Emergence of Hypervirulent Carbapenem-Resistant Klebsiella pneumoniae Coharboring a bla<sub>NDM-1</sub>-Carrying Virulent Plasmid and a bla<sub>KPC-2</sub>-Carrying Plasmid in an Egyptian Hospital

Mohamed Abd El-Gawad El-Sayed Ahmed,a,b,c,d Yanxian Yang,b,c Yongqiang Yang,b,c,e Bin Yan,b,c,f Guanping Chen,g Reem Mostafa Hassan,h Lan-Lan Zhong,b,c Yuan Chen,f Adam P. Roberts,i Yiping Wu,b,c Ruowen He,b,c Xiaoqiu Liang,k Mingyang Qin,a Xian Dang,a Liyan Zhang,i Hongyu Li,m Fan Yang,f Lingqing Xu,n Guo-Bao Tian,b,c,o

Department of Microbiology, School of Basic Medical Science, Xinxiang Medical University, Xinxiang, China
Department of Microbiology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China
Key Laboratory of Tropical Diseases Control (Sun Yat-sen University), Ministry of Education, Guangzhou, China
Department of Microbiology and Immunology, Faculty of Pharmaceutical Sciences and Drug Manufacturing, Misr University for Science and Technology, 6th of October City, Egypt
Department of Neonatal Surgery, Guangzhou Women and Children's Medical Center, Guangzhou, China
Sun Yat-Sen University School of Medicine, Guangzhou, China
Department of Clinical and Chemical Pathology, Faculty of Medicine, Cairo University, Cairo, Egypt
Department of Tropical Disease Biology, Liverpool School of Tropical Medicine, Liverpool, United Kingdom
Centre for Drugs and Diagnostics, Liverpool School of Tropical Medicine, Liverpool, United Kingdom
School of Laboratory Medicine, Chengdu Medical College, Chengdu, China
Department of Clinical Laboratory, Guangdong Provincial People's Hospital/Guangdong Academy of Medical Sciences, Guangzhou, Guangdong, China
Department of Clinical Laboratory, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, China
Department of Clinical Laboratory, The Sixth Affiliated Hospital of Guangzhou Medical University, Qingyuan People's Hospital, Qingyuan, China
School of Medicine, Xizang Minzu University, Xianyang, Shaanxi, China

Mingyang Qin,a Min Dai,k Liyan Zhang,i Hongyu Li,m Fan Yang,f Lingqing Xu,n Guo-Bao Tian,b,c,o

ABSTRACT The emergence of carbapenem-resistant Klebsiella pneumoniae (CRKP) isolates in Egyptian hospitals has been reported. However, the genetic basis and analysis of the plasmids associated with carbapenem-resistant hypervirulent K. pneumoniae (CR-HvKP) in Egypt have not been presented. Therefore, we attempted to decipher the plasmid sequences that are responsible for transferring the determinants of carbapenem resistance, particularly <i>bla<sub>NDM-1</sub></i> and <i>bla<sub>KPC-2</sub></i>. Out of 34 <i>K. pneumoniae</i> isolates collected from two tertiary hospitals in Egypt, 31 were CRKP. Whole-genome sequencing revealed that our isolates were related to 13 different sequence types (STs). The most prevalent ST was ST101, followed by ST383 and ST11. Among the CRKP isolates, one isolate named EBSI036 has been reassessed by Nanopore sequencing. Genetic environment analysis showed that EBSI036 carried 20 antibiotic resistance genes and was identified as a CR-HvKP strain: it harbored four plasmids, namely, pEBSI036-1-NDM-VIR, pEBSI036-2-KPC, pEBSI036-3, and pEBSI036-4. The two carbapenemase genes <i>bla<sub>NDM-1</sub></i> and <i>bla<sub>KPC-2</sub></i> were located on plasmids pEBSI036-1-NDM-VIR and pEBSI036-2-KPC, respectively. The IncFII-IncHI1B hybrid plasmid pEBSI036-1-NDM-VIR also carried some virulence factors, including the regulator of the mucoid phenotype (<i>mpA4</i>), the regulator of mucoid phenotype 2 (<i>mpA2</i>), and aerobactin (<i>luxABCD</i> and <i>luxA</i>). Thus, we set out in this study to analyze in depth the genetic basis of the pEBSI036-1-NDM-VIR and pEBSI036-2-KPC plasmids. We report a high-risk clone ST11 KL47 serotype of a CR-HvKP strain isolated from the blood of a 60-year-old hospitalized female patient from the intensive care unit (ICU) in a tertiary care hospital in Egypt, which showed the
cohabitation of a novel hybrid plasmid coharboring the \textit{bla}_{NDM-1} and virulence genes and a \textit{bla}_{KPC-2}-carrying plasmid.

