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FULL PAPER

Molecular epidemiology and resistome analysis of multidrug-resistant ST11 *Klebsiella pneumoniae* strain containing multiple copies of extended-spectrum β-lactamase genes using whole-genome sequencing

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SUMMARY

The aim of this work was to investigate the mechanism responsible for multidrug resistance in ST11 *Klebsiella pneumoniae* YMC 2013/7/B3993 containing multiple copies of ESBL genes using multiple parallel sequencing technology. In-depth analysis of the strain revealed multiple copies of ESBL genes, 2 copies of bla_{SHV-12} and 1 copy of $bla_{CTX-M-15}$. Furthermore, 1 copy of bla_{OXA-9} and 3 copies of bla_{TEM-1} were found. The insertion of Tn*1331* was detected, which consisted of bla_{OXA-9}, bla_{TEM-1}, *aac(6')-lb-cr,* and *aadA1* genes. The acquisition of multiple copies of resistance genes was due to the insertion of transposons in the bacterial genome and plasmid. The genotypic analysis revealed that the isolates belonging to ST11 showed severe resistance phenotypes and greater dissemination potential. To the best of our knowledge, this is the first report demonstrating multiple copies of same ESBL genes in *K. pneumoniae* ST11 isolate. Furthermore, massive parallel sequencing studies of genetic factors to enhance the fitness of this type strain would be warranted to determine whether ST11 *K. pneumoniae* can spread the KPC-type gene.

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INTRODUCTION

ST11 is the most frequent clone of *K. pneumoniae,* which is disseminated worldwide carrying the carbapenemase and ESBL genes and has been designated an epidemic clone III (ECIII) (Damjanova *et al*., 2008). ST11 is a single-locus variant of the international hyper-epidemic lineage ST258, showing rapid dissemination over other clones as good colonizers that capture and accumulate the resistance determinants. In addition, emergence of *K. pneumoniae* ST11 co-producing KPC-2 and 16S rRNA methylase *rmtB* is posing a greater threat to antimicrobial therapy and causing severe complications like bacteremia and UTI (urinary tract infection) (Ko *et al*., 2010; Li *et al*., 2008). In *K. pneumoniae,* multidrug resistance is offered by large conjugative plasmids along with increasing efflux pump activity and altered profile of outer membrane porins that control the entry of antibiotics into the cell wall (Institute of medicine (US) forum on Microbial Threats Workshop, 2010). To date, only 3 carbapenem-resistant *K. pneumoniae* have been sequenced and their resistance mechanisms illustrated and there are no studies on the mechanism responsible for the multidrug resistance of ST11 isolates producing ESBLs (Hudson *et al*., 2014; Zhu *et al*., 2013; Liu *et al*., 2012). Extended spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* constitutes one of the most common Gram-negative bacteria showing resistance to multiple antibiotics worldwide. These opportunistic pathogens carrying multiple antibiotic resistance genes are highly recurrent and are responsible for nosocomial infections. The prevalence of ESBL-producing *Klebsiella pneumoniae* has been reported to be 17.7% to 30% in Korea[1] and 23% of the *K. pneumoniae* produce Ambler Class A ESBLs, with predominant SHV-12 and CTX-M types (Kim *et al*., 2005).

Currently, massive parallel sequencing (MPS) is being increasingly used in clinical microbiology laboratories. The advent of this technology has helped us to understand the complexity of the resistance encountered by pathogens during antibiotic therapy.

This technology clarifies the mechanism offering resistance through phenotypic and genotypic co-relation (Capobianchi *et al*., 2013; Drögemüller *et al*., 2010; Didelot *et al*., 2012). The key objective of our study was to characterize the mechanism responsible for the multidrug resistance in ESBL-producing *K. pneumoniae* ST11 by using whole genome sequence analysis.

Key words:
ST11, Multiple parallel sequencing, Extended-spectrum β-lactamase.

