MULTILOCUS SEQUENCE TYPING OF *KLEBSIELLA PNEUMONIAE* PRODUCING EXTENDED SPECTRUM B-LACTAMASES ISOLATED FROM KURDISTAN REGION, IRAQ.

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Abstract:

This study was purposed to sequence analysis of ESBLs genotype of *K pneumonia*e using partial sequence and Multilocus sequence typing (MLST). A total of 275 *K. pneumoniae* isolatesinvolved three general hospitals in Duhok, Erbil, and Sulymania, from September 2010 to June 2011. The Minimum Inhibitory Concentration (MIC) was measured by Phoenix system that confirmed 187 ESBL producing isolates followed by the Double Disk Synergy Test (DDST). Then, 12 isolates were selectedaccording to sample diversity, high resistancy to β-lactam and cephalosporins and harboring combination of three genes (TEM, SHV, and CTX-M). Partial sequence analysis of TEM; showed two different genotypes regarding *bla*TEM as 9 isolates (75%) from different samples (wound, sputum and blood) from three provinces harbor TEM-1 gene and 3 isolates (25%) only from urine in three provinces harbor TEM-198 gene. SHV analysis revealed characterization of selected isolates into six different genotypes. The common genotype was *bla*SHV-11 involved five isolates from sputum and blood in Erbil and Sulymania provinces, and wound in Duhok province. Only one genotype as all 12 isolates (100%) from different samples and different provinces was found harbored CTX-M-15 gene. Multilocus sequence typing (MLST) study performed using seven housekeeping genes (gapA, infB, mdh, pgi, phoE, rpoB and tonB). A total of 8 different sequence types (STs) were identified;ST11 was dominant sequence type, accounting 41.6 %(5 isolates) and was harboring combination of TEM-1, SHV-11 and CTX-15 genes.

Key words: *Klebsiella pneumoniae*, ESBL,MLST.

Introduction

Klebsiella pneumoniae is an opportunistic pathogen responsible for a high proportion of nosocomial infections and are dramatically increasing resistant to multiple antimicrobial agents owing to the production of extended-spectrum beta-lactamases (ESBL) which confer high resistance to third and fourth generation cephalosporins (Brisse*et al.,* 2000;Paterson *et al.,* 2000; Paterson *et al*., 2004). Hospital outbreaks of *K. pneumoniae* isolates are frequent, and the inter-hospital dissemination of resistant strains has been described previously (Arlet*et al.,* 1994; Monnet *et al.,* 1997).

Multilocus sequence typing (MLST) is a nucleotide sequence-based method that is adequate for characterizing the genetic relationships among bacterial isolates (Maiden *et al.,* 1998; Enright and Spratt 1999;Feil*et al*., 2004). It provides unambiguous and portable data that allow the implementation of multiuser international databases (Jolley and Maiden 2004). For our best knowledge,Multilocus sequence typing has never been validated for the epidemiology of *K. pneumoniae*strains in our area using the diversity of some housekeeping genes (Paterson *et al.,* 2004). For this reason, this study was designated to develop an MLST scheme for clinical isolates of *K. pneumoniae* andits evaluation for molecular characterization of nosocomial dissemination.

Materials and Methods

Bacterial Isolation

Two hundred and seventy five (275) clinical isolates of *K. pneumoniae* werecollectedfrom various clinical specimens (urine, blood, sputum, and wound) from September 2010 to June 2011 included three general hospitals (Duhok, Erbil and Sulymania) at Kurdistan region/Iraq. All these isolates were characterized and identified using different conventional bacteriological and biochemical standard methods.

Antimicrobial Susceptibility and ESBLs Test Using the Phoenix System

The antimicrobial susceptibility and the ability to produce ESBLs of all 275 isolates were tested by the Phoenix system (Beckton Dickinson Diagnostic Systems, USA) using Gram-negative susceptibility card (GNS).

