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Carriage of distinct *bla*_{KPC-2} and *bla*_{OXA-48} plasmids in a single ST11 hypervirulent *Klebsiella pneumoniae* isolate in Egypt

Yanxian Yang^{1,2†}, Yongqiang Yang^{1,2,3†}, Mohamed Abd El-Gawad El-Sayed Ahmed^{1,2,4†}, Mingyang Qin⁵, Ruowen He^{1,2}, Yiping Wu^{1,2}, Xiaoxue Liang⁶, Lan-Lan Zhong^{1,2}, Ping Chen⁵, Baoguo Deng⁵, Reem Mostafa Hassan⁷, Weihong Wen⁸, Lingqing Xu⁸, Xubin Huang^{9*}, Lin Xu^{1,2,10*} and Guo-Bao Tian^{1,2,11*}

Abstract

Background: Carbapenem-resistant hypervirulent *K. pneumoniae* (CR-hvKP) causes serious infections with significant morbidity and mortality. However, the epidemiology and transmission mechanisms of CR-hvKP and the corresponding carbapenem-resistant plasmids require further investigation. Herein, we have characterized an ST11 *K. pneumoniae* strain EBSI041 from the blood sample encoding both hypervirulence and carbapenem resistance phenotypes from a patient in Egypt.

Results: *K. pneumoniae* strain EBSI041 showed multidrug-resistance phenotypes, where it was highly resistant to almost all tested antibiotics including carbapenems. And hypervirulence phenotypes of EBSI041 was confirmed by the model of *Galleria mellonella* infection. Whole-genome sequencing analysis showed that the hybrid plasmid pEBSI041-1 carried a set of virulence factors *rpmA*, *rpmA2*, *iucABCD* and *iutA*, and six resistance genes *aph(3')-VI*, *armA*, *msr(E)*, *mph(E)*, *qnrS*, and *sul2*. Besides, *bla*_{OXA-48} and *bla*_{SHV-12} were harboured in a novel conjugative IncL-type plasmid pEBSI041-2. The *bla*_{KPC-2}-carrying plasmid pEBSI041-3, a non-conjugative plasmid lacking the conjugative transfer genes, could be transferred with the help of pEBSI041-2, and the two plasmids could fuse into a new plasmid during co-transfer. Moreover, the emergence of the p16HN-263_KPC-like plasmids is likely due to the integration of pEBSI041-3 and pEBSI041-4 via IS26-mediated rearrangement.

Conclusion: To the best of our knowledge, this is the first report on the complete genome sequence of KPC-2- and OXA-48-coproducing hypervirulent *K. pneumoniae* from Egypt. These results give new insights into the adaptation and evolution of *K. pneumoniae* during nosocomial infections.

Keywords: *K. pneumoniae*, KPC-2, OXA-48, Hypervirulence, Egypt

*Correspondence: huangxb@mail.sysu.edu.cn; xulin@mail.sysu.edu.cn; tiangb@mail.sysu.edu.cn

[†]Yanxian Yang, Yongqiang Yang and Mohamed Abd El-Gawad El-Sayed Ahmed contributed equally to this work.

¹ Department of Microbiology, Zhongshan School of Medicine, Sun Yat-sen University, 74 Zhongshan 2nd Road, Guangzhou 510080, China

⁹ Department of Pulmonary and Critical Care Medicine, the First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China

Full list of author information is available at the end of the article

Background

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) is one of the most critical threats to global public health associated with significant morbidity and mortality [1–3]. *K. pneumoniae* producing KPCs, NDMs, and OXA-48-like carbapenemases have become rapidly disseminated worldwide [2]. The *bla*_{KPC} and *bla*_{NDM} genes in *K. pneumoniae* have been reported on multiple plasmid types, including IncF, IncA/C, IncR, IncX and IncL/M. Among



them, the IncF and IncA/C type plasmids are predominantly responsible for the transfer of *bla*_{KPC} and *bla*_{NDM}, respectively [4, 5]. Besides, the *bla*_{KPC} gene is strongly associated with Tn4401 flanking by ISKpn6 and ISKpn7, and the *bla*_{NDM} gene is closely related to Tn125 structure with two ISAbal125 elements [6, 7]. Unlike KPCs and NDMs, IncL group plasmid has been shown to be the major genetic carrier for *bla*_{OXA} in *K. pneumoniae* and the composite transposon Tn1999 is mainly responsible for integration of the *bla*_{OXA} gene [4]. Furthermore, dissemination of KPC-producing *K. pneumoniae* in worldwide is largely caused by expansion of the dominant ST258 clones [8]. Differently, the *bla*_{OXA} and *bla*_{NDM} genes are detected in various *K. pneumoniae* clones, in which ST11 is a major high-risk sequence type in many countries, such as China [9, 10], Australia [11], Poland [12], Spain [13] and Turkey [14]. Co-carriage of different carbapenems resistance genes is not common but renders clinical *K. pneumoniae* strains extremely highly resistant to different carbapenems, which leads to more difficult infection treatment [15, 16].

