

# Emergence of a Hypervirulent Carbapenem-Resistant *Klebsiella pneumoniae* Co-harboursing a *bla*<sub>NDM-1</sub>-carrying Virulent Plasmid and a *bla*<sub>KPC-2</sub>-carrying Plasmid in an Egyptian Hospital

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## ABSTRACT

The emergence of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) isolates in Egyptian hospitals has been reported. However, the genetic basis and the analysis of the plasmids associated with CR-hypervirulent-KP (CR-HvKP) in Egypt are not presented. Therefore, we attempt to decipher the plasmids sequences, which are responsible for transferring the determinants of carbapenem-resistance, particularly the *bla*<sub>NDM-1</sub> and *bla*<sub>KPC-2</sub>. Out of 34 *K. pneumoniae* 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 using Nanopore sequencing. Genetic environment analysis showed that EBSI036 carried 20 antibiotic resistance genes and was identified as CR-HvKP strain, it harboured four plasmids, namely; pEBSI036-1-NDM-VIR, pEBSI036-2-KPC, pEBSI036-3, and pEBSI036-4. The two carbapenemase genes, *bla*<sub>NDM-1</sub> and *bla*<sub>KPC-2</sub>, were located on plasmids pEBSI036-1-NDM-VIR and pEBSI036-2-KPC, respectively. The IncFIB:IncHI1B hybrid plasmid pEBSI036-1-NDM-VIR also carried some virulence factors, including regulator of the mucoid phenotype (*rmpA*), the regulator of mucoid phenotype 2 (*rmpA2*), and aerobactin (*iucABCD*, *iutA*). Thus, we set out this study to analyse in-depth the genetic basis of pEBSI036-1-NDM-VIR and pEBSI036-2-KPC plasmids. We reported for the first time a high-risk clone ST11 KL47 serotype of CR-HvKP strain isolated from the blood of a 60-year-old hospitalised female patient from the ICU in a tertiary-care hospital in Egypt, which showed the cohabitation of a novel hybrid plasmid co-harboursing the *bla*<sub>NDM-1</sub> and virulence genes, besides a *bla*<sub>KPC-2</sub>-carrying plasmid.

## IMPORTANCE

CRKP had been registered in the critical priority tier by the World Health Organization and became a significant menace to public health. Therefore, we set out this study to analyse

in-depth the genetic basis of pEBSI036-1-NDM-VIR and pEBSI036-2-KPC plasmids. Herein, we reported for the first time (to the best of our knowledge) a high-risk clone ST11 KL47 serotype of CR-HvKP strain isolated from the blood of a 60-year-old hospitalised female patient in a tertiary-care hospital from the ICU in Egypt, which showed the cohabitation of a novel hybrid plasmid co-harboursing the *bla*<sub>NDM-1</sub> and virulence genes, besides a *bla*<sub>KPC-2</sub>-carrying plasmid. Herein, the high rate of CRKP might be due to the continuous usage of carbapenems as empirical therapy, besides the failure to implement an antibiotic stewardship program in Egyptian hospitals. Thus, this study serves to alert the contagious disease clinicians to the presence of hypervirulence in CRKP isolates in Egyptian hospitals.

**KEYWORDS**, *Klebsiella pneumoniae*, NDM-1, KPC-2, Hybrid plasmid, Virulent plasmid, Egypt

Several studies have reported the emergence of carbapenem-resistant *K. pneumoniae* (CRKP) isolates in Egyptian hospitals (1-4); however, to the best of our knowledge, the genetic basis and the analysis of the plasmids associated with CR-hypervirulent-KP (CR-HvKP) in Egypt are not presented. Therefore, we sought to analyse in-depth the genetic basis of pEBSI036-1-NDM-VIR (a novel hybrid plasmid harbouring *bla*<sub>NDM-1</sub> and virulence genes) and pEBSI036-2-KPC plasmids (a *bla*<sub>KPC-2</sub>-carrying plasmid) which identified from a clinical *K. pneumoniae* strain in Egypt.

A total of 34 nonduplicate *K. pneumoniae* isolates were recovered from the blood of hospitalised patients in two tertiary care hospitals, namely; El-Demerdash hospital (Cairo, Egypt) and 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<sup>®</sup>2 and MALDI-TOF MS as *K. pneumoniae* causing bloodstream infections (BSIs), among which 31 were confirmed phenotypically and genotypically as CRKP isolates. Overall, the CRKP isolates were isolated from the blood of 55.9% (19/34) female and 44.1% (15/34) male hospitalised patients, aged from 9 days to 75yr. Minimum inhibitory concentrations of all the 34 isolates were determined for 17 antibiotics using the agar microdilution method according to (CLSI) (5), excepted for tigecycline and colistin using the broth microdilution method according to EUCAST (6). Out of 34 isolates, 91.2% (31/34) were resistant to ertapenem, whereas, 73.5% (25/34) and 61.8% (21/34) were resistant to imipenem and meropenem, respectively. However, all isolates were susceptible to colistin.

