\) \(\mu\)g/\(\mu\)l; meropenem, MICs \(\leq 0.047\) \(\mu\)g/\(\mu\)l). In addition, the five isolates were resistant to all aminoglycosides, except gentamicin, and to co-trimoxazole but remained susceptible to fluoroquinolones, tigecycline, and colistin (data not shown).

Whole-cell DNA extraction and ESBL- and carbapenemase-specific PCR, as previously described (6), revealed that the five isolates possessed only the \(\text{bla}_{\text{GES}}\) gene. However, sequencing of the PCR products on the two strands using an ABI 3100 automated Sanger sequencer (Applied Biosystems, Les Ulis, France) revealed a double peak (A or G) at position 508 in all five isolates. At this position, an AGC codon codes for Ser at Ambler’s position 170 and corresponds to the GES-6 variant whereas a GCC codon codes for Gly and corresponds to a GES-7 variant. Kieser’s extraction of natural plasmids revealed that all of the isolates possessed at least a ca. 80-kb plasmid that could be transferred by conjugation into E. coli as previously described (6, 7). Transconjugants expressed resistance patterns similar to those of the parental strains. PCR experiments confirmed the presence of the \(\text{bla}_{\text{GES}}\) gene in the transconjugants, and sequencing of the PCR


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G.C. and P.B. contributed equally to this work.

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products revealed the same double peak at position 508, suggesting the presence of two copies of the \textit{bla}\textsubscript{GES} gene on the same plasmid.

The entire genomes of \textit{E. cloacae} EB-1, EB-3, and EB-4 and of \textit{K. pneumoniae} EB-5 along with the natural plasmids pEB-1 and pEB-2 extracted from \textit{E. coli} transconjugants were sequenced using the Nextera XT v3 kit (Illumina, San Diego, CA, USA) according to the manufacturer’s recommendations and then run on MiSeq (Illumina) to generate paired-end 300-bp reads (6, 8).

\textit{De novo} assembly was performed by CLC Genomics Workbench v7.0.4 (Qiagen, Hilden, Germany). The acquired antimicrobial resistance genes and multilocus sequence types (MLSTs) were identified by uploading assembled genomes to the ResFinder server v2.1 (http://cge.cbs.dtu.dk/services/ResFinder-2.1) and MLST 1.8 (https://cge.cbs.dtu.dk/services/MLST/), respectively. The genome was annotated using the RAST server (9).

Plasmid pEB-1 was constructed and confirmed the presence of two copies of the \textit{bla}\textsubscript{GES} gene, \textit{bla}\textsubscript{GES-6} and \textit{bla}\textsubscript{GES-7} (Fig. 1). The two \textit{bla}\textsubscript{GES} genes were embedded together in a gene cassette array of a class 1 integron, along with four other gene cassettes, \textit{aacA4}, \textit{smr2}, \textit{dfrA1}, and \textit{addA1}. The 3’ conserved sequence was composed by the \textit{qacE} and \textit{sul1} genes as frequently described (10). The gene \textit{aacA4} encodes a 6'-N-aminoglycoside acetyltransferase \textit{[AAC(6’)-I]} conferring resistance to amikacin, tobramycin, and netilmicin but not to gentamicin, and \textit{dfrA1} and \textit{sul1} are responsible for trimethoprim and sulfonamide resistances, respectively, which is consistent with the observed phenotype of all isolates.

Plasmid pEB-1 was 78,907 bp in size and belonged to the IncM1 subgroup according to the IncRNA classification scheme, with 100%, 98%, and 98% IncRNA nucleotide sequence identity with pACM1, R69, and pFOX-7a, respectively (11–14). This plasmid contained open reading frames (ORFs) including genes involved in replication, mobilization, partitioning, and conjugation. Overall, the backbone of pEB-1 was similar to that of the IncL/M family plasmids described previously, e.g., pEL60 or pNDM-OM (Fig. 1), with the exception of the integration site of the resistance gene array (13).