Double Disk Synergy Test (DDST)

All ESBL producing isolates were tested by Double Disk Synergy Test (DDST) with disks containing cefotaxime (30 µg), ceftazidime (30 µg), and ceftriaxone (30 µg) whichwere placed 25 mm (centre to centre) from an Amoxicillin-clavulanic acid disk (30 and 10 µg, respectively) and incubated at 35°C overnight (Kaur *et al.,* 2013).

Multilocus sequence typing

Sevenhousekeeping genes were selected to establish the MLST. The primers used for amplification of the gene fragments are shown in table (1). PCR amplifications were carried out under the following conditions: Denaturation step at 95°C for 4 min, followed by 30 cycles of the following conditions: denaturation at 95°C for 1 min, annealing at 60°C for each primer set for 1 min, and extension at 72°C for 1 min. then the final extension step at 72°C for 5 mints. The PCR products were purified using a PCR purification kit (Qiagen-Germany), according to the manufacturer's recommendations, and then the products were sequenced on an ABI3130 DNA sequencer (Applied Biosystems, Singapore). MLST was performed as described by Diancourt*et al*. (2005) (http://www.pasteur.fr/recherche/genopole/PF8/ mlst/). Briefly stated, PCR fragments of the seven housekeeping genes rpoB, gapA,mdh, pgi, phoE, infB and tonB were obtained from chromosomal DNA and directly sequenced. Allelic profiles and sequence types (STs) were designatedatthewebsite (http://www.pasteur.fr/recherche/genopole/PF8/ mlst/Kpneumoniae.html).

Table.1 primers used for the amplification of the house keeping genes.

Locus	Putative function of gene	Primer sequence	Size (bp)	Temp (°C)
*rpo*B	Beta-subunit of RNA polymerase B	F: GGC GAA ATG GCW GAG AAC CA R: GAG TCT TCG AAG TTG TAA CC	501	50
*gap*A	Glyceraldehyde 3-Phosphatedehydrogenase	F: TGA AAT ATG ACT CCA CTC ACG G R: CTT CAG AAG CGG CTT TGA TGG CTT	450	60
mdh	Malatedehydrogenase	F: CCC AAC TCG CTT CAG GTT CAG R: CCG TTT TTC CCC AGC AGC AG	447	50
pgi	Phosphoglucoseisomerase	F: GAG AAA AAC CTG CCT GTA CTG CTG GC R: CGC GCC ACG CTT TAT AGC GGT TAA T F(seq): CTG CTG GCG CTG ATC GGC AT R(seq): TTA TAG CGG TTA ATC AGG CCG T	432	50
*pho*E	Phosphoporine E	F: ACC TAC CGC AAC ACC GAC TTC TTC GG R: TGA TCA GAA CTG GTA GGT GAT	420	50
*inf*B	Translation initiationfactor 2	F: CTC GCT GCT GGA CTA TAT TCG R: CGC TTT CAG CTC AAG AAC TTC F(seq): ACT AAG GTT GCC TCC GGC GAA GC	318	50
*ton*B	Periplasmic energy transducer	F: CTT TAT ACC TCG GTA CAT CAG GTT R: ATT CGC CGG CTG RGC RGA GAG	414	45

Detection of bla genes sequencing of ESBLs genes

The *bla*genes related to ESBL enzymes were assayed by PCR with the corresponding primers for the TEM, SHV, and CTX-M ESBL types that shown in table (2), as described previously by Yun-Tae *et al.*(2006).To specify the subtype of the *bla*genes, the amplified PCR products were sequenced on both strands, the amino acid sequences were deduced from the nucleotide sequences using the Mega and BioEdit programs and they were compared with the database of the website (http: //www.***blast***.ncbi.nlm.nih.gov).