All the isolates were assessed by whole-genome sequencing (WGS) using an Illumina HiSeq 2000 platform. *In silico* Multi-locus 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, where, ST11 is the dominant ST clone responsible for the prevalence of CRKP worldwide and is considered as an emerging high-risk clone (1, 7-9). According to the clinical data, the *K. pneumoniae* strain EBSI036 was isolated from the blood of a 60-year-old female patient two days after admission to the gastroenterology department of El-Demerdash hospital with symptoms of pneumonia, diarrhoea, and fever. The patient's symptoms improved following the administration of intravenous ceftriaxone and colistin, and she was discharged from the hospital eight days post-hospitalization. Of note, this strain co-harbours two carbapenemase genes, *bla*<sub>NDM-1</sub> and *bla*<sub>KPC-2</sub>. Besides, *bla*<sub>SHV-11</sub>, *oqx**B*, *oqx**A*, and *fos**A6*, were identified in EBSI036 chromosome. The plasmid-associated virulence determinants *rmpA/rmpA2*, *iucABCD*, and *iutA* in EBSI036 were predicted using the Virulence Factor Database (VFDB; <http://www.mgc.ac.cn/VFs/main.htm>). EBSI036 was determined as KL47 capsular serotype by using Kaptive software (<https://github.com/katholt/Kaptive>). The serotype K47 was the most reported type among CRKP infections in Asia (10-12). The virulence level of EBSI036 was confirmed using the *Galleria mellonella* larvae model as previously described (Figure S1) (10, 13). These results revealed that EBSI036 is a CR-hvKP strain.

As the EBSI036 co-harbours two carbapenem genes besides the plasmid-mediated virulence genes, we have further analyzed the characteristics of the related fully sequenced plasmids using a long-read MinION sequencer (Oxford Nanopore Technologies, Oxford, UK). 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 869bp),

pEBSI036-3 (10 060bp), and pEBSI036-4 (5 596bp) (Table S1). Twenty antimicrobial resistance genes, including six  $\beta$ -lactamases-encoding genes, were identified in EBSI036 using ABRicate version 0.5 (<https://github.com/tseemann/abrigate>) by aligning genome sequences to the ResFinder database. The two carbapenemase genes, *bla*<sub>NDM-1</sub> and *bla*<sub>KPC-2</sub>, were located on plasmids pEBSI036-1-NDM-VIR and pEBSI036-2-KPC, respectively.

Hybrid plasmids that harbour resistance and virulence genes in a single genetic environment have been reported recently in various *K. pneumoniae* isolates, including the high-risk clones ST23 and ST11 (14-16). Herein, the largest pEBSI036-1-NDM-VIR plasmid belongs to IncFIB: IncHI1B hybrid plasmid. BLASTn showed that pEBSI036-1-NDM-VIR shared >99% identity with plasmid pKpvST383L (CP034201.2), pKpvST147B\_virulence (CP040726.1), and p51015\_NDM\_1 (CP050380.1) with query coverages of 97%-99% (Figure S2). 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) (Figure 1). Most of the remaining sequences (~130 kb) of pEBSI036-1-NDM-VIR were similar to the virulence plasmid pJX6-1 with a length of 228,974 bp (CP064230.1) (Figure 1).

A ~38 kb MDR region in pEBSI036-1-NDM-VIR harboured carbapenemase-encoding gene *bla*<sub>NDM-1</sub> and another eight resistance genes *mph*(A), *sul1*, *dfrA5*, *aph*(3')-Ia, *armA*, *msr*(E), *mph*(E), and *qnrS*. A truncated transposons  $\Delta$ TnAs1 (Tn3 family, 6694 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, 880bp) were separated by two ORFs. *sul1* and *dfrA5* were surrounded by IS4321 (Family IS110, 1327bp),  $\Delta$ TnAs3 (Tn3 family, 18375 bp), and IS26 elements. This fragment with 15448 bp containing the above resistance genes was similar to plasmid pKpvST383L (Figure 1). The *aph*(3')-Ia gene was flanked by

IS26 elements, a similar structure was also found downstream of the resistance region in pKpvST383L. A segment IS26-*armA*-IS26-*msr(E)*-*mph(E)*-ORF-ORF-IS26- $\Delta$ Tn2 in pEBSI036-1-NDM-VIR was also found to be identical to the sequence in pKpvST383L with a reversion order. Besides, the *bla*<sub>NDM-1</sub> 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 *bla*<sub>NDM-5</sub> and *bla*<sub>OXA-9</sub>. Compared with plasmid p51015\_NDM\_1, the resistance region of plasmid pEBSI036-1-NDM-VIR lacked the *aph(3')*-*VI* and *sul2* genes (Figure 1). It is worth noting that in pEBSI036-1-NDM-VIR, the multidrug-resistant region contained six IS26 elements and other transposon elements. Some studies demonstrated that the resistance loci containing IS26 can be hotspots for the capture of further resistance genes to constitute a novel multi-drug resistant region (17).

