# Complete Sequence of a Plasmid co-harboring CTX-M-65, SHV-12, KPC-2, Rmtb and TEM-1 from a hyper-virulent Klebsiellarr pneumonia Clinical Strain

**Shen Wei[1,2], Wang Guang-Xi[1*], Zhou Ying-Shun[1*] and Tang Ling-Tong[1]**
1. Department of pathogenic biology; Southwest Medical University, Sichuan, CHINA
2.The First People's Hospital of Yibin, Sichuan, CHINA
*38592956@qq.com

## *Abstract*

*Plasmid Preparation Kit was to extract plasmid genome of multidrug-resistant Strain SWU01 of Klebsiellar pneumonia. High-throughout sequencing technique was performed for plasmid of SWU01 comprising of 162, 552 bp, which was referred as pSWU01. After analyzing, we found this plasmid harbors drug-resistant genes CTX-M-65, SHV-12, KPC-2, Rmtb, and TEM-1, toxin encoding gene including VagC, toxic poly-peptide, RelE toxin protein, virulence factor, transposon (Tn), insertion sequence(IS), Integron and Transfer position (Tra, Trb). BLAST search against NCBI Genebank revealed similarity between pSWU01 and drug-resistant plasmids. According to the research, pSWU01 is 85% identical t to the 151,466 bp of drug-resistant plasmid LJ04 (GeneBank Accession NO: KT185451.1), while 76% identical to the 136,848 bp of drug-resistant plasmid pkP1034 (GeneBank Accession NO: KP893385.1). Conclusion: Plasmid of multidrug-resistant Strain SWU01 for Klebsiellar pneumonia harbors rich drug-resistant elements, which contribute mainly to the multi-drug resistance of this strain.*

**Keywords:** Klebsiellar pneumonia, Plasmid, hyper-virulent multi-drug resistance, complete genome sequencing, drug-resistance encoding genes, transposons, Inserted Sequences

## Introduction

Klebsiellar pneumonia, an important opportunistic pathogen, is the causative agent of community-acquired infection. Due to the overuse of antibiotics, the isolating rate of multidrug-resistant klebsiellar pneumonia increases obviously, only lower than Escherichia coli[1]. Plasmid is a small circle of DNA that is independent of the main chromosome and capable of replication, which allows for the horizontal gene transfer (HGT) of bacteria drug-resistant gene and virulence encoding gene. In addition, it also contributes to the inter-species and intra-species spread of drug-resistant genes by horizontal transferring between identical or different genus of bacteria[2].

In recent years, hyper-virulent klebsiellar pneumonia with carbapenem-resistant gene is increasingly reported, which brings in serious challenges to clinical treatment. This research uses the third generation of High-through sequencing technique to conduct complete genome sequencing on the hyper-virulent strain (Strain SWU01) of klebsiellar pneumonia that was isolated from the patient in Intensive Care Unit (ICU), which would be important for the prevention and curing of hyper-virulent multidrug-resistant klebsiellar pneumonia.

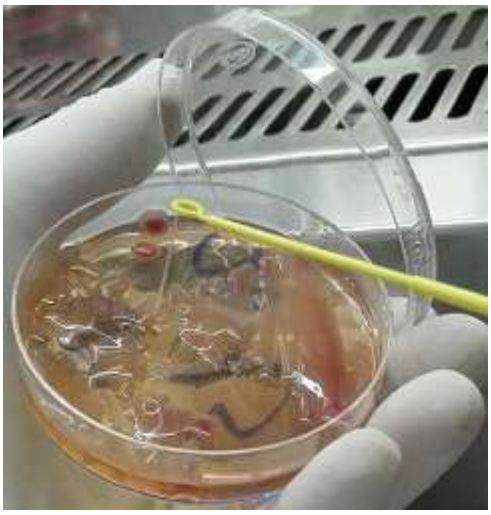
## Material and Methods

**Strain source and drug sensitive test:** Strain SWU01 of klebsiellar pneumonia was isolated from the patient blood in April 2015. Male patient, 72 years old, just finished operation of malignant digestive cancer, having severe pulmonary infection. Isolation of bacteria was performed in accordance with the 3rd version of National Clinical Laboratory Procedures. Equipment VITEK2-Compact from French BioMerieux Company was used for bacteria identification.

