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ORIGINAL ARTICLE

Quinolone Resistance among Extended-Spectrum β -Lactamases Producing *Klebsiella pneumoniae* in Sohag University Hospital, Upper Egypt

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Key words:

Klebsiellae pneumoniae; Quinolone; β –Lactamase

ABSTRACT

Background: *Extended-spectrum β-lactamases (ESBLs) production is the major resistance mechanism to β-lactam antibiotics in Enterobacteriaceae. Moreover, the emergence of quinolone resistance in ESBLs-producing isolates had become a global threat for treatment of infections caused by these bacteria.* ***Objectives:*** *We investigated the association between ESBLs production and quinolone resistance in Klebsiella pneumoniae isolates.* ***Methodology:*** *A total of 148 isolates of K. pneumoniae collected from Sohag University Hospital over the period of 10 months between June 2014 and April 2015. Identification of Klebsiella pneumoniae was performed with the Vitek 2 system (bioMerieux, France). Screening for extended spectrum beta lactamases (ESBLs) production was done with the Vitek 2 system (bioMérieux, France) and confirmed using the combined disk diffusion test (CDDT) .The ESBL's genotype was then analyzed by multiplex PCR of* bla_{TEM}, bla_{SHV} *and* bla_{CTX-M} *genes. Also multiplex PCR was used for detection of quinolone resistance determinant genes qnrA, qnrB and qnrS among ESBLs-producing K. pneumonia isolates.* ***Results****: In our study we found that among the 148 K. pneumoniae isolates; 121 (81.7%) were ESBLs producers according to CDDT results,* bla_{TEM} *was found in 44% of them, while* bla_{CTX-M} *found in 6%,* bla_{TEM} *and* bla_{CTX-M} *together found in 39% of the isolates, but in 7%* bla_{TEM}, bla_{SHV} *were found together, the three genes* bla_{TEM}, bla_{SHV} *and* bla_{CTX-M} *were found in 4% but none of the isolates carried* bla_{SHV} *gene alone or with* bla_{CTX-M}. *113(93%) of the 121 ESBLs producing isolates were found to carry one or more of quinolone resistance (QNR) genes qnrA, qnrB, qnrS. The three genes together were found in (53.9%) of the isolates, while qnrS was found in (29.1%), qnrA in (11%) of the isolates and (6%) carried both qnr B and S.* ***Conclusions:*** *Our results showed coexistence of ESBLs production and quinolone resistance in the majority of the K. pneumoniae isolates suggesting that more care should be taken for the choice of antibiotic therapy beside strict application of infection control policies to prevent dissemination of the multidrug resistant strains.*

INTRODUCTION

Klebsiella pneumoniae; is an opportunistic pathogen, it is a member of *Enterobacteriaceae* family responsible for many infections as pneumonia, urinary tract infections, abdominal infections, intra-vascular device infections, surgical site infections, soft tissue infections, and subsequent bacteraemia[1].

K. pneumoniae can also be found in the environment in association with plants, as well as in soil and in water[2].

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According to studies *K. pneumoniae* has been implicated in 7-12% of hospital-acquired pneumonia in ICUs in the United States, also accounting for 15, 32 and 34% of community-acquired pneumonia in Singapore, Africa and Taiwan, respectively[3].

Beta-lactam antibiotics are commonly used for the treatment of *Enterobacteriaceae* related infections. However, resistance to these agents has increased worldwide mostly due to β-lactamases production[4]. Among these enzymes, extended spectrum β-lactamases (ESBLs) have increased in response to the extensive use of extended-spectrum β-lactam antibiotics[5]. Based on several investigators idea, plasmid-encoded temoneira (TEM), sulfhydryl variable (SHV), and cefotaximase (CTX-M) are the most prevalent ESBLs genotypes[6]. In addition, ESBLs producing bacteria are typically

associated with multidrug resistance since multiple resistance genes often reside on the same plasmid[7].