Sequences of the other natural plasmids revealed a high degree of identity, except for pEB-4, which has another mercury operon.
TABLE 1 | Clinical data of the patients with GES-producing Enterobacteriaceae

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Country of origin</th>
<th>Date of hospitalization</th>
<th>Site of isolation</th>
<th>Underlying disease</th>
<th>Infection</th>
<th>Isolates</th>
<th>Plasmid</th>
<th>rep-PCR</th>
<th>MLST</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2</td>
<td>F</td>
<td>Israel</td>
<td>02/2007</td>
<td>Urine</td>
<td>Liver transplantation</td>
<td>Fever of unknown origin</td>
<td>E. coli</td>
<td>pEB-1</td>
<td>A</td>
<td>ST-346</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>F</td>
<td>Belgium</td>
<td>02/2007</td>
<td>Stool</td>
<td>Lung transplantation</td>
<td>Lung cancer</td>
<td>K. pneumoniae</td>
<td>EB-2</td>
<td>pEB-2 = pEB-1</td>
<td>A</td>
</tr>
<tr>
<td>C</td>
<td>3</td>
<td>M</td>
<td>Macedonia</td>
<td>11/2007</td>
<td>Urine</td>
<td>Congestive heart failure</td>
<td>Urinary tract infection</td>
<td>E. cloacae</td>
<td>EB-3</td>
<td>pEB-3 = pEB-1</td>
<td>A</td>
</tr>
</tbody>
</table>

Rep-PCR results were obtained for the three E. cloacae isolates with the semiautomated DiversiLab system (bioMérieux).<sup>a</sup> These plasmids are 100% identical to pEB-1.<sup>b</sup> pEB-1 ins Mer is 100% identical to pEB-1, except for the mercury transposon that was replaced with that of pSF088-1.<sup>c</sup> pEB-1 152bp is 100% identical to pEB-1, except for a 152-bp deletion between nucleotide positions 8823 and 8975 compared to pEB-1.<sup>d</sup> N.D., not done. Since isolate did not grow, the plasmid profile was not determined.<sup>e</sup> These plasmids are 100% identical to pEB-1, except for the mercury transposon that was replaced with that of pSF088-1.<sup>f</sup> pEB-1 ins Mer is 100% identical to pEB-1, except for the mercury transposon that was replaced with that of pSF088-1.<sup>g</sup> pEB-1 152bp is 100% identical to pEB-1, except for a 152-bp deletion between nucleotide positions 8823 and 8975 compared to pEB-1.<sup>h</sup> N.D., not done. Since isolate did not grow, the plasmid profile was not determined.<sup>i</sup> These plasmids are 100% identical to pEB-1, except for the mercury transposon that was replaced with that of pSF088-1.<sup>j</sup> pEB-1 ins Mer is 100% identical to pEB-1, except for the mercury transposon that was replaced with that of pSF088-1.<sup>k</sup> pEB-1 152bp is 100% identical to pEB-1, except for a 152-bp deletion between nucleotide positions 8823 and 8975 compared to pEB-1.

**REFERENCES**

9. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, inserted, while the rest of the plasmid was entirely identical. Semi-automated repetitive extragenic palindrome PCR (rep-PCR) typing (DiversiLab; bioMérieux) of the E. cloacae isolates (EB-1, EB-3, and EB-4) revealed that EB-1 and EB-3 were related (more than 95% identity) whereas EB-4 was unrelated to the two other strains (Table 1). These results were confirmed by MLST using the next-generation sequencing (NGS) results of each entire genome (Table 1). Thus, our results strongly suggest clonal spread of E. cloacae along with plasmid diffusion.

GES variants have already been described in association with other β-lactamases, such as carbapenemases like VIM, IMP, or OXA-23, especially in P. aeruginosa and in A. baumannii isolates (15–17). GES-1 and GES-5 were the two first GES variants identified in an epidemic P. aeruginosa clone ST255 in Spain (18). Another study highlighted the presence of GES-19 and GES-20 in K. pneumoniae and E. coli isolates from Mexico (19).

Molecular techniques such as PCR are not sufficient to identify GES carbapenemases, as sequencing of the entire gene is necessary. Only biochemical methods that address carbapenem hydrolysis may differentiate ESBL from carbapenemase variants (20). However, several studies report false-negative results from detection tests with GES-5- and GES-6-producing isolates (20–22).

To the best of our knowledge, this is the first description in Europe of clustered Enterobacteiraceae isolates carrying two GES β-lactamases, with one an ESBL and the other one a carbapenemase.

**Nucleotide sequence accession number.** The nucleotide sequence of the pEB-1 plasmid has been deposited in GenBank under accession number KX230795.

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