Seriously, carbapenem-resistant hypervirulent *K. pneumoniae* (CR-hvKP) has been increasingly reported in nosocomial infection and can cause higher mortality [17, 18]. The plasmid-mediated genetic factors conferring the hypervirulent phenotype including *rmpA* and *rmpA2* (regulators that increase capsule production), and several siderophore gene clusters [19]. Notably, a multinational prospective cohort study warns of the severity of carbapenem resistance in low-income and middle-income countries, including Egypt [3]. In the previous studies, the high prevalence of carbapenemase-mediated resistance in *K. pneumoniae* isolates in the clinical setting from Egypt was reported [20–23], but few studies have analyzed the genome characteristic of carbapenem-resistant *K. pneumoniae* by whole-genome sequencing. In this study, we report the in-depth characterization of co-producing *bla*_{KPC-2} and *bla*_{OXA-48} hypervirulent ST11 *K. pneumoniae* strain from Egypt. The dissemination of two plasmids carrying *bla*_{OXA-48} and *bla*_{KPC-2} and novel plasmid structures were identified.

Results

Strains characteristic

K. pneumoniae EBSI041 was collected from the blood sample of a male emergency ICU patient in March 2012 in Egypt. This strain showed high resistance to almost all tested antibiotics, including imipenem, meropenem, ertapenem, piperacillin-tazobactam, cefotaxime, ceftazidime, cefepime, aztreonam, gentamicin, amikacin, ciprofloxacin, fosfomycin, chloramphenicol, but sensitivity to colistin, tigecycline, tetracycline, and trimethoprim-sulfamethoxazole (Table S1). EBSI041 exhibited

extensive resistance to carbapenems with high resistance levels (MIC > 32 mg/L). In addition, it was also highly resistant to piperacillin-tazobactam, a β -lactam combination agent, with MIC > 512 mg/L, and cepheems cefotaxime (MIC > 256 mg/L), ceftazidime (MIC > 256 mg/L), cefepime (MIC = 256 mg/L). The virulence level of strain EBSI041 was tested in wax moth (*G. mellonella*) larvae. The larval mortality rate increased dramatically to 60% within 12 h post-infection with EBSI041. On being infected for 48 h at an inoculum of 1×10^4 colony-forming units (CFU), the survival of *G. mellonella* were 10 and 0% for EBSI041 and the hypervirulence control strains HvKP4, respectively (Fig. S1).

In vitro conjugation demonstrated that carbapenem resistance genetic factors in EBSI041 can be transferred to the recipient strains *E. coli* J53 and EC600. Further S1-PFGE and PCR assay revealed OXA-48-carrying plasmid (~90 kb) can be self-transferred and KPC-2-producing plasmid has no transferability. However, KPC-2-producing plasmid can be transferred to the recipient strains with the help of OXA-48-carrying plasmid, and the two plasmids may be fused a larger plasmid (~180 kb) carrying both *bla*_{OXA-48} and *bla*_{KPC-2} according to results of S1-PFGE (Fig. S2).

Genomic features of the carbapenem-resistant hypervirulent *K. pneumoniae* EBSI041

Genomic analysis showed EBSI041 included a 5,516,355 bp chromosome and seven plasmids: pEBSI041-1 (299,522 bp), pEBSI041-2 (97,179 bp), pEBSI041-3 (85,594 bp), pEBSI041-4 (45,103 bp), pEBSI041-5 (10,060 bp), pEBSI041-6 (5596 bp), pEBSI041-7 (1780 bp) (Table 1). EBSI041 carried 19 antibiotic resistance genes (ARGs), four of them were located on the chromosomes including the *bla*_{SHV-11}, *oqxB*, *oqxA*, and *fosA6* genes. The two carbapenemase genes, *bla*_{OXA-48} and *bla*_{KPC-2}, were identified in plasmids pEBSI041-2 and pEBSI041-3, respectively. In addition to resistance genes, eighty-six putative virulence genes were annotated in the genome of EBSI041, including genes coding for fimbriae, capsule, yersiniabactin, iron-enterobactin, mucoid and aerobactin. Most virulence factors were found on chromosome, except for seven genes (*rmpA*, *rmpA2*, *iucABCD* and *iutA*) in plasmid pEBSI041-1. The virulence of EBSI041 was demonstrated using the *Galleria mellonella* larvae model. EBSI041 was identified as resistant and virulent ST11 clone.

For source tracking bacterial pathogens, EBSI041 was found to be similar to a ST11 *K. pneumoniae* strain (WJTB01) isolated from bronchoalveolar lavage of patient in China, with 317 SNPs, based on SNP (sequence-based) strategy using BacWGSTdb 2.0 [24]. The core-genome-based MLST (cgMLST)