Nalidixic acid; a by-product of chloroquine synthesis, was marketed during the 1960s for oral treatment of urinary tract infections and is still available by prescription, several quinolones were invented since then, including flumequine which bearing a fluorine atom at position C-6, which was active against nalidixic acid resistant Enterobacteriaceae, but development of newer fluoroquinolones did not progress significantly till it was demonstrated that a fluorine atom substitutions at the C-6 and C-7 positions will improve antibacterial activity and pharmacological properties[8].

Since then, fluoroquinolones such as Ciprofloxacin have become established for treatment of urinary, respiratory, gastrointestinal, urogenital, intra-abdominal, and skin/skin structure infections in outpatients and hospitalised patients. Because of their extensive use; resistance to fluoroquinolones emerged rapidly[9].

In 1998; a plasmid mediated fluoroquinolone resistant *Klebsiella pneumoniae* clinical isolate was reported in USA[10], then fluoroquinolones; due to their extensive use, have become less effective against clinical isolates of most gram negative bacteria[11] . In addition to the role of plasmid, studies have shown that quinolone resistance can arise by mutations in topoisomerase subunits or changes in the expression of efflux pumps and porins that control the accumulation of these agents inside the bacterial cell[12].

METHODOLOGY

Over 10-months period between June2014 and April 2015 bacterial isolates of *Klebsiella pneumoniae* were collected from clinical specimens (urine, blood, pus, sputum, wound swab, endotracheal tube, stools and ascitic fluid) from inpatients and outpatients of Sohag University Hospital. Identification of *Klebsiella* primarily was done by conventional methods being gram negative diplobacilli In gram stained smear, lactose fermenters on Macconkey's agar with characteristic mucoid colonies then complete Identification of the species *K. pneumoniae* was performed using the Vitek-2 system *(bioMerieux, france)*.

Antimicrobial susceptibility testing

For initial screening the VITEK-2 Gram-negative antimicrobial susceptibility testing card *(AST-GN 73, bioMérieux)* was used to determine the susceptibility of *K. pneumoniae* isolates to different antimicrobial agents. VITEK-2 provides AST results for antimicrobials as susceptible, intermediate susceptible and resistant then confirmation was done using disc diffusion method (modified Kirby Bauer) method using antimicrobial susceptibility testing discs supplied by *(Oxoid, UK)* for the following antibiotics; Amikacin (AK 30µg), Amoxycillin/clavulanic acid (AMC 30 µg), Aztreonam (ATM 30 µg), Cefepime (FEP 30 µg), Cefotaxime (CTX 30 µg), Ceftazidime (CAZ 30 µg), Ceftriaxone (CRO 30 µg), Ciprofloxacin (CIP 5 µg), Gentamicin (CN 120 µg), Kanamycin (K 30 µg), Levofloxacin (LEV 5 µg), Nalidixic acid (NA 30 µg), Nitrofurantoin (F 300 µg), Norfloxacin (NOR 10 µg), Piperacillin (PRL 100 µg), Trimethoprim/Sulphamethoxazole (SXT 25 µg), Tigecycline (TGC 15 µg), Imipenem (IPM 10 µg), Piperacillin/Tazobactam (TZP 110 µg).

The results were interpreted according to CLSI guidelines [13]. In the present study, only resistant results were presented.

Detection of ESBLs producing *K. pneumonia* by *Combined disc diffusion test:*

For the confirmation of ESBLs production by the isolated ***K. pneumonia***; the combined disc diffusion test (CDDT) was used where a standardized suspension of the isolated bacteria was plated into Mueller-Hinton agar then antimicrobial discs CD10 (Cefpodoxime/ Clavulanic acid), CPD10 (Cefpodoxime10µg) *(Oxoid, UK)* were placed, after 24 hour incubation, the inhibition zone around each of the two discs is measured. An increase of inhibition zone diameter of CD10 disc by ≥ 5mm more than that of CPD10 disc indicates that the isolate is positive for ESBL production.