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MATERIALS AND METHODS

Bacterial strain

K. pneumoniae was isolated from the blood culture of a 70-year-old patient admitted to the intensive care unit

with bacterial meningitis and chronic renal failure from a tertiary care university affiliated hospital in Seoul, Korea. Bacteria identification was performed using VITEK 32 GN system (BioMérieux, Marcy l'Etoile, France), and was confirmed using the direct colony method with MALDI-TOF MS (Bruker Daltonics, Bremen, Germany).

Susceptibility tests, MIC determinations and conjugation studies

Antibiotic susceptibility was tested using the disc diffusion method containing piperacillin, ampicillin, piperacillin-tazobactam, ceftazidime, cefepime, imipenem, meropenem, ciprofloxacin, ceftazidime-clavulanate, ampicillin-sulbactam and aztreonam on Mueller-Hinton agar, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.

The MICs were determined by agar-dilution technique, and results were interpreted according to the CLSI guidelines. Screening of ESBL and AmpC β-lactamase production was carried out by double disk synergy test as described previously (Mohanty *et al.*, 2009; Jennifer *et al.*, 2005). Conjugation experiments were carried out using *E. coli* J53 (F^- met pro Azi^r) as the recipient. Transconjugants were selected on Mueller-Hinton agar plates containing 2 mg/L of cefotaxime and ceftazidime with 100 mg/L of sodium azide.

DNA isolation and Ion Torrent PGM sequencing

Genomic DNA and plasmid DNA were isolated using Wizard Genomic DNA purification Kit (Promega, Madison, WI, USA) and Plasmid plus Midi Kit (Qiagen, Valencia, CA, USA), respectively. DNA concentration was estimated using Qubit dsDNA BR assay kit (Molecular Probes, Eugene, OR, USA). Whole genome library was performed using Ionplus Fragment Library Kit and emulsion PCR was carried out using the Ion PGM Template OT2 400 kit (Life technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Sequencing of the libraries was carried out on a 318 chip v2 using the Ion Torrent PGM system and Ion Sequencing 400 kit (Life technologies, Carlsbad, CA, USA).

Sequence assembly, annotation, typing and resistome analysis

Reads from the Ion Torrent PGM system were assembled using MIRA plug-in using Ion Torrent software. Annotations were performed using the RAST annotation pipeline with manual scrutiny. Genomic analysis was performed using Geneious pro (Biomatters Ltd., AK, New Zealand). Multilocus sequence typing was performed by using MLST 1.7 web interface (https://cge.cbs.dtu.dk/services/MLST/) and confirmed using Institute Pasteur MLST databases. Resistance genes were screened using Resfinder (https://cge.cbs.dtu.dk/services/ResFinder/) and further verified using NCBI BLAST.

Analysis of OMPs

Bacterial cells were grown in high-osmolarity MHB to the logarithmic phase and were lysed by sonication. Unbroken cells were separated by centrifugation at 3,000 g for 5 minutes and outer membrane proteins (OMPs) were extracted with sodium lauroyl sarcosinate and recovered by ultracentrifugation, as described previously. The OMP profiles were determined using SDS-PAGE by Mini-Protean TGX gels followed by Coomassie blue staining (Bio-Rad, Hercules, CA, USA).

Phylogenetic analysis

The genome of YMC 2013/7/B3993 strain was used for phylogenetic analysis, along with 183 other *Klebsiella spp.* reference genomes that were available in NCBI as of November 2014. Ridom SeqSphere+ (Ridom GmbH, Germany) was used for the MLST and core-genome MLST analysis.