Table 1 Overall features of *K. pneumoniae* EBSI041

Clinical characteristics		Genome characteristics					
Country	Egypt	Parameter	Size (bp)	Plasmid_type	Resistance gene	Virulence gene	Accession no.
Unit	ICU emergency	Chromosome	5,516,355	–	<i>oqx</i> B <i>oqx</i> A <i>bla</i> _{SHV-11} <i>fos</i> A6	–	–
Gender	Male						
Type of Infection	Septicemia						
Sampling Site	Blood						
Date of Isolation	March 2012	pEBSI041-1	299,522	IncHI1B:IncFIB	<i>arm</i> A <i>msr</i> (E) <i>mph</i> (E) <i>qnr</i> S1 <i>aph</i> (3')-VI <i>sul</i> 2	<i>rmp</i> A <i>iuc</i> A <i>iuc</i> B <i>iuc</i> C <i>iuc</i> D <i>iut</i> A <i>rmp</i> A2	MW245019
		pEBSI041-2	97,179	IncL/M	<i>bla</i> _{OXA-48} <i>bla</i> _{SHV-12}		MW245020
		pEBSI041-3	85,594	IncR	<i>bla</i> _{KPC-2} <i>bla</i> _{SHV-12}		MW245021
		pEBSI041-4	45,103	IncFII	<i>bla</i> _{CTX-M-65} <i>fos</i> A3 <i>bla</i> _{TEM-1B} <i>rmt</i> B <i>cat</i> A2		MW245022
		pEBSI041-5	10,060	ColRNAI			–
		pEBSI041-6	5596	ColRNAI			–
		pEBSI041-7	1780	ColpVC			–

analysis showed that 67 strains were less than or equal to 50 alleles different from EBSI041. All strains were isolated from China, except one from USA. EBSI041 and HA_74 (PJOU01) were on the same branch of the phylogenetic tree (Fig. S3). ST11 *K. pneumoniae* strain HA_74 was isolated from China in 2015. However, because of limited sources from Egypt or Africa, we were not able to clarify the domestic transmission route.

The MDR pEBSI041-1 co-harbours virulence genes via recombination

The plasmid pEBSI041-1 is 299,522 bp in size and belongs to an IncFIB:IncHI1B type hybrid plasmid. A 27,568-bp multidrug-resistance (MDR) module in pEBSI041-1 harboured six resistance genes *arm*A, *msr*(E), *mph*(E), *qnr*S, *aph*(3')-VI and *sul*2. The *arm*A gene was mediated by an intact IS26 (IS6 family, 820 bp) element upstream. With a 2300-bp space to *arm*A downstream, a locus of *msr*(E) and *mph*(E) was flanked by an intact ISEc29 (IS4 family, 1325 bp) (Fig. 1). For the rest of ARGs, each of them was flanked independently by different IS elements. This module was almost identical to the sequences in plasmid p51015_NDM_1 (CP050380) which was identified in a human *K. pneumoniae* isolate in the Czech Republic. The only difference between them was an

inversion of a 7710-bp sequence bounded by two IS26 occurred in p51015_NDM_1. Besides this MDR module, pEBSI041-1 harbours the plasmid-located virulence factors, including regulator of the mucoid phenotype (*rmp*A), the regulator of mucoid phenotype 2 (*rmp*A2), aerobactin (*iuc*ABCD, *iut*A) (Fig. 1). A BLASTn search showed that a 36,929-bp sequence containing the virulence genes in pEBSI041-1 was identical to the sequences in pF16KP0084-1 (CP052159.1; South Korea). Furthermore, close to this sequence, a 37,030-bp sequence was also found to be identical to the sequence in pF16KP0084-1 with a reversion order, while the sequence harbours the virulence gene cluster *iro*BCD and *iro*N in pF16KP0084-1 was lacking in our plasmid (Fig. 1). Also, these two sequence fragments in pEBSI041-1 caused the main difference to plasmid pKpvST101_5 (CP031372.2; United Kingdom), indicating that the emergence of the MDR-virulent pEBSI041-1 was due to the transfer of virulence determinants into a pKpvST101_5-like MDR plasmid. Sequence alignments showed that pEBSI041-1 shared > 99% identity with plasmid pKpvST147B_virulence (CP040726.1; United Kingdom), pKpvST383L (CP034201.2; United Kingdom), and p51015_NDM_1 (CP050380.1) with query coverages of 96-99.5%, all of which are MDR-virulent hybrid plasmids (Fig. 1).

