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Original article

Epidemiology and Molecular typing of Klebsiella pneumonia with the Extended Spectrum of B lactamase specific genes causing nosocomial infection in Gaza strip

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ABSTRACT

Background &Objective: Klebsiella pneumonia causes different serious nosocomial infections for human and several strains became multiple drug resistance .This study was conducted to describe the epidemiology and molecular typing of Klebsiella pneumonia with the extended spectrum of B lactamase enzyme in Gaza strip .Methods :A cross-sectional survey was conducted during the period of December 2008 to November2009. One hundred and fifty clinical specimens were collected from patients admitted in different wards . Results : Sixty six percentage of the isolates were K.pneumonia .These were isolated from different infected sites : urine 24% , sputum 14%, wound 11% , stool11% , blood14% , cerebrospinal fluid 11% , skin16% . The ESBLs was detected in 67% of the strains ,53% strains were resistant for more than eight antibiotics , PCR demonstrated different patterns for the presence of SHV(80%) , TEM(60%) enzyme and CTX-M(20%), PFGE Showed 10 clusters of genetically unrelated strains with high prevalence of polyclonal strains of Klebsiella pneumonia. Antibiotic resistance was found against Cephalothin(95.0%),Cefotaxime(82.0%),Ceftazidime(59.0%),Ceftriax one(86.0%),Gentamicin(56.0%),Trimethoprim/sulphamethoxazole(47.0%)..Chloramphenicol(42%),Amikacin(33%),Aztreonam (32%) and Imipenem(0%). Interpretation, Conclusion : our findings showed that genetically-related isolates of K. pneumoniae producing SHV and TEM and CTX-M were present in Gaza Strip. Larger studies need to be done to better define the molecular epidemiology of ESBL producing K. pneumoniae and its clinical implications

Introduction

Klebsiella pneumoniae is a successful opportunistic pathogen associated with various ailments such as urinary tract infections, septicemia, respiratory tract infections and diarrhoea1 .K.pneumoniae is a part of commensally gastrointestinal flora in humans 1 .Antimicrobial resistance among the family of Enterobacteriaceae, especially in Klebsiella spp. represents a major problem in nosocomial infections, including urinary tract infections, respiratory tract infections and bacteremia, particularly in elderly or debilitated patients2 .These organisms elaborate plasmid encoded beta-lactamases, which can hydrolyze the amide bond in the beta-lactam ring of antibiotics. Although a variety of beta-lactamases have been described, the TEM and SHV enzymes are those most frequently observed among members of the family Enterobacteriaceae 3. Mutations in the genes encoding the TEM and SHV beta-lactamases can extend the spectrum of enzyme activity to include penicillin's, the extended-spectrum cephalosporin's (ESCs) (e.g., ceftazidime, cefotaxime and ceftriaxone) and aztreonam. Such enzymes are called extended-spectrum betalactamases (ESBLs). ESBLs are predominantly derivatives of TEM and SHV enzymes 3, however, some oxacillin hydrolyzing (OXA)4 , CTX-M5and AmpC-type beta-lactamases 3-6 also show activity against these antimicrobial agents. ESBLs are especially dangerous because they are plasmid-associated, and the plasmids may be exchanged among a variety of bacterial species. ESBL producing enterobacteriaceae. Resistance mediated by ESBL is of great concern for many reasons. First, ESBLs can hydrolyze broad-spectrum cephalosporin's and monobactams Second, these enzymes are encoded in plasmids that confer resistance to multiple antibiotics. Third, this type of resistance is not easily detected by routine antimicrobial susceptibility testing7. Fourth, severe infections caused by ESBL-Kp may be associated with a poor clinical outcome 8,9 Little is known about the antimicrobial resistance mechanisms of K. pneumoniae in Gaza strip. K. pneumoniae strains are found worldwide associated with pneumonia and urinary tract infections in nosocomial and community environments 9-11 .Here we report a phenotypic and genotypic analysis of clinical isolates of K. pneumoniae of nosocomial origin recovered in various hospitals in Gaza strip.

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Materials and Methods

1-Samples collection

A retrospective study was conducted in three governmental hospitals Al Shifa , Nasser and European Gaza Hospitals to assess the occurrence of nosocomial infections due to ESBL-Kp. All patients with positive cultures for K. pneumoniae during the period November 2007 and December 2008. were included. Only one clinical isolate per patient was included. Patients whose medical records unavailable were excluded.