RESULTS

The *K. pneumoniae* YMC 2013/7/B3993 strain was resistant to piperacillin, piperacillin-tazobactam, ceftazidime, amikacin, cefepime, cefoxitin, ciprofloxacin ampicillin, ampicillin-sulbactam and susceptible to imipenem, meropenem and ceftazidime-clavulanate (*Table 1*). Using 911,427 reads, we assembled the reads into 5,908,460 bp

Table 1 - *Antibiotic susceptibility of K. pneumoniae YMC 2013/7/B3993 with its genotypic explanation for the resistance observed.*

Antibiotics	*Class*	*MIC (mg/L)/ Interpretation*	*Enzymes/factors conferring resistance (gene locations*)*
Piperacillin	Ureidopenicillin	256/ R	TEM-1$^{(p/c)}$
Piperacillin-tazobactam	Ureidopenicillin+BLI	128/ R	TEM-1$^{(p/c)}$
Ceftazidime	Cephalosporin	256/ R	TEM-1$^{(p)}$, SHV-12$^{(p/c)}$, CTX-M-15$^{(p)}$, Loss of OmpK35 and OmpK36
Cefepime	Cephalosporin	94/ R	TEM-1$^{(p),}$ SHV-12$^{(p/c)}$, CTX-M-15$^{(p)}$
Imipenem	Carbapenem	0.25/ S	-
Meropenem	Carbapenem	0.25/ S	-
Ciprofloxacin	Quinolone	128/ R	QnrB66$^{(p)}$, OqxA$^{(p/c)}$, OqxB$^{(p/c)}$, Aac(6')-lb-Cr$^{(p/c)}$
Ceftazidime-clavulanate	Cephalosporin+BLI	1/ S	-
Cefoxitin	Cephalosporin	32/ R	TEM-1$^{(p/c)}$, Loss of OmpK35 and OmpK36
Ampicillin	Aminopenicillin	256/ R	TEM-1$^{(p/c)}$
Ampicillin -sulbactam	Aminopenicillin/BLI	128/ R	TEM-1$^{(p/c)}$

*(p) indicates the location of genes in plasmid and (p/c) indicates the location of the genes either in plasmid or chromosome. BLI, β-lactamase inhibitor.

chromosome with 188 contigs, consisting 5,714 coding sequences and 114 RNAs (33 rRNA and 81 tRNA). The average G+C content of the chromosome was 57%. This Whole Genome Shotgun project was deposited at DDBJ/ENA/GenBank under the accession no. LDWV00000000. In-depth analysis revealed multiple copies of ESBL genes including two copies of bla_{SHV-12} and one copy of $bla_{CTX-M-15}$ genes. In addition, we also found one copy of bla_{OXA-9} and three copies of bla_{TEM-1}. This strain belonged to ST11 which was confirmed by analyzing the housekeeping genes for MLST (*Figure 1*). An insertion of *Tn1331* was detected, which carried bla_{OXA-9}, bla_{TEM-1}, *aac(6')-lb-cr* and *aadA1* genes.

Thus genotypic and phenotypic relationships are summarized in *Table 1*. Ureidopenicillin and aminopenicillin resistance is predominantly offered by TEM-1 gene, which is likely a baseline overproduction due to either alteration in the gene expression or increase in the copy numbers in the plasmid as described below. bla_{SHV-12} and $bla_{CTX-M-15}$ are the ESBL determinants which confer resistance to 1st, 2nd and 3rd generation cephalosporins. MIC of ciprofloxacin in the strain was notably high, 128 mg/L, which might be due to the additive effect of both quinolone resistance-determining regions (QRDR) and plasmid-mediated quinolone resistance (PMQR). Mutations in QRDR were observed at Ser83Ile and Ser80Ile in *gyrA* and *parC* genes, respectively and PMQR analysis indicates the presence of *aac(6')-lb-cr* and *qnrB*, along with the genes for efflux pumps *oqxA* and *oqxB*. *In silico* analysis of the strain confirmed the presence of *ompA* porin gene alone, which was confirmed using SDS-PAGE (Data not shown). *ompK35* gene was interrupted by IS*1* and *OmpK36* (*OmpK36_v1* variant) with the amino acid substitution at Arg357His with a nucleotide substitution from A to T at -10 box, hindering its expression resulting in cefoxitin resistance. The prominent virulent genes found in other Enterobacteriaceae such as *magA* and *rmpA* gene and RNA methylases such as *apmA, armA, armA2, rmtA* and *rmtB* were absent in *K. pneumoniae* YMC 2013/7/B3993. However, virulence-associated VagCD operon was present. The strain also consists of *uge* (UDP galacturonate 4-epimerase), *Htra*, *PfrB* and *ccl* genes were present with iron acquisition yersiniabactin synthesis enzyme and yersiniabactin siderophore biosynthetic protein.