A group of virulence genes was detected in pEBSI036-1-NDM-VIR; *rmpA* and *rmpA2*, which are commonly attributed to the hypermucoviscous phenotype of *K. pneumoniae* and the *iucABCD* and *iutA*, associating with virulence, with increased colonisation and infection producing capabilities (18). The ~39 kb region harbouring virulence genes exhibited high similarity (99.9% identity and 98% query coverage) with pKpvST383L, pKpvST147B\_virulence, and p51015\_NDM\_1 (Figure 1).

The pEBSI036-2-KPC plasmid was determined to be an IncR: IncFII-type plasmid. Comparative analysis showed that pEBSI036-2-KPC had 98%-99% query coverages and 99.9% nucleotide identity with the following plasmids, pKP19-2029-KPC2 (CP047161.1), p69-2 (CP025458.1), and p16HN-263\_KPC (CP045264.1). The pEBSI036-2-KPC plasmid carried carbapenemase-encoding gene *bla*<sub>KPC-2</sub> and three  $\beta$ -lactamases-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 main resistance regions (Figure 2). The *bla<sub>KPC-2</sub>* and *bla<sub>SHV-12</sub>* genes were separated by sequence  $\Delta$ Tn*AsI*-IS26- $\Delta$ Tn3-ISK*pn27*, and ISK*pn6* was located downstream of *bla<sub>KPC-2</sub>*. The downstream of *bla<sub>KPC-2</sub>* contained a *mer* operon responsible for mercuric resistance and transposons elements ( $\Delta$ Tn*AsI*-IS26- $\Delta$ Tn*As3*). This segment carrying *bla<sub>KPC-2</sub>*, *bla<sub>SHV-12</sub>*, and a *mer* operon, was highly similar to other plasmids such as pKP1034 (19). 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 IS26 fragments (Figure 2). The basic structure of pEBSI036-2-KPC is similar to plasmid pKPC2\_040035 (CP028796.1) (99.98% identity and 88% query coverage), except for two regions. One 10,379-10,795 locus carried *fosA3*, which was flanked by IS26. The other region contained  $\Delta$ ISC*fr3*-ISK*pn26*-IS26-*catA2*-IS26-IS5075- $\Delta$ Tn3-IS26 structure with a length of 15,042bp, which was the same as plasmid p3\_L382 (CP033962.1) with 100% query coverage and 99.99% nucleotide identity (Figure 2). Both *fosA3* and *catA2* were flanked by IS26 as previously reported (19, 20). That evidence emphasizes the role of insertion elements such as IS26 in insertion and deletion of resistance genes again.

In conclusion, to the best of our knowledge, this is the first report of a high-risk clone ST11-KL47 of CR-HvKP strain isolated from the blood of a patient from the ICU in Egypt, co-harboring two plasmids, one is a novel hybrid plasmid harbouring the carbapenemase gene *bla<sub>NDM-1</sub>* and virulence genes, and the other is a *bla<sub>KPC-2</sub>*-carrying plasmid. 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 were deposited in GenBank with accession numbers MT648512 and MT648513.

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## Disclosure statement

The authors report no conflicts of interest in this work. All authors have read and approved the manuscript.

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## Figure Legends

**FIG 1** Structure analysis of pEBSI036-1-NDM-VIR. Major structural features of plasmid pEBSI036-1-NDM-VIR were compared with plasmids pKpvST101\_5 (GenBank accession number CP031372.2) and pJX6-1 (GenBank accession number CP064230.1). The comparative schematic diagram of resistance region and virulence region in plasmids pEBSI036-1-NDM-VIR, pKpvST383L (GenBank accession number CP034201.2), pKpvST147B\_virulence (GenBank accession number CP040726.1) and p51015\_NDM\_1 (GenBank accession number CP050380.1) were shown respectively. Grey shading indicates shared regions with a high degree of homology. Red and purple represent the antibiotic resistance and virulence genes, respectively, and yellow is the insertion sequences and transposons.

**FIG 2** Sequence alignment analysis among plasmids pEBSI036-2-KPC, pKPC2\_040035 (GenBank accession number CP028796.1) and p3\_L382 (GenBank accession number CP033962.1). Red and green represent the antibiotic resistance and heavy metal resistance genes, respectively. And yellow is the insertion sequences and transposons.

## Supplementary Materials

**Table S1** Overall features of the *K. pneumoniae* EBSI036 genome

**Table S2** Putative virulence genes detected on the *K. pneumoniae* EBSI036 chromosome

**FIG S1** Virulence potential of *K. pneumoniae* strain EBSI036 as depicted in a *Galleria mellonella* infection model with an inoculum of  $1 \times 10^4$  CFU.

**FIG S2** Sequence alignment analysis among plasmids pEBSI036-1-NDM-VIR and pKpvST383L (GenBank accession number CP034201.2), pKpvST147B\_virulence (GenBank

345   accession number CP040726.1) and p51015\_NDM\_1 (GenBank accession number  
346   CP050380.1).

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