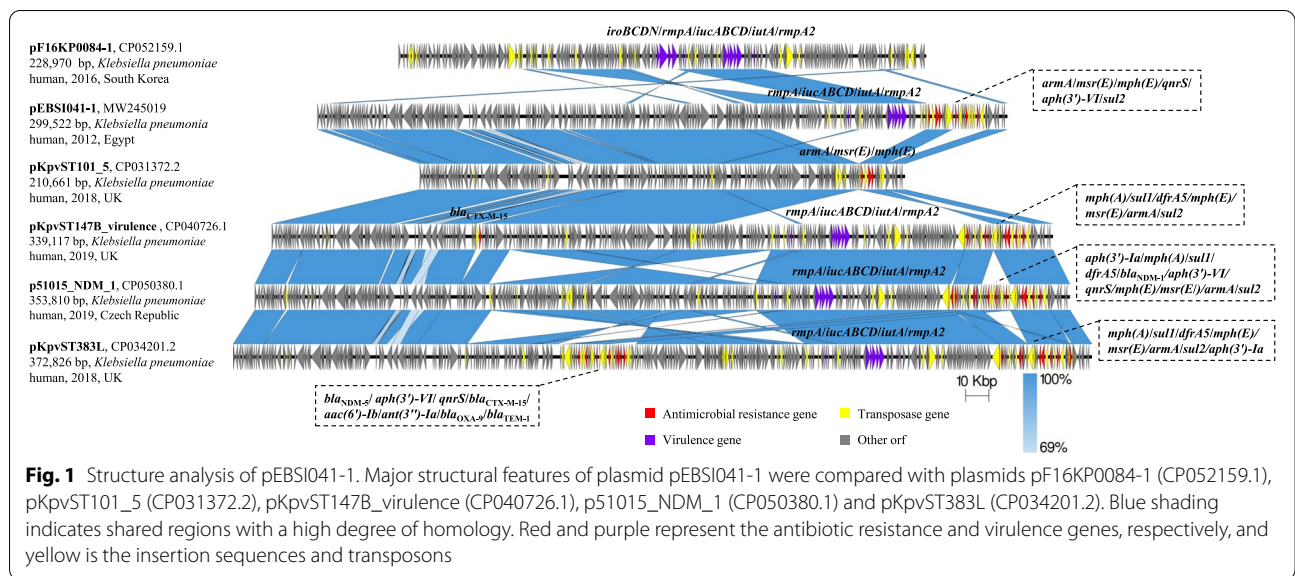


Fig. 1 Structure analysis of pEBSI041-1. Major structural features of plasmid pEBSI041-1 were compared with plasmids pF16KP0084-1 (CP052159.1), pKpvST101_5 (CP031372.2), pKpvST147B_virulence (CP040726.1), p51015_NDM_1 (CP050380.1) and pKpvST383L (CP034201.2). Blue shading indicates shared regions with a high degree of homology. Red and purple represent the antibiotic resistance and virulence genes, respectively, and yellow is the insertion sequences and transposons

The bla_{OXA-48}-carrying pEBSI041-2 harbours an exogenetic chromosome-located fragment

The IncL/M-type plasmid pEBSI041-2 was 97,179 bp in size with an average GC content of 52.45% and contained 134 open reading frames (ORFs). It possessed a complete array of genes involved in replication (*repA* gene), stabilization (*stbAB* genes), toxin-antitoxin system (*pemIK* genes) and conjugation (*traHIJKLMNOPQR* and *traUWXYZ* genes). The *bla_{OXA-48}* gene, encoding the class D carbapenemase OXA-48, was found in this plasmid. The *bla_{OXA-48}* gene was surrounded by multiple IS elements, namely Δ IS10A (IS4 family, 1329 bp), IS1R (IS1 family, 768 bp) and IS10A (IS4 family, 1329 bp)

(Fig. 2). pEBSI041-2 exhibited a high similarity (80% coverage, 99.58% identity) with plasmid pOXA-48_1639 (CP025105.1), which also encodes OXA-48 and identified from *E. coli*. Besides, pEBSI041-2 was also similar to plasmids pOXA-48_920 (LR025095.1) and pUR17313-1 (KP061858.1), which are from *K. pneumoniae* and *Enterobacter cloacae* respectively. This indicates that pOXA-48_1639-like plasmids have been widely spread among bacteria of different species. However, compared with pOXA-48_1639, an 18,779-bp sequence harbouring *bla_{SHV-12}* was missing in pEBSI036-2. The *bla_{SHV-12}* gene was surrounded by two IS26 (IS6 family, 820 bp) elements, and this 6100-bp fragment was