Conjugation studies were performed to localize the resistance genes and to determine the transferable ability of resistance genes between the strains. In spite of repeated attempts only one plasmid was transconjugated that was resistant to cefotaxime and ceftazidime. Resistance transfer was confirmed by comparing the antibiotic zone diameter between parent strain, *E. coli* J53 and the transconjugant (Data not shown). E-test indicated that the MIC of ceftazidime, cefotaxime and ciprofloxacin was higher in transconjugant (CAZ-24 mg/L; FOX-32 mg/L; CIP-0.19 mg/L) when compared to the Wild type *E. coli* J53 (CAZ-1 mg/L; FOX-0.25 mg/L; CIP-0.0.023 mg/L). Massive parallel sequencing of this plasmid revealed the presence of $bla_{CTX-M-15}$, bla_{TEM-1}, *strA, strB, qnrB66, sul2* and *dfrA14* indicating that the β-lactamases and aminoglycoside resistance determinants were encoded in plasmids that could have been transferred to *Escherichia coli* by conjugation. The localization of another ESBL gene bla_{SHV-12} was not clear. However we could see two copies of bla_{SHV-12} gene in strain YMC 2013/7/B3993, of which one was flanked by *IS26*. Both copies of this gene were confirmed using PCR and analysis of the nucleotides surrounding both the bla_{SHV-12} genes (*Figure 2*). The average G+C content of the isolated plasmid was 50% with 2,770 coding sequences and 23 RNAs. The whole genome sequence of conjugated plasmid was mapped against the *E. coli* J53 and the genomic DNA contamination which