One hundred fifty different clinical specimens (24%urine ,14%sputum , 11% wound, 11%stool 14%blood ,11% CSF and 16skin) were obtained from patients admitted to different wards of the participated hospitals All the hospitals had capacity of 320 -180 beds and provide inpatient services and an outpatient services.

2-Microbiological analysis

The clinical specimens were inoculated directly on MacConkey and Cystine-Lactose-Electrolyte Deficient (CLED) agar (Oxoid Ltd., Basingstoke, Hampshire, England). Cultures were incubated at 37 ◦C for 24 h. The bacteria were identified by their characteristics appearance on their respective media and by the pattern of biochemical reactions using the standard procedures. After overnight incubation suspected colonies large pink colonies , these strains were identified directly by API system (Biomereux , France)

3-Antibiotic susceptibility analysis

Antibiotic susceptibility profiles of the strains were determined by the disc diffusion methods , plates of Mueller Hinton agar (Sanofi diagnostic Pasteur France) were inoculated with a bacterial suspension equivalent to a 0.5 McFarland standard and incubated aerobically at 37Co for 18hr .The results were expressed as susceptible or resistant according to the criteria adopted by CLSI(Clinical Laboratory Standard 2005) , the antibiotic tested were Ampicillin , Cefotaxime , Ceftriaxon , Ceftazidime ,Gentamicin , Amikacin , Imipenem , Aztereoname ,Chloramphenicol ,Ciprofloxacin ,Nalidixic acid , Tetracycline, Trimthoprime Sulphamethoxazole

4-Molecular biology analysis

All the isolated Klebsiella strains were kept frozen at −20 ◦C in nutrient agar (Oxoid) until ESBL detection were done. Reference K. pneumonia strain (ATCC 700603) was cultured for quality control throughout the study.

ESBL production was detected by double disk diffusion synergy test , disks of Cefotaxime (30ug) and Ceftazidime(30ug) were placed 20 and 30mm (center to center) from a disk with Amoxicillin –Clavulenic acid(20 and 30ug respectively , K. pneumonia strains producing the known enzyme TEM ,SHV and CTX –M-were used for quality control ESBL detection

PCR detection and sequencing of ESBLs: Genomic DNA was prepared by boiling the isolates and used as the templates for PCR reactions. Amplification of TEM ,SHV and CTX-M ESBL genes was performed on Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) with primers and cycling conditions given in the following pattern:

The amplification primers for SHV genes [blaSHV SHV-F-S1 5'-ATTTGTCGCTTCTTTACTCGC-3', SHV-R-S2 5'-TTTATGGCGTTACCTTTGACC-3'Annealing temperature C060 yielding a Product size 1051]used for PCR have been described previously (9).as have those for TEM [bp)blaTEM TEM-F 5'-ATGAGTATTCAACATTTCCGTG-3', TEM-R 5'-TTACCAATGCTTAATCAGTGAG-3' Annealing temperature C055 yielding a Product size (bp) 861] while for those CTX-M [blaCTX-M 5'-TTTGCGATGTGCAGTACCAGTAA-3', 5'-CGATATCGTTGGTGGTGCCATA-3' Annealing temperature C051 yielding a Product size (bp) 544].

The PCR product were separated on 1 per cent agarose gels and visualized under UV light. The PCR products were purified with a QIAquick PCR purification kit (Qiagen,Valencia, CA, USA) and were sequenced on an ABI Prism 3100 automated sequencer (Applied Biosystems). Genomic fingerprinting by pulsed field gel electrophoresis (PFGE): Agarose plugs containing chromosomal DNA were prepared as described by Yuan et al11. The chromosomal DNA was digested overnight with 30 U of Xbal (New England Biolabs, Ipswich, MA, USA). PFGE was run in a CHEF-DRIII apparatus (Bio-Rad, Hercules, CA, USA) at 6V/cm for 22 h. The pulse times were 5-40s. The banding patterns were interpreted according to the criteria of Tenover 12 13.

A standard reference of E.coli [ATCC 25922] sensitive to all antimicrobial drugs being tested was used as a control in this study .

Results

Clinical specimen isolates

Out of 150 clinical specimens ,66(44%) of clinical specimens were belonging to K. pneumonia The other species isolated were belonged to K .oxytoca ,E.coli ,Citrobactert freundii and Serratia fonticola.

Fourteen percentage of specimens were isolated from sputum, 24% from urine ,11% from wound ,11% from stool ,11% from CSF ,14% from blood and 15% from skin .