With respect to drug sensitive test, E-test from French BioMerieux Company was used to test the minimal inhibitory concentration (MIC) of 18 kinds of antibiotics, including AMK,AMP, SAM, ATM,ETP, NIT,SXT, CIP,TZP, GEN,FEP,CRO, CAZ,CTT, CZO,TOB,IMP and LVX. The drug sensitive test result was referred to the M100-S22 document of Clinical and Laboratory Standards Institute (CLSI) in 2013[3]. E.coli ATCC25922, Staphylococcus aureus ATCC25923, Pseudomonas aeruginosa ATCC27853 and E.coli ATCC35218 were used for contrast of drug sensitive test, all of which come from Hangzhou Binhe Microorganism Reagent Company.

**Identification of hyper-virulent strain:** As the picture 1 show, this strain is high mucinous strain, positive in string test. After the specific PCR of hyper-virulent gene CTX-M-65, SHV-12, KPC-2, Rmtb and TEM-1, it was confirmed that this strain does express relevant hyper-virulent gene, which ascertains this strain to be hyper-virulent.

**Sequencing and Assembling:** First of all, SOAPdenovo (v2.04) was used for the preliminary assembling of Illumina sequencing data. Then blasR was used to compare with the sequencing data of Pacbio, assembling scaffold by the overlapping region. Then Celera Assembler 8.0 was used for the follow-up assembling. After all the assembling of scaffold, Illumina data was used for proofreading. Meanwhile, GapCloser v1.12 (related software of SOAPdenovo) was used for gap closing. The complete genome sequencing was conducted by the MajorBio Company in Shanghai.



**Picture 1: Positive for string test**

**Function annotation of plasmid genome:** After the complete genome sequencing of plasmid, software of Glimmer 3.02, GeneMark and Z-Curveprogram were used for gene prediction. The searching of IS structure was based on the IS Finder database (http://www-is.biotoul.fr). Gene function could be predicted by BlastP search against non-redundant protein sequence database of National Center for Biotechnology Information (NCBI) (http://www.blast/ncbi.nlm.nih.gov) and protein database of KEGG in Japan (http://www.genome.jp/kegg). InterProScan could be used to identify functional domain of genes to be further confirmation of functions.

**SWU01 plasmid sequence:** Blastn was used to compare pSWU01 sequences with the sequences of plasmids recorded by NCBI (http://www.blast/ncbi.nlm.nih.gov). Cluster analysis was performed with Fast Minimum Evolution Method.

**Sequence Submitting:** After the gene function annotation of pSWU01 sequence, the data were submitted to NCBI GeneBank.

## Results

**Drug-resistance test of SWU01 of klebsiellar pneumonia:** For all the 18 kinds of antibiotic, SWU01 of *Klebsiellar* shows high resistance, higher MIC than the maximum of E-test, including AMK, AMP, SAM, ATM, ETP, NIT, SXT, CIP, TZP, GEN, FEP, CRO, CAZ, CTT, CZO, TOB, IMP and LVX.

**Multilocus Sequence Typing (MLST):** Comparing complete genome sequence by BLAST, we found there are 7 house-keeping genes, rpoB, gapA, infB, mdh, pgi, tonB and phoE, details in Table 1[4].Submitting these house-keeping genes to MLST database, allele numbers could be identified, and the MLST typing of this strain was identified to ST11[5].

**Analysis on pSWU01:** The total length of pSWU01 is 162552 bp, GC content 52.76%. It harbors 250 Open Reading Frames, 70 of which are genes relevant to drug-resistance, (including 36 transfer-encoding genes for conjugate plasmid, 6 drug-resistant encoding genes, 4 virulence encoding genes, 8 Tn, 15 IS and 1 integrase encoding gene for Type I Integron). Detail position and function annotation of these 70 drug-resistance genes were listed in Table 2. The 250 genes were annotated and uploaded on NCBI Genbank (GeneBank Accession NO: CP006657).