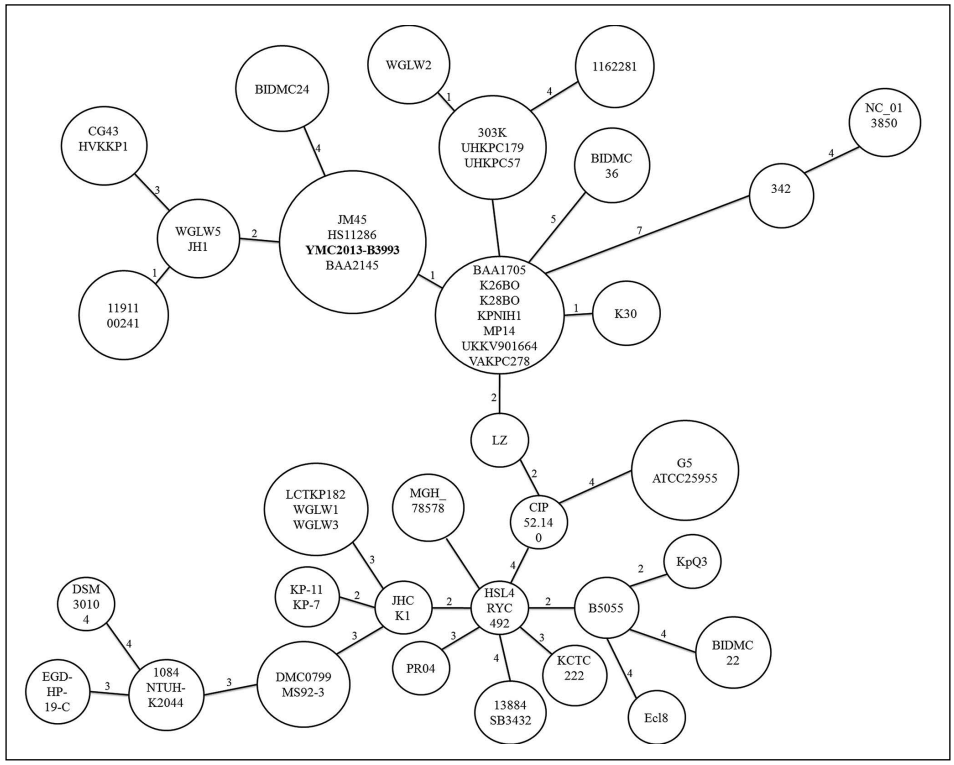

Figure 1 - *Klebsiella pneumoniae phylogeny: Whole-genome phylogeny of YMC 2013/7/B3993 relative to other strains. The tree demonstrates the genome of YMC 2013/7/B3993 is similar to other sequenced genomes i.e. JM45, HS11286 and ATCC BAA2146, all belonging to ST11 clone of K. pneumoniae.*

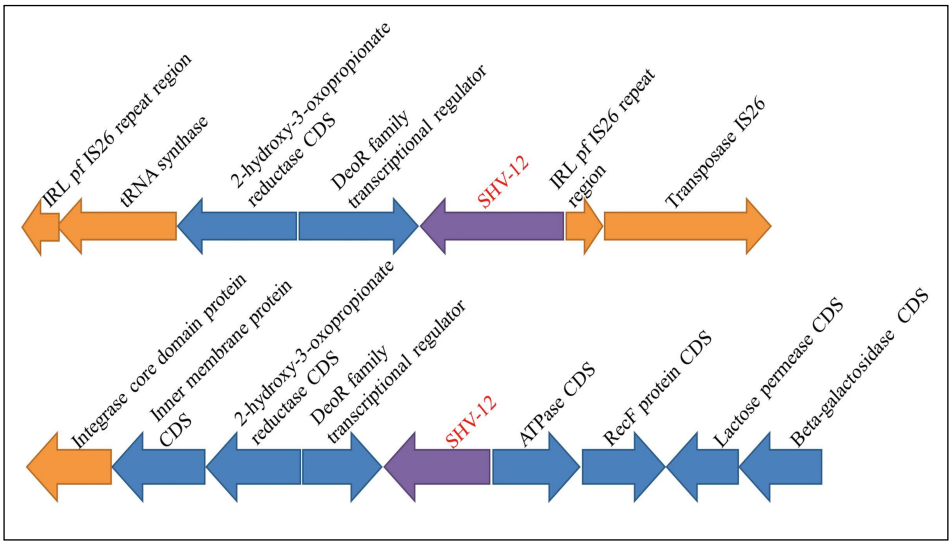

Figure 2 - *Contigs carrying the dual copy of ESBL Resistance Genes in YMC 2013/7/B3993 K. pneumoniae: Contigs carrying the Dual copy of SHV-12 ESBL resistance genes flanked by mobile elements (orange arrows).*