Table.2 PCR amplification and sequencing of ESBLs genes

ESBLs primer	Primer sequence	Size (bp)	PCR cycles
TEM	F: CGCCTGTGATTATCTCCCT R: ATCGTTGTCAGAAGTAAGTTGG F(Seq): CGCCTGTGATTATCTCCCT R(Seq): ATCGTTGTCAGAAGTAAGTTGG	1080	1cycle of 5 min at 94°C:30 cycles of (30 sec at 94°C, 1 min 30 sec at 45°C, 1 min at 72°C); cycle of 10 min at 72°C
SHV	F: CGCCTGTGATTATCTCCCT R: CGAGTAGTCCACCAGATCC F(Seq): CGCCTGTGATTATCTCCCT R(Seq): CGAGTAGTCCACCAGATCC	861	1cycle of 5 min at 94°C:30 cycles of (30 sec at 94°C, 30 sec at 48°C, 1 min at 72°C); cycle of 10 min at 72°C
CTX-M	F: CGCTGTTGTTAGGAAGTGTG R: GGCTGGGTGAAGTAAGTGAC F(Seq): CGCTGTTGTTAGGAAGTGTG R(Seq): GGCTGGGTGAAGTAAGTGAC	551	1cycle of 5 min at 94°C:30 cycles of (30 sec at 94°C, 1 min at 58°C, 1 min at 72°C); cycle of 10 min at 72°C

Resulst and Discussion

Partial sequence analysis of TEM; showed two different genotypes regarding *bla*TEM as 9 isolates (75%) from different samples (wound, sputum and blood) from the three provinces harbor TEM-1 gene and 3 isolates (25%) only from urine samples of the three provinces harbor TEM-198 gene. Results obtained from SHV analysis revealed the characterization of the selected isolates into six different genotypes. The most abundant genotype was *bla*SHV-11 involved five isolates from sputum and blood in Erbil and Sulymania provinces, and wound in Duhok province. Only one genotype as all 12 isolates (100%) from different samples and different provinces was found harbored CTX-M-15 gene.

MLST using seven housekeeping genes (gapA, infB, mdh, pgi, phoE, rpoB and tonB) was performed on 12 ESBLs producing *K. pneumoniae* selected isolates obtained from different samples and different provinces according to the protocol described for *K.pneumoniae*MLST website: (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html)

Results from table (3) revealed that eight different sequence types (STs) were identified in this study.

ST11 was the dominant sequence type accounting for 41.6 % (5 isolates). All 5 isolates having ST11 (allelic profile 3-3-1-1-1-1-4) were obtained from sputum and blood (Erbil and Sulymania) and wound (Duhok) table (3). These isolates were also found to have the same genotype regarding ESBLs (TEM-1, SHV-11, and CTX-M-15), While the other 7 isolates belonged to 7 different sequence types including; ST14, ST15, ST37, ST48, ST268, ST413, and ST514.

In South Korea ST11 was also found to be the most dominant type in ESBLs producing *K. pneumoniae* isolates obtained from urine and bacteremia (Ko*et al*., 2010). In China ST11 was also reported as the dominant clone of ESBL-producing *K. pneumoniae* (Qi *et al.,* 2011). ST11 has been also described in different European countries such as Hungary, the Netherlands, France and Spain (Damjanova*et al.,* 2008; Oteo*et al.,* 2009; Toth*et al*., 2010).

All eight STs obtained from the 12 different isolates regarding ESBLs genotype were found to be 100% CTX-M-15 and the isolates obtained from wound, sputum and blood were 100% TEM-1

ESBLs producing *K. pneumoniae* isolates obtained from urine samples were found to have TEM-198 (table.3).

These results probably could represent regional dissemination of selected isolates of *K. pneumoniae* among the three provinces. Similar findings were recorded in the Midwestern USA (Kitchel*et al*., 2009).