**pSWU01 Comparing:** Picture 2 shows positions of transfer-encoding genes for conjugate plasmid, drug-resistance encoding gene, virulence encoding gene, Tn and IS on PSWU01 (see Picture 2).

Comparing PSWU01 with drug-resistant plasmids, pSWU01 is 85% identical t to the 151,466 bp of drug-resistant plasmid LJ04 (GeneBank Accession NO: KT185451.1) , while 76% identical to the 136,848 bp of drug-resistant plasmid pkP1034 (GeneBank Accession NO: KP893385.1), see in Picture 3.

## Discussion

Klebsiellar pneumonia is important opportunistic pathogen for nosocomial infection. According to recent papers. There are more and more reports on new drug-resistant gene, which shows the increasing problems of drug-resistance for klebsiellar pneumonia[6-8]. Plasmid is a small circle of DNA that is independent of the main chromosome and capable of replication. It could encode drug-resistant gene and virulence gene, it also allows for the horizontal gene transfer (HGT) of bacteria drug-resistant gene and virulence encoding gene, which contributes to the inter-species and intra-species spread of drug-resistant genes[9]. In accordance with its function, plasmid could be classified into Fertility (F), Resistance(R), catabolic and pathogenic. In addition, there is a kind of plasmid called chimeric plasmid or mosaic cointegrate, such as pTN48[10], pSMS35_130[11], pCooKm[12], and pB3[13].

SWU01 of klebsiellar pneumonia was isolated from the patient blood who just finished operation of malignant digestive cancer and got severe pulmonary infection. It is a multidrug resistant strain, which shows resistance to the majority of antibiotics. After complete genome sequencing of pSWU01, a total number of 70 genes relevant to drug-resistance were found. Due to the conjugate plasmid transfer-encoding gene, it could allow for the horizontal transfer of drug-resistant gene, which makes the spread of drug-resistant bacteria (see in tra /trb locus of Table 1 and Picture 1).