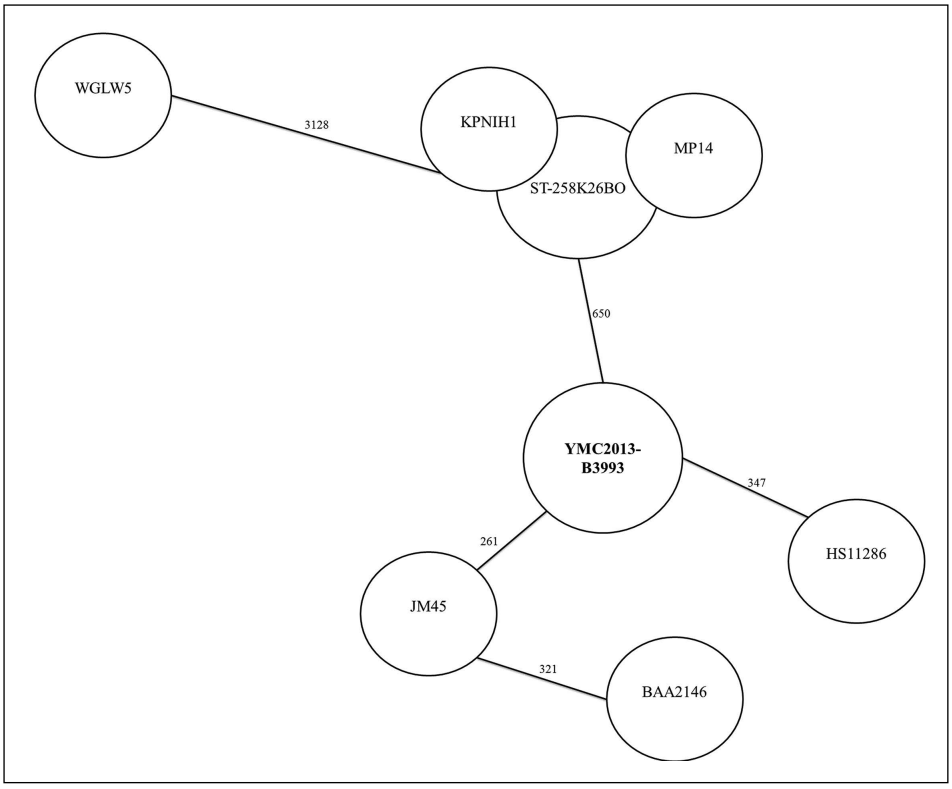

Figure 3 - *Core-Genome MLST of YMC 2013/7/B3993 and closely related K. pneumoniae strains: Core-genome MLST of YMC 2013/7/B3993 with other closely related strains from ST11 and ST12. Tree created with task template K. pneumoniae+3597 targets HS11286 with K. pneumoniae accessory 1628 targets HS11286. Our strain shares close relationship with other 3 ST11 K. pneumoniae consisting of Carbapenemase gene along ESBLs.*

might have occurred during DNA extraction was carefully removed from the parent strain.

Resistance genes were further examined for its relationship with other strains. The gene *aac(6')-Ib-cr*, *aadA1*, *bla*$_{OXA-9}$, and *bla*$_{TEM-1}$ were present in close proximities within the structure of *Tn1331*. The GenBank indicated that this was identical to the multi resistance plasmid pJHCMW1 (Genbank accession number AF479774) in *K. pneumoniae* isolated from neonates with meningitis in 1986 (Woloj *et al*., 1986). However, this plasmid was not transferred in spite of repeated attempts. The amikacin resistance intrinsic to the plasmid pJHCMW1 was also noticed in YMC 2013/7/B3993, which must have been contributed from the same plasmid mentioned above.

DISCUSSION

Outbreaks of ESBL-producing *K. pneumoniae* strains are a major concern in nosocomial infections. ESBLs are enzymes manifesting considerable hydrolytic activity on a wide variety of β-lactam antibiotics including oxyimino-cephalosporins and aztreonam (Shahid *et al*., 2004). Poor hygiene and improper handling of pathogenic strains has led to emerging resistance in the developing countries. As a result, strains once susceptible have evolved as resistant strains (Laxminarayan *et al*., 2006).

To date, 3 strains belonging to ST11 have been sequenced, which contain both ESBL and carbapenemase genes. Core-genome sequence typing revealed that YMC 2013/7/

Table 2 - *Resistance analysis of related Klebsiella pneumoniae strains: Type, Resistance genes and plasmid.*