**IMPORTANCE** CRKP has been registered in the critical priority tier by the World Health Organization and has become a significant menace to public health. The emergence of CR-HvKP is of great concern in terms of both disease and treatment. In-depth analysis of the carbapenemase-encoding and virulence plasmids may provide insight into ongoing recombination and evolution of virulence and multidrug resistance in \textit{K. pneumoniae}. Thus, this study serves to alert contagious disease clinicians to the presence of hypervirulence in CRKP isolates in Egyptian hospitals.

**KEYWORDS** \textit{Klebsiella pneumoniae}, NDM-1, KPC-2, hybrid plasmid, virulent plasmid, Egypt

Several studies have reported the emergence of carbapenem-resistant \textit{Klebsiella pneumoniae} (CRKP) isolates in Egyptian hospitals (1–4); however, to the best of our knowledge, the genetic basis and analysis of the plasmids associated with CR-hypervirulent \textit{K. pneumoniae} (CR-HvKP) in Egypt have not been presented. Furthermore, carbapenem resistance has been reported to be associated with increased length of hospital stay and mortality of bloodstream infection (BSI) patients in low- and middle-income countries (5). Therefore, we sought to analyze in depth the genetic basis of pEBSI036-1-NDM-VIR (a novel hybrid plasmid harboring \textit{bla}_{NDM-1} and virulence genes) and pEBSI036-2-KPC (a \textit{bla}_{KPC-2}-carrying plasmid), which have been identified in a clinical \textit{K. pneumoniae} strain from a blood sample of a patient in Egypt.

A total of 34 nonduplicate \textit{K. pneumoniae} isolates were recovered from the blood of hospitalized patients in two tertiary care hospitals, namely, El-Demerdash Hospital (Cairo, Egypt) and the National Cancer Institute (Cairo, Egypt), in the period between June 2017 and March 2018 as a part of a study for the monitoring of antimicrobial resistance. Our isolates were selected based on their clinical characteristics, where all of them were primarily identified by Vitek 2 and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) as \textit{K. pneumoniae} causing bloodstream infections (BSIs), among which 31 were confirmed phenotypically and genotypically as CRKP isolates. Overall, the 34 isolates were isolated from the blood of 55.9% (19/34) female and 44.1% (15/34) male hospitalized patients from 9 days to 75 years of age. MICs of all 34 isolates were determined for 17 antibiotics using the agar microdilution method according to CLSI (6), except for tigecycline and colistin, for which MICs were obtained by the broth microdilution method according to EUCAST (7). Out of 34 isolates, 91.2% (31/34) were resistant to ertapenem, while 73.5% (25/34) and 61.8% (21/34) were resistant to imipenem and meropenem, respectively. However, all isolates were susceptible to colistin.

All isolates were assessed by whole-genome sequencing (WGS) using an Illumina HiSeq 2000 platform. In silico multilocus sequence typing showed that our isolates belong to 13 different sequence types (STs). The most prevalent ST was ST101 (13/34 [38.2%]) followed by ST383 (5/34 [14.7%]). One isolate, EBSI036, belongs to ST11: ST11 is the dominant ST clone responsible for the prevalence of CRKP worldwide and is considered an emerging high-risk clone (1, 8–10). Among 31 CRKP isolates, the prevalence of carbapenemase genes \textit{bla}_{NDM-1} and \textit{bla}_{OXA-48} was 45.2% (14/31) in addition, four strains carried both genes. Moreover, strain EBSI036 coharbors \textit{bla}_{NDM-1} and \textit{bla}_{KPC-2}.