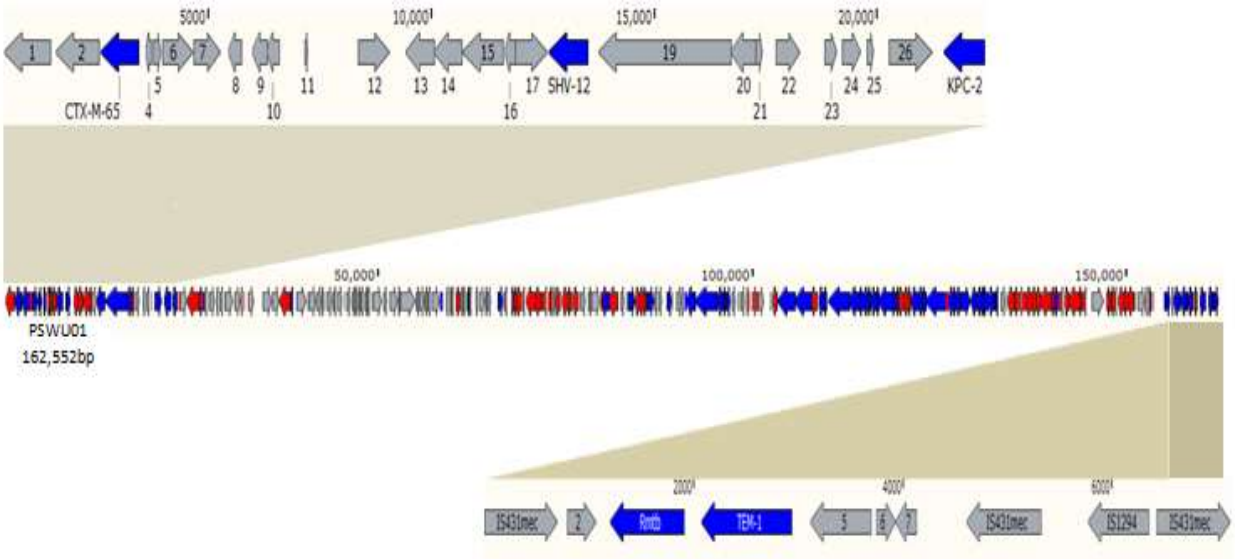
**Table 1**
**MSLT Typing Result**

| ORF | Starts | Ends | Length (bp) | Gene Name | Allele NO |
|---|---|---|---|---|---|
| Orf04167 | 4190835 | 4191353 | 501 | rpoB | 1 |
| Orf00655 | 665159 | 665608 | 450 | gapA | 3 |
| Orf03373 | 3360760 | 3361077 | 318 | infB | 3 |
| Orf03431 | 3418166 | 3418642 | 477 | mgh | 1 |
| Orf04212 | 4250312 | 4250743 | 432 | pgi | 1 |
| Orf01757 | 1727270 | 1727683 | 414 | tonB | 4 |
| Orf05079 | 6135128 | 5135547 | 420 | phoE | 1 |

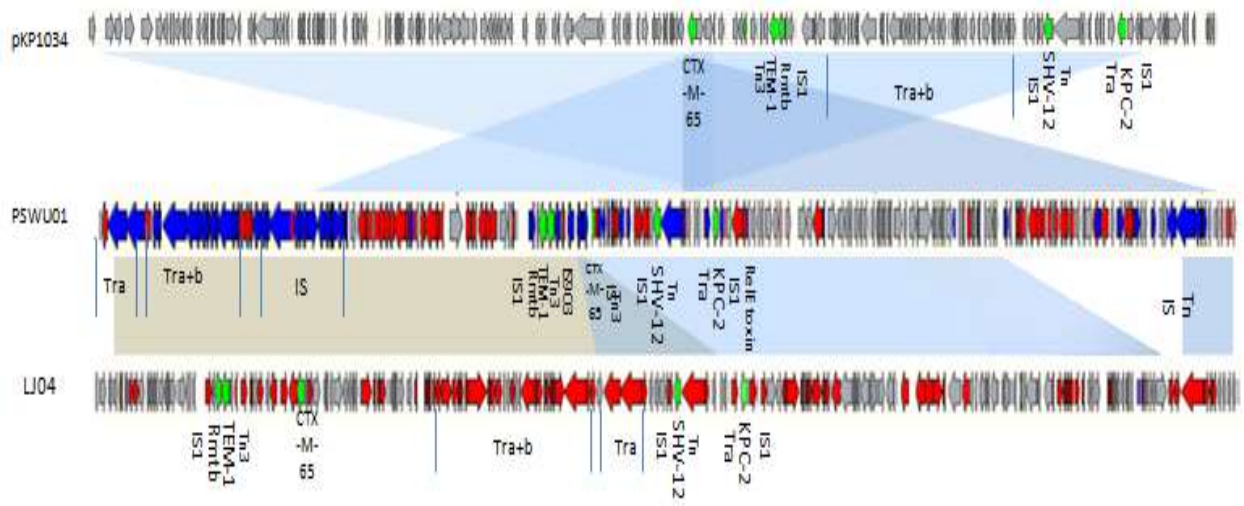
**Table 2**
**Position and function annotation of pSWU01, transfer-encoding genes for conjugate plasmid, drug-resistance encoding gene, virulence encoding gene, Tn and IS**