Antibiotic resistance pattern .

The resistance pattern of 66 Klebsiella pneumonia isolated from different hospitals against Eleven chosen antimicrobial agents is presented in Table 2. Resistance pattern was found amikacin 33% cefotaxime 82%, aztreonam 69%, ceftazidime59%,ceftriaxone86%,cephalothin95%,chloramphenicol56%,ciprofloxacin42%,gentamicin 56%, trimethoprim–sulphamethoxazole 47% and Imipenem (0%) .A total of 53%of the tested strains were resistant to eight antibiotics ,all of the strains were susceptible to Imipenem .

Beta lactamase enzyme

A total of 92(82%) strains were positive for ESBL of these 78 (69.6%) were detected by both double disc synergy test and Vitek ESBL test .Also 9(8%) strains only by the antimicrobial discs Ceftazidime and Ceftazidime/ Clavulanic acid of Vitek ESBL test ,while the remaining 5)4.5%) strains were detected only by the antimicrobial discs Cefotaxime , Cefotaxime/ Clavulenic acid of Vitek ESBL test ESBL producers showed enhanced inhibition to extended-spectrum B-lactams, ceftazidime 6/10 (60%) and cefotaxime 7/10 (70%) by the double disk diffusion method and demonstrated a 5-mm or greater increase in the zone

diameter of ceftazidime/ cefotaxime plus inhibitor (calvulanate) with respect to ceftazidime/cefotaxime alone. All ESBL producers were K. pneumoniae. Of the 10 ESBL producing K. pneumoniae, tested 9/66 (14%) were from sputum, 16(24%) from urine and 7(11%) from pus culture. Resistance to the antimicrobial agents tested is shown to be much higher in the ESBL producing Klebsiella spp. (84–95%) than non-ESBL producing isolates (13–58%) (data not shown) . ESBL producing Klebsiella spp. was detected in a total of 67% . PCR demonstrate different patterns for presence of SHV(80%) , TEM(60%) enzyme and CTX-M(20%) , PFGE demonstrated 10 clusters of genetically unrelated strains of the isolates. SHV and TEM genes were detected in 40% of the K. pneumoniae isolates; SHV ,TEM and CTX-M 10% SHV and CTX-M20 ,PFGE analysis was used to establish the genetic relatedness of 66 of the K. pneumoniae isolates producing four different types of B-lactamases PFGE profile types were identified; 52% included only a single isolate (non-clustered) genetically unrelated to other isolates in the study. The remaining 48% profile types, defined clusters I to X, included two or more isolates that were genetically related (with >80% similarity) or indistinguishable .48% were depreesed mutants while 11% were plain ESBL producers and 28% were plain for the presence of plasmid mediated AmpC B-Lactamase (table4).

Molecular typing patter4ns

PCR demonstrated different banding patterns of Klebsiella strains for genes of SHV ,TEM and CTX-M Showed dissimilarities in dendograms in fig1 lane 1; 100 pb DNA ladder ,lane 2 blood culture isolates showing pattern II ,lane3 and4 urine culture isolates showing pattern III and IV ,lane 5 blood culture isolate pattern V ,lane 6 wound pattern VI , lane 7 pattern VII , lane 8 urine culture pattern VIII , lane9 wound isolate pattern IX and lane 10blood culture pattern X

Results Tables

Table I The distribution of *Klebsiella pneumonia* isolated strains from clinical specimens

Clinical site of specimens	Total no of isolates N=66	Percentage
Urine	16	24
Sputum	9	14
Wound	7	11
Stool	7	11
Blood	9	14
CSF	7	11
Skin	11	16

Table II The Genotype analysis for SHV and TEM enzymes and INT genes by PCR

Strain code number	SHV	TEM	CTX-M
K-10	Neg	Pos	Neg
K-19	Pos	Neg	Neg
K-44	Pos	Neg	Neg
K-15	Pos	Neg	Pos
K-1	Pos	Pos	Neg
K-6	Pos	Pos	Neg
k-56	Pos	Pos	Pos
k-3	Pos	Pos	Neg
K-47	Pos	Neg	Neg
K-66	Neg	Pos	Neg
Percentage	80%	60%	20%