According to the clinical data, the \textit{K. pneumoniae} strain EBSI036 was isolated from the blood of a 60-year-old female patient 2 days after admission to the Gastroenterology Department of El-Demerdash Hospital with symptoms of pneumonia, diarrhea, and fever. The patient’s symptoms improved following the administration of intravenous ceftriaxone and colistin, and she was discharged from the hospital 8 days posthospitalization. With the further genome analysis of EBSI036, the plasmid-associated virulence determinants \textit{mpa}/\textit{mpaA2}, \textit{iucA}/\textit{BCD}, and \textit{iutA} in this strain were predicted using the Virulence Factor Database ([http://www.mgc.ac.cn/VFs/main.htm]). EBSI036 was
determined as a KL47 capsular serotype by using Kaptive software (https://github.com/katholt/Kaptive). The serotype KL47 was the most reported type among CRKP infections in Asia (11–13). The virulence level of EBSI036 was confirmed using the Galleria mellonella larva model as previously described (11, 14) (see Fig. S1 in the supplemental material). These results revealed that EBSI036 is a CR-HvKP strain.

As EBSI036 coharbors two carbapenem genes besides the plasmid-mediated virulence genes, we have further analyzed the characteristics of the related fully sequenced plasmids by using a long-read MinION sequencer (Oxford Nanopore Technologies, Oxford, United Kingdom). Genomic analysis showed that EBSI036 included a 5,513,124-bp chromosome and four plasmids, namely, pEBSI036-1-NDM-VIR (347,365 bp), pEBSI036-2-KPC (129,869 bp), pEBSI036-3 (10,060 bp), and pEBSI036-4 (5,596 bp) (see Table S1 in the supplemental material). Twenty antimicrobial resistance genes, including six β-lactamase-encoding genes, were identified in EBSI036 by using ABRicate version 0.5 (https://github.com/tseemann/abricate) and aligning genome sequences to the ResFinder database. Of these, blaNDM-1, oqxAB, oqxA, and fosA6, were identified in the EBSI036 chromosome. The two carbapenemase genes blaNDM-1 and blaKPC-2 were located on plasmids pEBSI036-1-NDM-VIR and pEBSI036-2-KPC, respectively. In addition, 86 putative virulence genes were annotated in the genome of EBSI036, including genes coding for fimbriae, capsule, yersiniabactin, iron-enterobactin, mucoid, and aerobactin (see Table S2 in the supplemental material).

Hybrid plasmids that harbor resistance and virulence genes in a single genetic environment have been reported recently in various K. pneumoniae isolates, including the high risk of virulence clone ST23 and multidrug resistance (MDR) clone ST11 (15–17). Herein, the largest pEBSI036-1-NDM-VIR plasmid belongs to an IncFIB:IncHI1B hybrid plasmid. BLASTn showed that pEBSI036-1-NDM-VIR shared >99% identity with plasmids pKpvST383L (CP034201.2), pKpvST147B_virulence (CP040726.1), and pS1015_NDM_1 (CP050380.1), with query coverages of 97 to 99% (see Fig. S2 in the supplemental material). The backbone region of pEBSI036-1-NDM-VIR almost covered the complete sequence of the MDR plasmid pKpvST101_5, with a length of 210,661 bp (CP031372.2) (Fig. 1). Most of the remaining sequences (~130 kb) of pEBSI036-1-NDM-VIR were similar to those of the virulence plasmid pJX6-1, with a length of 228,974 bp (CP064230.1) (Fig. 1).

An ~38-kb MDR region in pEBSI036-1-NDM-VIR harbored carbapenemase-encoding gene blaNDM-1 and another eight resistance genes: mph(A), sul1, dfrA5, aph(3’)-la, armA, mrs(E), mph(E), and qnrS. A truncated transposon, ΔTn411 (Tn3 family [6,694 bp]), and IS26 elements (IS6 family [820 bp]) were located upstream of mph(A). The mph(A) gene and the downstream complete IS6100 sequence (family IS6 [880 bp]) were separated by two open reading frames (ORFs). sul1 and dfrA5 were surrounded by IS4321 (family IS110 [13,27 bp]), ΔTn33 (Tn3 family [18,375 bp]), and IS26 elements. This fragment with 15,448 bp containing the resistance genes mentioned above was similar to plasmid pKpvST383L (Fig. 1). The aph(3’)-la gene was flanked by IS26 elements; a similar structure was also found downstream of the resistance region in pKpvST383L. The segment IS26-armA-IS26-mrs(E)-mph(E)-ORF-ORF-IS26-ΔTn2 in pEBSI036-1-NDM-VIR was also found to be identical to an inverted sequence in pKpvST383L. In addition, the blanDM-1 and qnrS genes were on either side of this fragment, while they were absent in pKpvST383L. By comparing the complete sequences of pEBSI036-1-NDM-VIR and pKpvST383L, it was found that pKpvST383L had another resistance region (26,683 bp) carrying blanDM-5 and blanOXA-9. Compared with plasmid pS1015_NDM_1, the resistance region of plasmid pEBSI036-1-NDM-VIR lacked the aph(3’)-VI and sul2 genes (Fig. 1). In pEBSI036-1-NDM-VIR, the MDR region contained six IS26 elements and other transposon elements. Some studies demonstrated that the resistance loci containing IS26 can be hot spots for the capture of further resistance genes to constitute a novel MDR region (18).