Fig. 1: Combined disc diffusion test (CDDT) showing ESBLs producing *K. pneumoniae.*

Fig. 2: Combined disc diffusion test (CDDT) showing non-ESBL producing *K. pneumonia*

Multiplex PCR for amplification of ESBLs genes:

All phenotypically ESBLs producing *K. pneumoniae* isolates according to CDDT were analyzed for detection of the genes bla_{SHV}, bla_{TEM}, and bla_{CTX-M} by Multiplex PCR assay using primers presented in **table 1** [14]. Rapid DNA extraction was performed by a boiling technique that includes a heating step at 100°C of a single colony in a total volume of 100 µL of distilled water followed by a centrifugation step of the cell suspension [15]. Multiplex PCR reactions were carried out in a final 50µL volume containing 5µL of 1X PCR reaction buffer, 5µL deoxynucleoside triphosphates mix (dNTPs), 2µL DNA solution, 1µL MgCl2, 2µL of each gene-specific primer, 0. 5µL Taq DNA polymerase, and 24.5 µL PCR water all supplied from ***(invitrogen)***. PCR amplifications were performed on a T-Gradient thermal cycler ***(Biometra, USA***). PCR amplification conditions were as follows; initial heating at 95°C for 10 minutes, followed by denaturation at 95°C for 1 minute, annealing at 54°C for 1 minute, extention at 72°C for I minute for 35 cycles then final extension at 72°C for 10 minutes. PCR amplicons were separated electrophoretically on a 1% agarose gel and stained with ethidium bromide. A marker (EzWayTM DNA Ladder 100bp, 10 bands) from *(KOMA BIOTECH INC.)* was used to assess PCR product size, then the gel was exposed to UV rays of the transluminator and photographed.

Table 1: Primers used for ESBLs production genes amplification.

Gene name	Primer name	Amplicon size(bp)	Nucleotide sequence (5'-3')
bla-TEM	bla-TEM-F	445	TCGCCGCATACACTATTCTCAGAATGA
	bla-TEM-R	445	ACGCTCACCGGCTCCAGATTTAT
bla-CTX-M	bla-CTX-M-F	593	ATGTGCAGCACCAGTAATGTCATGGC
	bla-CTX-M-R	593	TGGGT CAA CTA CGTTACCAGAA CCAGCGG
bla-SHV	bla-SHV-F	973	TCTCCCTGTTAGCCACCCTG
	bla-SHV-R	973	CCACTGCAGCAGCTGCACGTT

Multiplex PCR of quinolone resistance genes:

Rapid DNA preparation was performed by a boiling technique as previously mentioned [15]. On the basis of a sequence alignment of the qnrA-, qnrB- and qnrS- genes, pairs of primers were designed to amplify fragments with sizes of 580, 264 and 428 bp, respectively (Table 2) [16]. Then amplification and detection were done as previously mentioned.

Table 2: Primers used for QNR genes amplification

Gene name	Primer name	Amplicon size	Nucleotide sequence (5'-3')
qnrA	qnrA-F	580	AGAGGATTTCTCACGCCAGG
	qnrA-R	580	TGCCAGGCACAGATCTTGAC
qnrB	qnrB-F	264	GGCATCGAAATTCGCCACTG
	qnrB-R	264	TTTGCTGTCCGCCAGTCGAA
qnrS	qnrS-F	428	GCAAGTTCATTGAACAGGGT
	qnrS-R	428	TCTAAACCGTCGAGTTCGGCG

RESULTS

148 *K. pneumoniae* isolates were isolated from different clinical specimens including: urine (44.9%), blood (15.8%), pus (10.6%), sputum (10.6%), wound swab (7.6%), endotracheal tube (4.5%), stools (3%), ascitic fluid (3%).