| locus tag | Start | End | Length (bp) | Gene Name | Function annotation |
|---|---|---|---|---|---|
| **orf00002_1** | 2572 | 1604 | 969 | IS903 | IS |
| **orf00003_1** | 3467 | 2607 | 861 | CTX-M-65 | ESBL |
| **orf00006_1** | 3993 | 4697 | 705 | IS431mec | IS |
| **orf00007_1** | 4703 | 5308 | 606 | Tn3 | Tn |
| **orf00008_1** | 5759 | 5484 | 276 | IS1 | IS |
| **orf00012_1** | 7972 | 7268 | 705 | IS431mec | IS |
| **orf00013_1** | 8386 | 9090 | 705 | IS431mec | IS |
| **orf00019_1** | 13530 | 12670 | 861 | SHV-12 | ESBL |
| **orf00020_1** | 16737 | 13771 | 2967 | Tn1721 | Tn |
| **orf00021_1** | 17301 | 16741 | 561 | Tn21 | Tn |
| **orf00027_1** | 20288 | 21268 | 981 | TraN | Transfer position |
| **orf00028_1** | 22399 | 21518 | 882 | KPC-2 | carbapenemase |
| **orf00029_1** | 23228 | 22725 | 504 | IS1 | IS |
| **orf00034_1** | 26964 | 26734 | 231 | VagC | Toxin protein |
| **orf00049_1** | 38761 | 38330 | 432 | ImpA | ImpA |
| **orf00081_1** | 58469 | 58627 | 159 | small toxic polypeptide | Small toxic poly-peptide |
| **orf00092_1** | 62057 | 62377 | 321 | RelE toxin protein | Toxin protein |
| **orf00098_1** | 66817 | 66113 | 705 | IS431mec | IS |
| **orf00126_1** | 80934 | 79963 | 972 | parB | Virulence factor |
| **orf00131_1** | 83532 | 84482 | 951 | Tnpa | transposase |
| **orf00135_1** | 85996 | 86976 | 981 | IS5Y | IS |
| **orf00138_1** | 88654 | 89358 | 705 | IS431mec | IS |
| **orf00142_1** | 91070 | 91246 | 177 | | integron |
| **orf00143_1** | 92432 | 91428 | 1005 | IS4321R | IS |
| **orf00144_1** | 95477 | 92511 | 2967 | Tn21 | Tn |
| **orf00145_1** | 96302 | 95598 | 705 | IS431mec | IS |
| **orf00146_1** | 96279 | 96527 | 249 | TraI | Transfer position |
| **orf00147_1** | 96547 | 97293 | 747 | Trax | Transfer position |
| **orf00165_1** | 105758 | 103509 | 2250 | TraI | Transfer position |
| **orf00166_1** | 107974 | 105758 | 2217 | TraD | Transfer position |
| **orf00168_1** | 109699 | 108965 | 735 | TraT | Transfer position |
| **orf00169_1** | 109996 | 109721 | 276 | TraS | Transfer position |
| **orf00170_1** | 113091 | 110275 | 2817 | TraG | Transfer position |
| **orf00171_1** | 114461 | 113088 | 1374 | TraH | Transfer position |
| **orf00172_1** | 114840 | 114448 | 393 | TrbF | Transfer position |
| **orf00173_1** | 115108 | 114794 | 315 | TrbJ | Transfer position |
| **orf00174_1** | 115643 | 115098 | 546 | TrbB | Transfer position |
| **orf00175_1** | 115911 | 115630 | 282 | TraQ | Transfer position |