Table III the Antibiotic susceptibility profiles of *K.pneumonia* cases

Antibiotic	Percentage of susceptibility	Percentage of Resistance
Aztreonam	68	32
Amikacin	67	33
Cephalothin	5	95
Trimthoprime/sulphamethoxazole	53	47
Cefotaxime	18	82
Ceftazidime	41	59
Ceftriaxone	14	86
Chloramphenicol	58	42
Ciprofloxacin	50	50
Gentamicin	44	56
Imipenem	100	0

Table IV. Characteristics of ESBL-producing isolates from stool samples of patients from Gaza Strip isolates

CodeN0.	CTX-M	TEM	SHV	CAZ	CTX	SXT	IMIP	AZ	CIP	C	AN	GN	CF	CRO
K-10	Neg	Pos	Neg	R	R	R	S	R	S	R	R	R	R	S
K-19	Neg	Neg	Pos	S	S	R	S	S	S	R	S	R	R	S
K-44	Neg	Neg	Pos	S	S	S	S	R	S	R	S	R	R	R
K-15	Pos	Neg	Pos	R	R	R	S	S	R	R	S	R	S	R
K-1	Neg	Pos	Pos	S	R	R	S	S	R	S	S	R	R	R
K-6	Neg	Pos	Pos	S	R	R	S	S	S	S	S	S	R	R
K-56	Pos	Pos	Pos	R	R	S	S	S	S	S	S	S	R	R
K-3	Neg	Pos	Pos	R	R	S	S	S	R	S	S	S	R	R
K-47	Neg	pos	Pos	R	R	S	S	R	R	S	R	S	R	R
K-66	Neg	Pos	Neg	R	R	S	S	S	R	S	R	S	R	R
Percentage	20	60	80											

Table V Screening for the Mechanism ESBL production

	Percentage of different mechanisms by B-Lactame Resistance		
	Plasmid mediates AMP –B Lactamase	ESBL Production	ESBL Producer depressed mutant
Number of Strains tested N=66	26(39%)	10(15%)	30(46%)

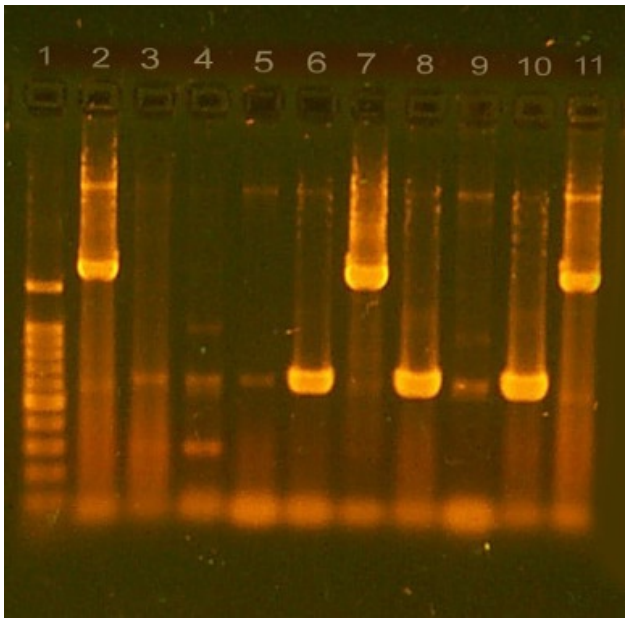

Figure (1): PCR patterns for TEM gene of Klebsiella spp. strains. Lane 1, 100 pb DNA ladder; lane 2, blood culture isolate (K-10) showing pattern II; lanes 3 and 4, urine culture isolates (K-19, K-44) showing pattern III and IV; lane 5, blood culture isolate (K-15) showing pattern V; lane 6, wound swab isolate (K-1) showing pattern VI; lane 7, sputum culture isolate (K-6) showing pattern VII; lane 8, urine culture isolate (K-56) showing pattern VIII; lane 9, pus culture isolate (K-3) showing pattern IV; lanes 9 and 10, blood culture isolates (K-47, K-66) showing pattern XIII and XI.