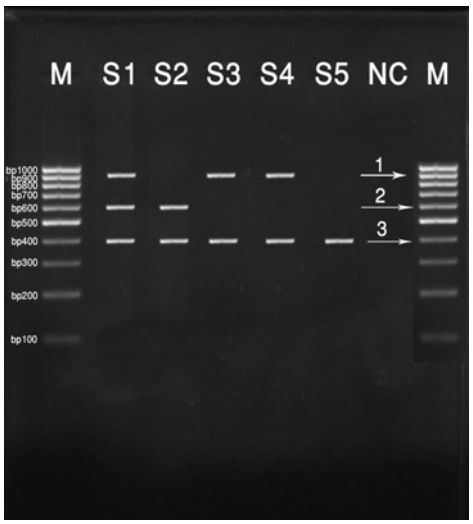
Table (3): comparison of antibiotic resistance between non- ESBLs and ESBLs producing *K. pneumoniae* isolates

Antibiotic	% resistance		P value
	ESBL Isolates	Non-ESBL Isolates	
Cefepime	86.9	2.8	< 0.05
Cefotaxime	95.6	10.5	< 0.05
Ceftriaxone	96.7	4.8	< 0.05
Ceftazidime	89.1	14.4	< 0.05
Piperacillin	93.4	7.5	< 0.05
Amoxicillin/clavulanate	48.9	50	NS
Piperacillin/tazobactam	32.6	5.7	< 0.05
Aztreonam	93.4	8.6	< 0.05
Imipenem	4.00	0.00	NS
Tigecycline	0.00	0.00	
Gentamicin	75.0	2.8	< 0.05
Amikacin	52.1	3.8	< 0.05
Nalidixic acid	95.9	9.6	< 0.05
Ciprofloxacin	92.2	10.5	< 0.05
Levofloxacin	90.1	7.6	< 0.05
Ofloxacin	91.5	9.6	< 0.05
Norfloxacin	88.6	5.7	< 0.05
Trimethoprim/Sulphamethoxazole	77.1	17.3	< 0.05
Nitrofurantoin	54.3	27.8	< 0.05

NS: not significant

Table (4): Distribution of *K. pneumoniae* isolates according to extended spectrum β-lactamases genotype.

ESBLs gene	% of ***K. pneumoniae*** isolates
bla_{TEM} +ve	44%
bla_{CTX-M} +ve	6%
bla_{SHV} +ve	0.00%
bla_{TEM} +ve & bla_{CTX-M} +ve	39%
bla_{TEM} +ve & bla_{SHV} +ve	7%
bla_{SHV} +ve & bla_{CTX-M}	0.00%
bla_{TEM} +ve & bla_{SHV} +ve& bla_{CTX-M}	4%



M: marker NC: negative control S: sample

Fig. 3: Electrophoresis of Multiplex PCR product showing the distribution of ESBLs genes in our *K. pneumonia* isolates:

-Arrow 1 point to the 937 bp corresponding to bla_{SHV} gene molecular weight.

-Arrow 2 point to the 593 bp corresponding to bla_{CTX-M}gene molecular weight.

- Arrow 3 point to the 445 bp corresponding to bla_{TEM}gene molecular weight.

Table(5):Distribution of *K. pneumoniae* isolates according to Quinolone resistance genotype.

Qnr gene	% of *K. pneumoniae* isolates
qnrA & qnrB & qnrS	**53.9**
qnrA & qnrS	**0.00**
qnrB & qnrS	**6**
qnrA & qnrB	**0.00**
qnrA	**11**
qnrB	**0.00**
qnrS	**29.1**

M: marker NC: negative control S: sample

Fig. 4: Electrophoresis of Multiplex PCR product showing the distribution of qnr genes in our *K. pneumonia* isolates:
-Arrow 1 point to the 580 bp corresponding to qnrA molecular weight.
-Arrow 2 point to the 428 bp corresponding to qnrS molecular weight.
-Arrow 3 point to the 264 bp corresponding to qnrB molecular weight.

DISCUSSION

Quinolones are clinically very important group of antibiotics for treatment of bacterial infections. The wide use of this group in both human and animals leads to increased bacterial resistance worldwide[17-19]. Quinolones kill bacteria by inhibiting the DNA gyrase enzyme which consists of A and B subunits. Mutations in *gyr*A are the most common mechanism involved in quinolone resistance among bacteria [19].