Table.3 MLST of 12 selected ESBL producing *K. pneumoniae* isolates obtained from different sources and different provinces in Kurdistan region/Iraq

No. of Isolates	Source	Province	TEM	SHV	CTX-M	Allelic Profile	ST
Isolate Kp1	Urine	Duhok	TEM-198	SHV-125	CTX-M-15	2-9-2-1-13-1-16	37
Isolate Kp2	Wound		TEM-1	SHV-11	CTX-M-15	3-3-1-1-1-1-4	11
Isolate Kp3	Sputum		TEM-1	SHV-148	CTX-M-15	2-1-2-1-7-1-81	268
Isolate Kp4	Blood		TEM-1	SHV-12	CTX-M-15	2-5-2-2-7-1-10	48
Isolate Kp5	Urine	Erbil	TEM-198	SHV-28	CTX-M-15	1-1-1-1-1-1-1	15
Isolate Kp6	Wound		TEM-1	SHV-63	CTX-M-15	2-1-1-1-8-1-9	514
Isolate Kp7	Sputum		TEM-1	SHV-11	CTX-M-15	3-3-1-1-1-1-4	11
Isolate Kp8	Blood		TEM-1	SHV-11	CTX-M-15	3-3-1-1-1-1-4	11
Isolate Kp9	Urine	Sulymania	TEM-198	SHV-148	CTX-M-15	2-6-1-3-8-1-113	413
Isolate Kp10	Wound		TEM-1	SHV-28	CTX-M-15	1-6-1-1-1-1-1	14
Isolate Kp11	Sputum		TEM-1	SHV-11	CTX-M-15	3-3-1-1-1-1-4	11
Isolate Kp12	Blood		TEM-1	SHV-11	CTX-M-15	3-3-1-1-1-1-4	11

From this study it could be speculated that ST11 to be a good colonizer to capture plasmids as these isolates are easily transmitted between patients (Qi *et al.,* 2011). Results from table (3) shows that ST15 is a single locus variant (infB) of ST14. ST15 was detected in China in just one hospital in Hangzhou and spreads regionally (Qi*et al*., 2011).

The dissemination in both Europe and Asia may indicate that ST11 is one of the pandemic clones although there are limited epidemiological data on the worldwide distribution of *K. pneumoniae (*ko*et al.,* 2010*)*.

In conclusion, these isolates are worrying due to their rapid transmission by plasmid or mobilization of genetic elements between hospitalized patients causing sporadic spreading and worse effects, these bacteria may not be detected in routine antibiotic susceptibility testing. We should therefore pay careful attention should be paid to this problem from the public health point of view through monitoring closely and strict infection control measures should be adopted to control nosocomial infections.

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پوختە

ئارمانجا ڤێ لێکولینێ ئەوە بۆ شلوقەکرنا زنجیرا جینوتایب یا ئەنزیمی بتالاکتیمیز و بریکا بکارئینانا ملتی لوکس(MLST). بۆ ڤێ لێکولینێ هژمارا ٢٥٧نەخوشێن جوداکری یێن (*K. pneumoniae*) هاتبوونە کومکرن ژ سێ نەخوشخانێن گشتی یێن دهوك و هەولێر و سلێمانیێ هەر ژ هەیڤا ٩\٢٠١٠ تا هەیڤا ٦\٢٠١١ پەیتێ کێم یێ (MIC) ژڤان جوداکەران هاتبو پیڤان بکارئینانا سیستەمێ فینکس. بتنێ ١٨٧ ژ جوداکەرێن (*K. pneumoniae*) هاتنە هەست پێکرن ژلایێ ڤی سیستەمی. هەروەسا ب ریژا ١٠٠٪ ژڤان جوداکەران هاتنە هەست پێکرن ژ لایێ دبل دسك. شلوقەکرنا زنجیرا سکوینس یا TEM دیارکر دوو جیاوازی یێن جینوتایب *bla*TEM ژ (٩) جوداکەرا (٧٥٪) ژناڤ نموونەیێن جورەجور یێن (برین بلغم و خزین) ژناڤ سێ بەندەرێن جینا TEM-1 و (٣) جوداکەر (٢٥٪٩ بتنێ ژ نموونەیێن میزێ یێن سێ بەندەرێن جینا TEM-198 بوون. ئەو ئەنجامێن دەرکەتین ژ شلوقەکرنا SHV ئاشکراکر سالوخەتێن جوداکەرێن هەلبژارتی بو شەش جینوتایب جورەوجور. جینوتایب یێن ژ خرەکا مشەتر ئەو بوون یێن -*bla*SHV 11 وبخوڤە دگرت پێنج جوداکەر ژ جووریێ (خوین و بلغم) ل باژێریێ هەولێر و سلێمانیێ وپێنج ژ جوریێ (برین) ل باژێریێ دهوکێ. بتنێ ئێك جینوتایب ژ کوما ١٢ جوداکەر بریژا ١٠٠٪ ژنموونە و باژارێن جورەوجور هاتە دیتن جوریێ هەلگریێ ژ جینا CTX-M-15. توێژینا ملتی لوکس هاتەکرن بریکا بکارئینانا حەفت خانیێن پاراستنا جینان (gapA, infB, mdh, pgi, phoE, rpoB and tonB) لسەر ١٢ جوداکەرێن ژێگرتی ژ (*K. pneumoniae*) ژ تێکرایێ ٨ (STs) یێن جوریێ زال یێ ئێك لدویڤ ئێك، لدویڤ ٤١.٦ ٪ (٥ جوداکەر) و بەندەرێن پێکڤە یێن جینێن (CTX-15 , SHV-11 , TEM-1) هەروەسا ئەو ٧ جوداکەر نیشانا ٧ جورێن ئیك ل دویڤ ئێك یێن جودانە.