		ß-Lactam																*Aminoglycoside*								
	MLST	DHA-1	CTX-M-24	CTX-M-14	CTX-M-15	KPC-2	KPC-3	NDM-1	OXA-1	OXA-9	SHV-1	SHV-11	SHV-36	SHV-12	SHV-99	VEB-3	TEM-1	LEN-22	aac(6')-Ib	aadA1	aadA2	aadB	aph(3')-Ia	aph(3')-Ic	aph(4)-Ia	strA	strB
KP52.145(PRJEB99)	66																										
KCTC2242(PRJNA162147	375														•							•					
HS11286 (PRJNA84387)	11			•		•						•					•		•		•					•	•
BAA214 (PRJNA138848)	11				•			•		•		•					•		•							•	•
JM45 (PRJNA215235	11		•			•						•				•	•				•		•			•	
YMC2013/7/B3993	11				•					•				•			•		•	•						•	•
KPNIH1(PRJNA73191	258						•			•		•					•		•		•		•				
K30BO(PRJNA180981)	512						•					•							•		•						
KpQ3 (PRJNA182046)	256	•							•			•							•	•	•		•		•		
MGH78578 (PRJNA57619)	38											•		•			•		•	•		•		•		•	•
NTUHK2044 (PRJNA59073)	23											•	•														
SB3432 (PRJNA203334	67											•															
ATCC13884 (PRJNA41361)	67										•																
342 (PRJNA59145)	146																	•									

B3993 falls in a clad containing fellow ST11 strains such as ATCC BAA-2146 (bla_{NDM-1} with $bla_{CTX-M-15}$), JM45 (contains bla_{KPC-2} with $bla_{CTX-M-24}$) and HS11286 (contains bla_{KPC-2} with $bla_{CTX-M-14}$); and this clad is located close to ST258 and ST512 strains (*Figure 3*). Interestingly, the three resistance clads contain the maximum number of resistance genes including ESBLs, AmpC β-lactamases and carbapenemases (*Table 2*).

The combination of carbapenemase and ESBL genes in ST11 clones indicates that the strains with only ESBL