β-Lactamase production is the most common mechanism for resistance to β-lactam antibiotics in Enterobacteriaceae especially *Klebsiella pneumoniae* isolates [20].

Extended spectrum ß-lactamases are enzymes produced by enterobacteriace eg., some *K. pneumoniae* isolates. These enzymes inactivate penicillins, expanded- spectrum cephalosporins, monobactams including older beta-lactam antimicrobial agents. ß-lactamases are inactivated by clavulanic acids, sulbactam and tazobactam (Kaur and Aggarwal)[21].

The prevalence of ESBL producing *K. pneumoniae* isolates varies greatly in different geographic areas; it ranges from 0% in Iceland to 83.3% in Romania [22]

As regards the prevalence of ESBL production in *Klebsiella pneumoniae* in our study it was (81.7%). In other studies carried out in Egypt; the prevalence ranged from 23.81% [23] to 67% [24].

Also we compared the prevalence of ESBL producing isolates of *K. pneumoniae* according to our study with that in different countries as Iran where the prevalence was 60% in 2013 [14] and increased to 79.7% in 2015 [25].

However, lower prevalence rates of ESBLs producing *K. pneumoniae* was reported by other studies. The prevalence was in the range of 7.5% to 22.8% and

8.5% to 29.8% in Taiwan and Korea respectively [26, 27]. A multicenter study in mainland of China showed that the prevalence of ESBL production in *K. pneumoniae* isolates increased from 11% in 1994 to 34% in 2001[28]. In Pakistan, the prevalence of the ESBLs producing *K. pneumoniae* was 36% [29].

As regards the distribution of ESBLs genes in *K. pneumoniae* isolates in our study; bla_{TEM} was found in 44% of the isolates, while bla_{CTX-M} was found in 6%, bla_{TEM} and bla_{CTX-M} together were found in 39%, but in 7% bla_{TEM} and bla_{SHV} were found together, the three genes bla_{TEM}, bla_{SHV} and bla_{CTX-M} were found in 4% but none of the isolates carried bla_{SHV} gene alone or with bla_{CTX-M}.

Younes Ghasemi et al [14] reported in Iran that the distribution of ESBLs genes in their *K. pneumoniae* isolates was; TEM 39%, CTX-M and TEM 39%, TEM, SHV and CTX-M 13%, which agree with us in that blaTEM is the most common genotype. Our study disagree with the study reported by Aisha et al[24] where BlaSHV was found as a predominant gene responsible for ESBLs production and the study reported by Mehdi Goudarzi et al [26] where bla_{CTX-M} was found to be the predominant gene.

Our results showed that ESBL producing isolates were significantly more resistant to all tested antibiotics, except for Imipenem and Tigecycline, compared to the non-ESBL producing isolates ($P < 0.05$); which agree with all studies carried worldwide. This could be due to the presence of plasmids which frequently carry both ESBL and other antibiotic resistance genes.

Ninety three percent of our ESBLs harboring *K. pneumoniae* isolates have been found to be resistant to fluroquinolones which agree with many studies. In two studies in Korea; the percentage of quinolone resistant *Klebsiella pneumoniae* among its ESBLs producers was 40*%* [30] and 22% [31], while in a study was done in Iran the percentage was 65% in 2014 [32]. In an Egyptian study done in 2015 by Rasha H. El-Mahdy, the percentage was 100% [33]. However, Eftekhar et al., did not find a relationship between ESBL production and ciprofloxacin resistance, in a limited number of *K. pneumoniae* urinary isolates [34].

Imipenem or Tigecycline is still the most reliable and effective antimicrobial treatment option for ESBLs producing *K. pneumoniae* isolates which agree with Ghafourian et al[35], Aisha et al[24] and many other studies.

CONCLUSION

Coexistence of quinolone resistance with ESBLs production is a serious public health problem and requires continuous surveillance, monitoring, revision of the antibiotic use and strict applications of infection control policies.

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