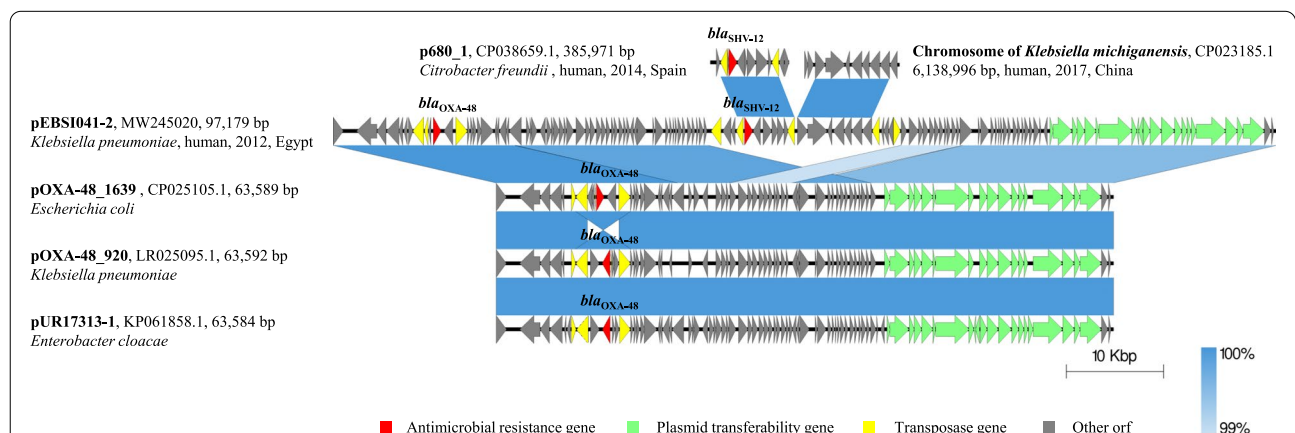
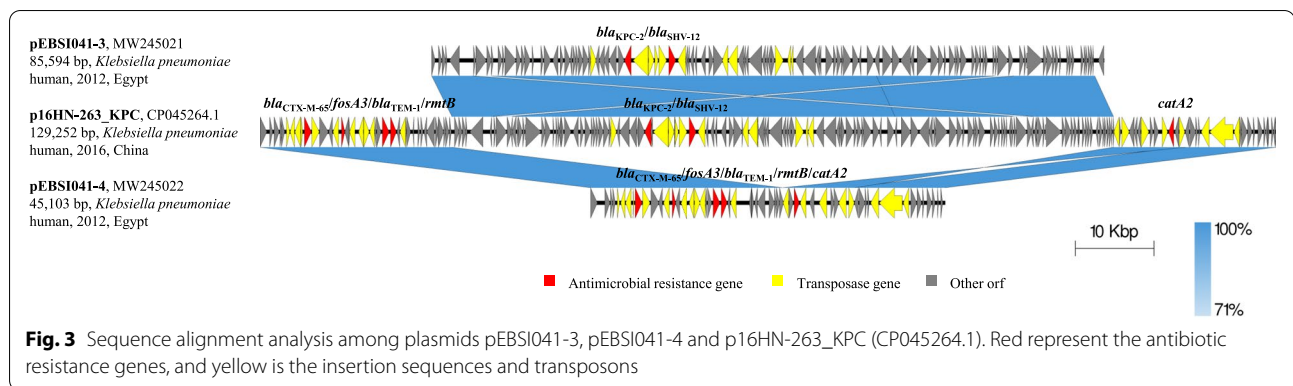


Fig. 2 The comparative schematic diagram of plasmid pEBSI041-2. pEBSI041-2 was compared with three OXA-48-carrying plasmids pOXA-48_1639 (CP025105.1), pOXA-48_920 (LR025095.1) and pUR17313-1 (KP061858.1). Besides, plasmid p680_1 (CP038659.1) and the chromosome of *K. michiganensis* strains (CP023185.1) were analyzed. Red represent the antibiotic resistance, and yellow is the insertion sequences and transposons. Plasmid transferability genes were shown in green



similar to plasmid p680_1 (CP038659.1) from *Citrobacter freundii*. A BLASTn search did not find the homologous plasmid-located sequence to the remaining region (47,727–55,495 bp) while exhibited limited similarities (~50% coverage, >99% identity) to the chromosome of some *K. michiganensis* strains (CP023185.1; CP022348.1; CP003683.1) (Fig. 2). The mobilization of *bla*_{SHV-12} can be attributed to the gene exchange by recombination, while the acquisition of exogenous chromosome sequence needs to be further considered. Indeed, the conjugation experiment demonstrated that pEBSI041-2 transferred from the donor strain *K. pneumoniae* EBSI041 to the recipients *E. coli* J53 and EC600.

The transfer of non-conjugative *bla*_{KPC-2}-carrying pEBSI041-3 needs the help of pEBSI041-2

The pEBSI041-3 was an IncR-type plasmid. In pEBSI041-3, two antibiotic resistance genes *bla*_{KPC-2} and *bla*_{SHV-12} were found, which were separated by a fragment of *ISKpn27-ΔTn3-IS26-ΔTnAs1* (Fig. 3). Besides, an *ΔISKpn6* (IS1182 family, 1540 bp) was located in upstream of *bla*_{KPC-2}. The pEBSI041-3 acquired *bla*_{KPC-2} by a transposon unit with the core structure of *ΔISKpn6-bla*_{KPC-2}-*ISKpn27* as pKPC-LK30 (KC405622.1) [25] and pKPC-L111 (CP030134.1) [15] and captured *bla*_{SHV-12} with an IS26-interrupted *TnAs1* (Tn3 family, 6694 bp) element. The complete transfer operon (locus *tra-trb*) was not detected in pEBSI041-3, except *traA* and *traM*, which may explain why pEBSI041-3 does not transfer conjugatively to the recipients *E. coli* J53 and EC600. However, the results of vitro conjugation and PFGE experiments showed pEBSI041-3 can be transferred with the help of conjugative plasmid pEBSI041-2. Moreover, pEBSI041-2 and pEBSI041-3 may fuse into a larger plasmid (~180 kb) carried both *bla*_{OXA-48} and *bla*_{KPC-2} genes during co-transfer (Fig. S2).

Potential recombination of plasmids pEBSI041-4 and pEBSI041-3

Plasmid pEBSI041-4 was identified as an IncFII type. A 20,894 bp multi-drug resistance region in pEBSI041-4 harboured five resistance genes, including *bla*_{CTX-M-65}, *bla*_{TEM-1B}, *catA2*, *fosA3* and *rmtB*. Those genes were separated by eight IS26 elements, one *IS903B* (IS5 family, 1057 bp) element, one *ISKpn26* (IS5 family, 1196 bp) element, one *ISCfr3* (ISNCY family, 1081 bp) element and a few truncated insertion sequences and transposons (Fig. 3).