A set of virulence genes was detected in pEBSI036-1-NDM-VIR with increased colonization and infection-producing capabilities, including mmpA and mmpA2 for the hypermucoviscous phenotype and iucABCD and iutA, associated with virulence (19). The ~39-kb region harboring virulence genes exhibited high similarity (99.9% identity and
Comparative analysis showed that the IncR:IncFII-type plasmid pEBSI036-2-KPC had 98 to 99% query coverages and 99.9% nucleotide identity to the following plasmids: pKP19-2029-KPC (CP047161.1), p69-2 (CP025458.1), and p16HN-263_KPC (CP045264.1). The pEBSI036-2-KPC plasmid carried the carbapenemase-encoding gene.
**bla**<sub>KPC-2</sub> and three β-lactamase-encoding genes: **bla**<sub>CTX-M-65</sub>, **bla**<sub>TEM-1B</sub>, and **bla**<sub>SHV-12</sub>. The pEBSI036-2-KPC plasmid had additional resistance genes: **catA2**, **fosA3**, and **rmtB**. These resistance genes were located in two regions (Fig. 2). The **bla**<sub>KPC-2</sub> and **bla**<sub>SHV-12</sub> genes were separated by sequence Δ**TnAs1-**IS<sub>26</sub>-Δ**TnAs3**-IS<sub>Kpn27</sub>, and IS<sub>Kpn6</sub> was located downstream of **bla**<sub>KPC-2</sub>. Sequence downstream of **bla**<sub>KPC-2</sub> contained a mer operon responsible for mercuric resistance and transposon elements (Δ**TnAs1-**IS<sub>26</sub>-Δ**TnAs3**). This segment carrying **bla**<sub>KPC-2</sub>, **bla**<sub>SHV-12</sub>, and a mer operon, was highly similar to those of other plasmids, such as pKP1034 (20). There was another MDR region (15,254 bp) that consisted of **bla**<sub>CTX-M-65</sub>, **fosA3**, **bla**<sub>TEM-1B</sub>, and **rmtB** genes and five IS<sub>26</sub> fragments (Fig. 2). The basic structure of pEBSI036-2-KPC was similar to that of plasmid pKPC2_040035 (GenBank accession no. CP028796.1) (99.98% identity and 88% query coverage), except for two regions. One locus (10,379 to 10,795 bp) carried **fosA3**, which was flanked by IS<sub>26</sub>. The other region contained a Δ**ISCr3-**IS<sub>Kpn26-**IS26-catA2-IS26-IS5075-**ΔTn3-IS26** structure with a length of 15,042 bp, which was the same as plasmid p3_L382 (CP033962.1), with 100% query coverage and 99.99% nucleotide identity (Fig. 2). Both **fosA3** and **catA2** were flanked by IS<sub>26</sub>, as previously reported (20, 21). That evidence emphasizes the role of insertion elements such as IS<sub>26</sub> in regulating insertion and deletion of resistance genes again.

In conclusion, we have reported a high-risk clone of ST11 KL47 of a CR-HvKP strain isolated from the blood of a patient from an ICU in Egypt, which coharbors two plasmids: one is a novel hybrid plasmid harboring the carbapenemase gene **bla**<sub>NDM-1</sub> and virulence genes, and the other carries **bla**<sub>KPC-2</sub>. Further countrywide surveillance studies are needed to elucidate the rate of prevalence of this high-risk clone in Egypt and its burden on hospital-acquired infections.

**Accession numbers.** The sequences of the plasmids pEBSI036-1-NDM-VIR and pEBSI036-2-KPC have been deposited in GenBank under accession no. MT648512 and MT648513.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**FIG S1**, TIF file, 0.3 MB.
**FIG S2**, TIF file, 2.3 MB.
**TABLE S1**, DOCX file, 0.03 MB.
**TABLE S2**, XLS file, 0.04 MB.

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