| | | | | | |
|---|---|---|---|---|---|
| **orf00176_1** | 116770 | 116027 | 744 | TraF | Transfer position |
| **orf00177_1** | 117023 | 116763 | 261 | TraE | Transfer position |
| **orf00178_1** | 118897 | 117047 | 1851 | TraN | Transfer position |
| **orf00179_1** | 119316 | 118894 | 423 | TraF | Transfer position |
| **orf00180_1** | 119713 | 119342 | 372 | TrbE | Transfer position |
| **orf00181_1** | 120348 | 119710 | 639 | TrbC | Transfer position |
| **orf00184_1** | 122286 | 121294 | 993 | TraV | Transfer position |
| **orf00185_1** | 122915 | 122283 | 633 | TraW | Transfer position |
| **orf00186_1** | 123298 | 122912 | 387 | TraI | Transfer position |
| **orf00187_1** | 125922 | 123295 | 2628 | TraC | Transfer position |
| **orf00189_1** | 126303 | 126082 | 222 | TraR | Transfer position |
| **orf00190_1** | 126953 | 126438 | 516 | TraV | Transfer position |
| **orf00191_1** | 127201 | 126950 | 252 | TrbG | Transfer position |
| **orf00192_1** | 127410 | 127213 | 198 | TrbD | Transfer position |
| **orf00193_1** | 127987 | 127397 | 591 | TraP | Transfer position |
| **orf00194_1** | 129404 | 127977 | 1428 | TraB | Transfer position |
| **orf00195_1** | 130132 | 129404 | 729 | TraK | Transfer position |
| **orf00196_1** | 130685 | 130119 | 567 | TraE | Transfer position |
| **orf00197_1** | 131018 | 130707 | 312 | TraL | Transfer position |
| **orf00198_1** | 131398 | 131033 | 366 | TraA | Transfer position |
| **orf00199_1** | 131614 | 131432 | 183 | TraY | Transfer position |
| **orf00200_1** | 132439 | 131753 | 687 | TraJ | Transfer position |
| **orf00201_1** | 133013 | 132630 | 384 | TraM | Transfer position |
| **orf00216_1** | 140936 | 140409 | 528 | SSB | DNA-binding protein |
| **orf00240_1** | 155279 | 155139 | 141 | IS1d | IS |
| **orf00241_1** | 155270 | 155974 | 705 | IS431mec | IS |
| **orf00243_1** | 157179 | 156424 | 756 | Rmtb | Aminoglycoside 16S rRNA methylase |
| **orf00244_1** | 158209 | 157349 | 861 | TEM-1 | None-ESBL |
| **orf00245_1** | 158964 | 158392 | 573 | Tn3 | Tn |
| **orf00248_1** | 160590 | 159886 | 705 | IS431mec | IS |
| **orf00249_1** | 161621 | 161046 | 576 | IS1294 | IS |
| **orf00250_1** | 161701 | 162405 | 705 | IS431mec | IS |




**Picture 2: Positions of drug resistant elements on pSWU01**




**Picture 3: Gene Map of pSWU01, LJ04 and pkP1034. All the dark areas reveals the high similarity of the overlapping zones. All green arrows represent drug resistant gene. All the IS, Tn and Integron as well as Transfer position of pSWU01 were marked as blue arrow. All the IS, Tn and Integron as well as Transfer position of LJ04 were represented as red arrow. There is no special color for the genes of pkP1034 except the drug resistant genes in green. Comparing Gene Map of pSWU01with LJ04 and pkP1034 (All the three plasmid expressing SHV-12 and KPC-2 are identical, the 116093-125882 of pkP1034, 76602-86331 of LJ04, and 12670-22399 of pSWU01. All the three plasmid expressing CTX-M-65 are identical, 72833-73683 of pkP1034, 26781-27655 of LJ04, and 2607-3467 of pSWU01. For 82728-84513 of pkP1034 and 156424-158209 of pSWU01, both of them express RmtB and TEM-1, they are overlapped but in different positions.**

Therefore, pSWU01 is a kind of conjugative plasmid (F plasmid). The drug-resistant genes on pSWU01 are β-lactam resistant gene (TEM-1, CTX-M-65, SHV-12), aminoglycoside resistant gene rmtB, and Carbapenemase resistant gene KPC-2. The drug-resistance encoding gene hosting on pSWU01 contributes genetically to multidrug resistance of its hosting strain. In addition, as the Table 1 and Picture 1 show, pSWU01 harbors many tranfer-encoding gene for conjugate plasmid, Tn and IS, which indicates that it is both conjugative plasmid and drug resistance plasmid. Therefore, pSWU01 should be classified into chimeric plasmid or mosaic cointegrate.