The segment contained genes *bla*_{CTX-M-65}, *bla*_{TEM-1B}, *fosA3*, and *rmtB* was similar to F33:A-B-type plasmid pHN7A8, which isolated from an *E. coli* in China [26]. The pEBSI041-4 shared 99.98% identity with pHN7A8 (JN232517.1) with query coverages of 71%. Compared to pHN7A8, pEBSI041-4 has an additional resistance gene *catA2* flanked by two IS26 elements. Besides, the downstream region of *catA2* has *ISKpn26*, *ISCfr3* and IS26 elements. The presence of multiple insertion sequences, especially IS26, indicates that recombination may repeatedly have occurred in this multi-drug resistance region.

Moreover, the combination of pEBSI041-3 and pEBSI041-4 was almost identical to p16HN-263_KPC (CP045264.1) (Fig. 3). The p16HN-263_KPC was collected from *Klebsiella pneumoniae* of bloodstream infection in China and shared high similarity with pKP1034 (KP893385.1) and p69-2 (CP025458.1) [27, 28]. Those KPC-2-producing plasmids belonged to IncR-F33:A-B-type and carried all resistance genes in pEBSI041-3 and pEBSI041-4, except p69-2 without *fosA3*. The comparison suggested that pEBSI041-3 and pEBSI041-4 might have undergone rearrangement by recombination to form p16HN-263_KPC-like plasmids.

Discussion

ST11 is the dominant clone of carbapenemases-producing *K. pneumoniae* in Asia, especially in China, and ST11 clone was reported to account for up to 60% of carbapenem-resistant *K. pneumoniae* [9, 29, 30]. Recently, ST11 has been reported in several clinical infection cases from African countries, such as Egypt [31, 32] and Tunisian [33]. And in ST11 carbapenem-resistant *K. pneumoniae* isolates, the most predominant carbapenemase genes are *bla*_{KPC-2}, *bla*_{NDM-1} and *bla*_{OXA-48} [10, 34]. The emerging threat of carbapenem resistance of *K. pneumoniae* in Egyptian hospitals has been highlighted over recent years [3, 35]. To the best of our knowledge, this is the first report on the complete genome sequence of KPC-2- and OXA-48-coproducing virulent *K. pneumoniae* from Egypt.

The uncommon co-carriage of genes encoding different classes of carbapenemases endowed EBSI041 with high carbapenems resistance. Not restricted in this study, the co-harboring *bla*_{KPC-2} and *bla*_{OXA-48} in *K. pneumoniae* isolates were also found from other clinical setting [15]. The presence of carbapenemase genes on mobile elements greatly promotes the spread and stacking of carbapenems resistance. The *bla*_{OXA-48}-carrying pEBSI041-2 was identified as an IncL/M-type plasmids, which are commonly self-conjugative among *Enterobacteriaceae* according to the previous studies [36]. It's worth noting that non-conjugative pEBSI041-3 carried *bla*_{KPC-2} gene, was successfully transferred with the help of pEBSI041-2. The results warn that the mechanisms of *bla*_{OXA-48} or *bla*_{KPC-2} -carrying plasmid transfer need to be further studied to better control the spread of carbapenemase-producing *K. pneumoniae*.

The genetic structure of *bla*_{OXA-48} or *bla*_{KPC-2} -carrying plasmid in this study is different from that of reported plasmids due to the presence of multiple transposons and insertion sequences. The *bla*_{OXA-48}-carrying pEBSI041-2 was similar to other IncL/M plasmids previously sequenced, the majority of which only carry *bla*_{OXA-48} [15]. However, pEBSI041-2 harboured an exogenous chromosome-located fragment and acquired additional resistance gene *bla*_{SHV-12} due to the recombination of the IS26-like elements. In pEBSI041-3, the *bla*_{KPC-2} gene was located on a transposon unit with the core structure of Δ ISKpn6-*bla*_{KPC-2}-ISKpn27 as that reported [15, 25]. Further, pEBSI041-3 and MDR plasmid pEBSI041-4, which carried eight IS26 elements, almost constitutes another KPC-2-producing plasmids [27, 28]. Therefore, the novel genetic structure of these plasmids are likely to be created by IS-mediated recombination.

Conclusion

This study reported the co-carriage of distinct *bla*_{KPC-2} and *bla*_{OXA-48} plasmids in a single ST11 hypervirulent *Klebsiella pneumoniae* isolate in Egypt. The recombination and rearrangement of MDR plasmids and virulent plasmids have occurred during evolution. These results give new insights into the adaptation and evolution of *K. pneumoniae* plasmids during nosocomial infections in Egypt.