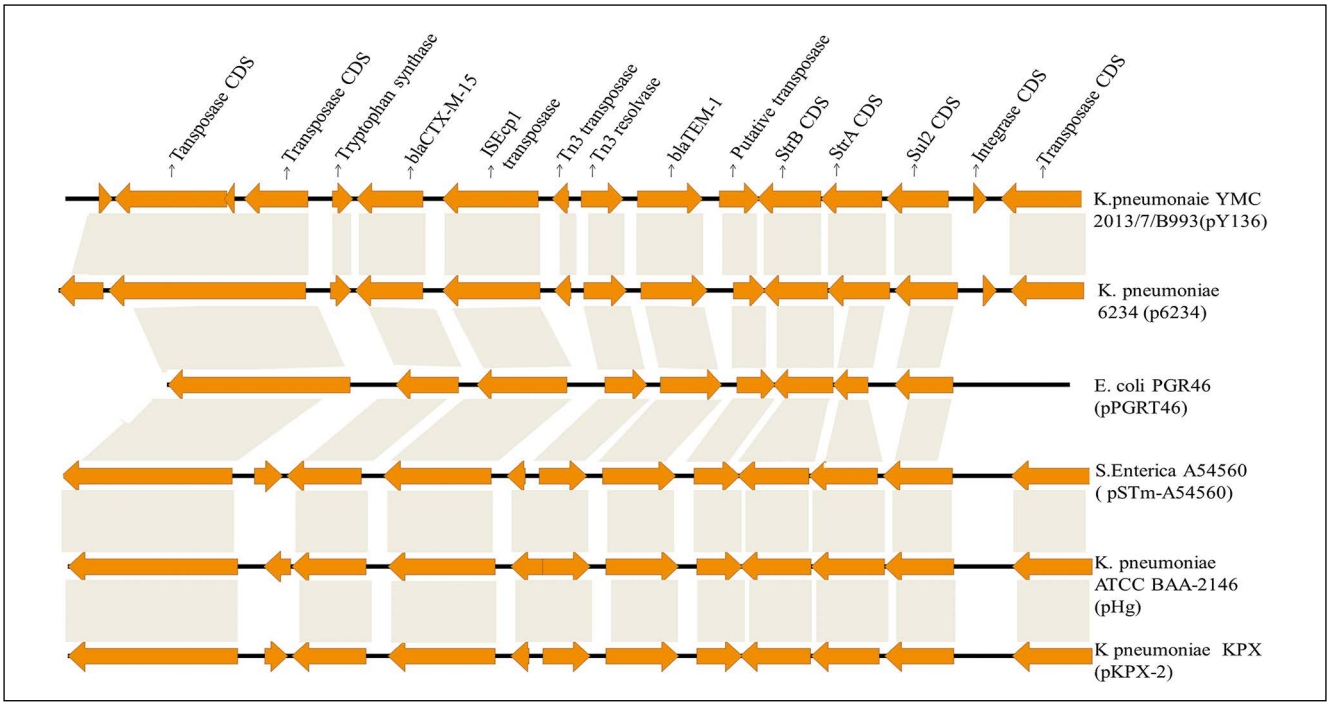

Figure 4 - *Comparison of plasmid resistome: Transconjugated plasmid carrying blaCTX-M-15 and blaTEM-1 was sequenced and compared with other plasmids. 13.6Kb of the plasmid shares 100% identity with the other plasmids found in K. pneumoniae, E. coli and Salmonella enterica.*

Fos	*Quinolone*					*Phenicol*			*RIf*	*Tetracycline*				*Trimetho-prim*			*Sulfon-amide*		*Plasmids*														
FosA	QnrB66	QnrB4	aac(6')Ib-cr	oqxA	oqxB	catA1	catB3	floR	ARR-3	tet(A)	tet(C)	tet(D)	tet(G)	dfrA1	dfrA12	dfrA14	sul1	sul2	HI1B	A/C2	CoI(KPHS)	CoIRNAI	F1A(HI1)	F1B(pKP)	F1B(K)	F1B(pOil)	FII(K)	FII	Col(MGD2)	FII(Y)	R	X3	Q2
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genes can acquire the carbapenemase genes in future. Tóth *et al*. reported that ST11 and ST258 belong to the clone complex CC258, along with ST 270, ST340, ST379, ST407 and ST418 (Tóth *et al*., 2010). Epidemiological data indicate that CC258 spread rapidly across the world in a similar fashion as the CC17 of vancomycin-resistant *Enterococcus faecium* and CC22 of carbapenem-resistant *Acinetobacter baumannii (*Willems *et al*., 2005; Fu *et al*., 2010). From this we can conclude that the ST11 clone is a good colonizer to capture the plasmids, which results in multiple resistances.

Previous studies reported that $bla_{CTX\text{-}M\text{-}14}$ or $bla_{CTX\text{-}M\text{-}15}$ were notoriously detected in most *K. pneumoniae* isolates in South Korea. YMC 2013/7/B3993 strain can be now termed a unique ESBL strain containing multiple copies of ESBL gene.

Transconjugation studies confirmed the presence of ESBL gene $bla_{CTX\text{-}M\text{-}15}$ which is flanked by mobile elements like *Tn2* and transposase *ISCR2*. Comparative genome studies showed that the 13.6kb of this plasmid was identical to the *K. pneumoniae* plasmids, p6234, pHg, pKPX-2, *Salmonella enterica* plasmid pSTm-A54560 and *E. coli* pPGRT46 isolated in Columbia, USA, Taiwan, Sub-Saharan Africa and Nigeria respectively (*Figure 4*).

The non-conjugated plasmid in our strain was identical to pJHCMW1, a plasmid isolated from an outbreak strain in USA in 1986. Thus, global spread of this resistance is observed and worrisome due to the mobility of this plasmid between genus levels and uncommon broad host range dissemination.

To our knowledge, this is the first study demonstrating the massive parallel sequencing of ST11 strain containing multiple ESBL genes. Expansion of ST11 clones and plasmids that have acquired the transposons seems to have fueled dissemination of the KPC-type gene.

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