BLAST search against NCBI Genebank revealed similarity among pSWU01, drug-resistant plasmids LJ04 and plasmid pkP1034. There are overlapping of drug-resistant gene SHV-12, KPC-2 and CTX-M-65. All the three plasmid expressing SHV-12 and KPC-2 are identical, the 116093-125882 of pkP1034, 76602-86331 of LJ04, and 12670-22399 of pSWU01. All the three plasmid expressing CTX-M-65 are identical, 72833-73683 of pkP1034, 26781-27655 of LJ04, and 2607-3467 of pSWU01. For 82728-84513 of pkP1034 and 156424-158209 of pSWU01, both of them express RmtB and TEM-1,they are overlapped but in different positions.In addition, all the three plasmids express huge amount of Tn, IS and Transfer position, enabling fast spread of drug-resistant gene among bacteria communities.

This research is the first finding of pSWU01 in size of 162552 bp (see in plasmid sequence molecular evolution analysis on NCBI). There are abundant drug-resistant elements on pSWU01, including plasmid transfer encoding genes, drug-resistant gene, Tn and Is, indicating its complicated genetic background. With the continuous evolution of drug-resistant strain in clinical environment, there certainly would be more and more drug-resistant elements, surely there would be more and more multidrug resistant strains. This requires our retrospect on the situation of antibiotic-overuse, and it also raises the standards for research on drug-resistant gene.

## References

1. Kong H.S. et al, Mohnarin report of 2011: monitoring of bacterial resistance in east China, *Chinese Journal of Nosocomiology*, **22(22)**, 4971-4976 **(2012)**

2. Da Silva G.J. and Mendonca N., Association between antimicrobial resistance and virulence in Escherichia coli, *Virulence*, **3(1)**, 18-28 **(2012)**

3. Clinical and Laboratory Standards Institute, Performance standards for antimicrobial susceptibility testing, Twenty-Second informational supplement, M100-S22, Wayne, PA, CLSI **(2013)**

4. Gu D.X., Virulence factors and antimicrobial susceptibility characteristics of Klebsiella pneumoniae in intestinal tract from various populations, Zhejiang University, 6-25 **(2015)**

5. Cheng L. et al, Investigation on the genotyping, virulence genes and serotype characteristics of Klebsiella pneumoniae producing KPC-2, *Chinese Journal of Clinical Laboratory Science*, **33(8)**, 591-595 (**2015)**

6. Pereira P.S. et al, Update of the molecular epidemiology of KPC-2-producing Klebsiellar pneumonia in Brazil, spread of

clonal complex 11 (ST11, ST437 and ST340), *J Antimicrob Chemother*, **68(2)**, 312 -316 **(2013)**

7. Hirsch E.B. and Tam V.H., Detection and treatment options for Klebsiellar pneumonia carbapenemases (KPCs): an emerging cause of multidrug-resistant infection, *J Antimicrob Chemother*, **65(6)**, 1119 -1125 **(2010)**

8. Zhu J.M. et al, Novel KPC variant from a carbapenem-resistant strain of K. pneumonia in a Chinese hospital, *Infect Control Hosp Epidemiol*, **32(10)**, 1050 -1052 **(2011)**

9. Zhu Jian-Ming et al, Resistant Elements in plasmid of extensive drug resistant Klebsiellar pneumonia, *Disease Surveillance*, **30(2)**, 134-139 **(2015)**

10. Villard-Pomares T. et al, Complete nucleotide sequence of plasmid pTN48, encoding the CTX-M-14 Extended-Spectrum β-Lactamase from an Escherrichia coli O102-ST405 strain, *Antimicrob Agents Chemother*, **55(3)**, 1270 -1273 **(2011)**

11. Fricke W.F. et al, Insights into the environmental resistance gene pool from the genome sequence of the multidrug-resistant environmental isolate Escherrichia coli SMS-3 -5, *J Bacteriol*, **190(20)**, 6779 -6794 **(2008)**

12. Froehlich B. et al, The pCoo plasmid of enterotoxigenic Escherrichia coli is a mosaic cointegrate, *J Bacteriol*, **187(18)**, 6509 -6516 **(2005)**

13. Heuer H. et al, The complete sequences of plasmids pB2 and pB3 provide evidence for a recent ancestor of the IncP-1βgroup without any accessory genes, *Microbiology*, **150**, 3591-3599 **(2004)**