Materials and methods

Bacterial strain

K. pneumoniae EBSI041 was collected from the blood sample of a patient in Egypt. The clinical strain was initially isolated on MacConkey agar (Oxoid, UK). Species identification was determined primarily with an automated VITEK[®]2 AST-16 Gram-negative susceptibility card (bioMérieux, Marcy-l'Étoile, France) and confirmed by matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS). Investigation of carbapenemase production in the routine hospital laboratory procedure using the Modified Hodge Test (MHT) [37] and Carba NP test [38] showed that EBSI041 was resistant to carbapenems. We tested EBSI041 for carbapenems-resistant genes and confirmed that it carried *bla*_{OXA-48} and *bla*_{KPC-2}. Then, EBSI041 was selected for whole-genome sequencing for further identification. Ethical approval for this study was given by Zhongshan School of Medicine of Sun Yat-sen University under approval number 068. All methods involved in this study were carried out in accordance with relevant guidelines and regulations.

Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) were determined for the following 17 different antibiotics: cefotaxime (CTX), ceftazidime (CAZ), cefepime (FEP), colistin (CT), tigecycline (TGC), imipenem (IMP), ertapenem (ETP), meropenem (MEM), ciprofloxacin (CIP), fosfomicin (FOS), trimethoprim-sulfamethoxazole (SXT), piperacillin-tazobactam (PTZ), amikacin (AMK), gentamicin (GEN), chloramphenicol (CHL), tetracycline (TET), and aztreonam (ATM) for EBSI041 using the agar microdilution method excepted for colistin using the broth microdilution method. *Escherichia coli* ATCC 25922 strain was used as the reference control. MICs were interpreted following the Clinical and Laboratory Standards Institute (CLSI 2018) [39] guidelines, except for tigecycline and colistin, which were interpreted using the EUCAST 2019 guidelines (ECOFFs; <http://www.eucast.org/>).

In vitro conjugation and S1-PFGE

The horizontal transfer of plasmids was examined by in vitro conjugation using *K. pneumoniae* EBSI041 as a donor and *E. coli* strain EC600 and J53 as a recipient, respectively. Briefly, the EBSI041, EC600 and J53 strains were cultured to OD₆₀₀ 0.4–0.6, mixed in a 1:1 donor-to-recipient ratio, plated onto Luria-Bertani (LB) agar plates and incubated at 37 °C overnight. One ml of sterile saline was used to remove the conjugation mix from the LB agar plates. Transconjugants were then selected by plating LB agar plates containing rifampicin (Rif; 500 µg/ml) and imipenem (Imp; 2 µg/ml) for EC600, and sodium azide (NaN₃; 100 µg/ml) and imipenem (Imp; 2 µg/ml) for J53. The transfer of the plasmid was checked by PCR analysis and MICs. The *bla*_{KPC-2} and *bla*_{OXA-48} genes were confirmed by PCR and sequencing with primers KPC-A (TGTAAGTTACCGCGCTGAGG), KPC-B (CCAGACGACGGCATAGTCATF) [40], and OXA-A (TTGGTG GCATCGATTATCGG), OXA-B (GAGCACTTCTTT TGTGATGGC) [41]. And S1-PFGE was used to determine the sizes and numbers of plasmids harboured by the isolate EBSI041 and transconjugants [42].

Galleria mellonella virulence assay

The virulence of strain EBSI041 was tested using the wax moth (*Galleria mellonella*) larvae model. Briefly, 30 larvae weighing about 300 mg were randomly selected for each isolate and maintained on woodchips in the dark at 15 °C until being used. Overnight cultures of *K. pneumoniae* strains were washed with phosphate-buffered saline (PBS) and further adjusted with PBS to concentrations of 1 × 10⁶ CFU/mL (10 µl for injection). Colony counts were conducted by serial dilution with final plating on LB agar. The *G. mellonella* were infected with the tested bacteria, as previously described [43]. PBS injection controls and the negative controls (receiving no injection) were used to evaluate trauma and attrition, respectively. EC600 strain was used as non-virulent control, while, HvKP4 strain was used as the hypervirulent control [18]. HvKP4, a ST11 carbapenem-resistant hypervirulent *K. pneumoniae* outbreak strain, was isolated from China. The larvae were incubated at 37 °C in the dark and observed every 12 h for 7 days. We recorded the survival rate of the *G. mellonella* over 48 h post-infection. Results were not included if greater than or equal to two larvae died in either of the control groups. All experiments were done in triplicate.

Whole-genome sequencing (WGS)

The long-read MinION sequencer (Oxford Nanopore Technologies, Oxford, UK) was performed to sequencing the EBSI041 strain with a mean read length of 24 Kbp. De novo hybrid assembly was performed using Unicycler

v0.4.3 [44]. Complete circular contigs were then corrected using Pilon v1.22 with Illumina reads (Illumina, USA). In silico multilocus sequence typing (MLST) was performed by MLST 1.8 (<https://cge.cbs.dtu.dk/services/MLST/>). The antibiotic resistance genes and virulence genes were identified using ABRicate version 0.5 (<https://github.com/tseemann/abricate>). Insertion sequence (IS) elements were determined with ISFinder (<https://www-isfinder.biotoul.fr>). The SNP (sequence-based) and core-genome-based MLST (cgMLST) strategies on BacWGSTdb 2.0 were used for source tracking bacterial pathogens, and the phylogenetic tree was generated and visualized by Grapetree [24, 45].