الخلاصة

تهدف هذه الدراسة الى تحليل التتابعات الجزيئية للانماط الجينية للانزيم بتا لاكتميز في بكتريا*K pneumoniae*مستخدمةتنميط التتابعاتالمتعددة والجزئيةللموقع الجيني(MLST).جمعت ٢٧٨ عزلة سريرية شملت الادرار، القشع، مسحات الجروح وزروع الدم من ثلاث مستشفيات عامة في محافظات اقليم كوردستان: دهوك و أربيل والسليمانية من شهر ايلول ٢٠١٠حتى شهر حزيران ٢٠١١.تم قياس التركيز المثبط الادنىMICبأستخدام جهاز Phoenixللتاكد من انتاجها لانزيماتالبيتالاكتيميز الواسعة الطيف(ESBLs)ثمتلاهااختبارالقرص الثنائي المتساند(DDST).تم اختيار ١٢ عزلة من بكتريا *K pneumoniae* المنتجة لانزيمات البيتا لاكتميز الواسعة الطيف طبقا الى التباين في مصادر العينات ومقاومتها العالية تجاه مضادات البيتا لاكتموالسيفالوسبورينات وامتلاكها للمؤشرات الجينية .(TEM, SHV, and CTX-M) اشارت النتائج الى ان الجين *bla*TEM تواجد بنمطين مختلفين حيث كانت ٩(٧٥٪) من عزلات الجروح، القشع والبلغم ومن المحافظات الثلاثة وتمتلك النوع TEM-1و٣ (٢٥٪) عزلات من عينات الادرار ومن المحافظات الثلاثة وتمتلك الجين TEM-198.اما الجين *bla*SHV فقط اظهر ست انماط جينية مختلفة وكان النط الجيني SHV-11 هو الاكثر تواجدا حيث كانت ٥ عزلات (٤١.٦٪) من عينات القشع والدم من محافظتي أربيل والسليمانية وعينة واحدة فقط من الجروح من محافظة دهوك. اما النمط الجيني *bla*CTX-M فقد اعطى نمطا واحدا في العزلات المختلفة وهو النمط.gene CTX-M-.15.تمت دراسة تنميط تتابعات متعدد الموقع الجيني بأستعمال سبع جينات (Housekeeping genes) (*gapA, infB, mdh, pgi, phoE, rpoB, tonB*) واظهرت النتائج ثمان انماط تتابعيه متاينة ,وكان النمط التتابعي الاكثر سيادة هو ST-11 في ٥ عزلات (٤١.٦٪) والتي تمتلك النمط الجيني TEM-1, SHV-11 and CTX-15