Discussion

This study was conducted because there was little available information regarding antimicrobial susceptibility and resistance mechanisms of K. pneumoniae isolates in our region and this pathogen remains one of the major ESBL-producing strains worldwide12 in addition, an increase in hospital costs is associated with ESBL producing K. pneumoniae13 .B-lactamases (ESBLs) are plasmid-encoded enzymes, which can arise from point mutations in the TEM, SHV and CTX-M-lactamase genes and can hydrolyze B- lactams, including third-generation cephalosporin's such as ceftriaxone, ceftazidime, cefotaxime and the monobactam and aztreonam 14,15.The phenotype and genotype of 66 clinical K. pneumoniae isolates responsible for hospital-acquired infections isolated between November 2007 and December 2008 in Palestinian hospitals ESBL production was detected in 67% of the isolates. Over the past decade, several studies have assessed the occurrence of ESBLs among Enterobacteriaceae recovered from hospitalized patients16.From 1997 to 1999, ESBL detection rates in Klebsiella spp. were 45.4% in Latin America, 24.6% in the Western Pacific region, 22.6% in Europe, 7.6% in the United States and 4.9% in Canada17, but other studies reported a rate of 3.5% in Cameroon[18], 44.0% in Singapore19, 60.5% in Turkey20 and 29–69% in Slovakia21. Our study provides insights into the problem of resistance in bacterial Gram-negative enteric pathogens in inpatients .To our knowledge this is the first study in Gaza strip determining antimicrobial susceptibility patterns of nosocomial infection related to k.. pneumoniae . Results have demonstrated that in general, K.pneumonia have high rates of resistance to the used antibiotics. ESBL producing Klebsiella spp. showed high level resistance (82–95%) to cephalosporins ,chloramphenicol(42%), gentamicin(56%) and trimethoprim–sulphamethoxazole(47%). It means that the use of these antibiotics for treatment of infection caused by ESBL producing strains may result in failure in a significant proportion of cases. The clinical relevance of ESBLs has been well documented by numerous published reports describing clinical failures with the use of third generation cephalosporins22 .Thus, the problem of ESBLs is clinically important, yet remains relatively unappreciated by most clinicians23. Only 50% of the isolates presented resistance to ciprofloxacin, but resistance to Aminoglycosides Gentamicin and Amikacin were56%and 33% respectively). K. pneumoniae isolates resistant to these antibiotics are less common (1%, And 12% respectively) elsewhere in Europe 24 .Many factors have contributed to such high rates of resistance, including misuse of antibiotics by health professionals and unskilled practitioners, misuse of antibiotics by the public (antibiotics can be purchased without prescription), poor drug quality, unhygienic conditions accounting for the spread of resistant bacteria, and inadequate antimicrobial surveillance program24.The fact that pathogen was isolated year-round in the study may be indirect evidence of colonization of the patients by this organism, forming a reservoir of infection. This is worsened by the finding that the genes encoding for ESBLs can be transferred between strains and other bacterial species, causing infection to the host when defenses are weak, or being nosocomially transmitted to other immunocompromised patients .Therefore, it is not surprising that patients who developed bacteremia had a prolonged duration of hospitalization compared to those who did not develop bacteremia. This concurs with previous studies in which colonization and infection by ESBL-producing Enterobacteriaceae have been associated with lengthy hospitalization, especially in intensive care or oncology units 25,26.Ceftazidime resistant K.pneumonia has been isolated .This work documents the substantial diversity of B-lactamases produced by K. pneumoniae and identified several B- lactamases are recorded among the 66 isolates producing SHV non-ESBL enzymes in the first period, blaSHV genes were identified. The importance of ESBL enzymes as a serious clinical problem for the treatment of patients with K. pneumoniae expressing these resistance determinants is of concern; however, the clinical importance of the non-ESBL enzymes is yet to be revealed 21,24.All the 66 isolates were typed by PFGE. The selected 10 strains from bacterial isolates of patients demonstrated different PFGE patterns. The patterns of the strains showed that four clusters of either two or three strains demonstrated more than 50% similarity in their PFGE patterns. However the strains with similar PFGE patterns did not have any relationship with each other .Therefore these strains not belonged to a single clone and not due to the spread of an epidemic strain of multidrug –resistant K. pneumonia .

Conclusion

This is one of the first reports from Gaza Strip in which the causative organism of an interhospital infection with multidrug-resistant K. pneumoniae associated with a high mortality rate has been characterized at the molecular level. Knowing the resistance mechanisms in this type of clinical isolate will allow the proposal of improved therapeutic measures and the rational use of antibiotics, which are indispensable tools in fighting infectious diseases produced by bacteria. More-prudent use of antibiotics and control of the spread of these resistant organisms are necessary.

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Conflict of interest :

None of the authors has a conflict of interest:

Authors' contributions :

All authors participated in the conception and design of the study, acquisition of data, analysis and interpretation of data, and drafting of the manuscript. Both authors read and approved the final manuscript.

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