Nucleotide accession numbers

The annotated sequences of all four plasmids have been deposited in the GenBank nucleotide sequence database under accession numbers MW245019 (pEBSI041-1), MW245020 (pEBSI041-2), MW245021 (pEBSI041-3) and MW245022 (pEBSI041-4).

Abbreviations

CRKP: Carbapenem-resistant *Klebsiella pneumoniae*; CR-hvKP: Carbapenem-resistant hypervirulent *K. pneumoniae*; ICU: Intensive Care Unit; S1-PFGE: S1 nuclease pulsed field gel electrophoresis; ARGs: Antibiotic resistance genes; MDR: Multidrug-resistance; ORFs: Open reading frames; MALDI-TOF MS: matrix-assisted laser desorption ionisation-time of flight mass spectrometry; MICs: Minimum inhibitory concentrations; WGS: Whole-genome sequencing; MLST: Multilocus sequence typing.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12864-021-08214-9>.

Additional file 1: Table S1 Minimum inhibitory concentrations (MICs) of *K. pneumoniae* EBSI041 strain and transconjugants. **Figure S1** Virulence potential of strain EBSI041 as depicted in a *Galleria mellonella* infection model with an inoculum of 1 × 10⁴ CFU. **Figure S2** The S1-PFGE map of *K. pneumoniae* EBSI041 strain and transconjugants. Transconjugant a and b were *E. coli* J53 as the recipient strains, transconjugant c and d were *E. coli* EC600 as the recipient strains. **Figure S3** The phylogenetic tree of 67 *K. pneumoniae* strains based on core-genome-based MLST (cgMLST) analysis using BacWGSTdb 2.0 (threshold 50). The tree was generated and visualized by Grapetree.

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Authors' contributions

Conceptualization, Guo-Bao Tian, Xubin Huang and Lin Xu; Data curation, Yanxian Yang and Yongqiang Yang; Funding acquisition, Guo-Bao Tian, Yongqiang Yang, Lingqing Xu and Lin Xu; Investigation, Yanxian Yang, Yongqiang Yang, Reem Mostafa Hassan and Mohamed Abd El-Gawad El-Sayed Ahmed; Methodology, Yanxian Yang, Mingyang Qin, Ruowen He, Yiping Wu, Xiaoxue Liang; Resources, Guo-Bao Tian, Reem Mostafa Hassan and Mohamed Abd El-Gawad El-Sayed Ahmed; Visualization, Yanxian Yang; Writing-original draft, Yanxian Yang and Yongqiang Yang; Writing-review & editing, Guo-Bao Tian, Xubin Huang, Lin Xu, Mohamed Abd El-Gawad El-Sayed Ahmed, Lan-Lan Zhong, Ping Chen, Baoguo Deng, Weihong Wen and Lingqing Xu; Supervision,

Guo-Bao Tian. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The newly sequenced plasmid sequences are available in GenBank under accession numbers MW245019 (pEBSI041-1) (<https://www.ncbi.nlm.nih.gov/nuccore/MW245019>), MW245020 (pEBSI041-2) (<https://www.ncbi.nlm.nih.gov/nuccore/MW245020>), MW245021 (pEBSI041-3) (<https://www.ncbi.nlm.nih.gov/nuccore/MW245021>) and MW245022 (pEBSI041-4) (<https://www.ncbi.nlm.nih.gov/nuccore/MW245022>). The data in the present study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Ethical approval for this study was given by the Ethics Committee of Zhongshan School of Medicine of Sun Yat-sen University under approval number 068 and sample collection was approved by the Ethics Committee of Souad-Kafai teaching hospital in Misr University for Science and Technology. Informed consent was provided by the guardians of the patients in the study. All methods involved in this study were carried out in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹Department of Microbiology, Zhongshan School of Medicine, Sun Yat-sen University, 74 Zhongshan 2nd Road, Guangzhou 510080, China. ²Key Laboratory of Tropical Diseases Control (Sun Yat-sen University), Ministry of Education, Guangzhou 510080, China. ³School of Pharmaceutical Sciences (Shenzhen), Sun Yat-sen University, Guangzhou 510006, China. ⁴Department of Microbiology and Immunology, Faculty of Pharmaceutical Sciences and Drug Manufacturing, Misr University for Science and Technology, Cairo, 6th of October City, Egypt. ⁵Department of Pathogen Biology, School of Basic Medical, Xinxiang Medical University, Xinxiang 453003, China. ⁶School of Laboratory Medicine, Chengdu Medical College, Chengdu 610500, China. ⁷Department of Clinical and Chemical Pathology, Faculty of Medicine, Cairo University, Cairo, Egypt. ⁸Department of Clinical Laboratory, The Sixth Affiliated Hospital of Guangzhou Medical University, Qingyuan People's Hospital, Qingyuan 511518, China. ⁹Department of Pulmonary and Critical Care Medicine, the First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China. ¹⁰Research Center for Clinical Laboratory Standard, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China. ¹¹School of Medicine, Xizang Minzu University, Xianyang 712082, Shaanxi, China.

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