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RESEARCH ARTICLE

Detection of CTX-M and CTX-M1 Genes in *Klebsiella* spp. Isolation from Clinical and Environmental Samples in Thi-Qar Province.

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Abstract

The aim of this study was to identify the prevalence of some extended-spectrum β-lactamases genes (ESBLs) (bla_{CTX-M} , bla_{CTX-M1}) in *Klebsiella* spp. isolated from clinical specimens (Burns , wounds , urinary tract infections, respiratory tract infections and otitis media patients and vaginal infection) and hospitals environments . A total of 554 samples (468 clinical and 86 environmental samples) during the period from October 2013 to March 2014 were collected. Only 45(8.1%) samples were gave positive growth for as following: 2⁄48 (4 %) from burns , 7⁄40 (17 %) from wounds , 29 ⁄315 (9.20%) from urinary tract infections , 4 ⁄ 25 (16%) from respiratory tract infections , 3⁄86 (3,48%) from hospitals environments. All the 45 isolates were submitted to molecular detection of some MBLs genes (bla_{CTX-M} , bla_{CTX-M1}) by using PCR technique . The results showed that only 28 (62,22%) isolates were carried these genes. and 21 isolates were carried both genes.

INTRODUCTION

Extended-spectrum β-lactamases (ESBLs) resistant Gram-negative bacteria have been associated with increased mortality, long-period of hospitalization and hospital costs (Schwaber *et al*., 2006). In humans, *Klebsiella spp.* may colonize the skin, pharynx, or gastrointestinal tract, as well as they colonize sterile wounds, urine and may be regarded as normal flora in many parts of the colon, intestinal tract and biliary tract (Podschun and Ullmann 1998 ; Brisse *et al*., 2006).

Extended spectrum β-lactamases (ESBLs) are a group of enzymes with the ability to hydrolyze and cause resistance to the oxymino- cephalosporins (i.e.cefotaxime, ceftazidime, ceftriaxone, cefuroxime and cefepime) monobactams (i.e. aztreonam) (Peirano and Pitout, 2010).

ESBLs produced mostly by members of Enterobacteriaceae have emerged as serious nosocomial pathogens globally (Moland *et al*.,2002).

Prevalence of ESBL in many parts of the world was (10-40%) among *E. coli* and *Klebsiella pneumoniae* (Rupp and Paul, 2003). The prevalence of ESBLs in Europe is higher than in the USA but lower than in Asia and South America (Girlich *et al*., 2004). In 2007 in Asia pacific region was found to harbour plasmid borne ESBLs 62% and 75% in *E. coli* and *Klebsiella* spp. respectively (Bell *et al*., 2007).

In the past several years, the emergence of new variants of ESBL producers, especially CTX-M has suggested the involvement of the co-resistance to other drug classes during endemic condition. This co-resistance is due to the transmission of different types of resistance genes within the same clone. Several studies showed that bla_{CTX-M} genes are commonly found on large plasmids that often carry other genes conferring resistance to other antimicrobial agents including aminoglycosides, fluoroquinolones, chloramphenicols, tetracyclins and others (particularly, bla_{OXA}-1, bla_{TEM}-1, tetA, aac(6′)-lbcr) (Livermore *et al*., 2007 ; Harajly *et al*.,2010).

ESBLs of the CTX-M type were rare until the end of the 1980s, but Japan, Argentina and Germany reported almost concomitantly findings of this ESBL type. Predominance of CTX-M B-lactamases may be due to the selective pressure of increased use of ceftriaxone (Pinheiro *et al*., 2008 ; Samaha-Kfoury and Araj, 2003).

The genes encoding CTX-Ms have been mobilised from *Klyuviera* spp. by several genetic events and mechanisms (Perez *et al*., 2007).With the emergence of the CTX-M, there has been a marked shift in the epidemiology of ESBLs (Coque *et al*., 2008).

CTX-M type ESBLs typically hydrolyze Cefotaxime and Ceftriaxone more efficiently than Ceftazidime but point mutations around the active site belonging to the CTX-M-1 and CTX-M-9 groups having ability to hydrolyze Ceftazidime significantly (Pitout ,2010).

CTX-M B-lactamases (i.e. 'active on **C**efo**T**a**X**ime, first isolated in Munich') were first reported from Japan in 1986 (the enzyme was initially named TOHO-1 and was later changed to CTX-M) (Matsumoto *et al*.,1988). More than 80 CTX-M enzymes are currently known, they are currently divided into 5 clusters on the basis of amino acid sequence: CTX-M-1,CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 (Al-Agamy *et al*. 2009 ; Smet *et al*., 2010). named after the enzyme first discovered for each lineage (Pagani *et al*., 2003). Despite their name, a few are more active on ceftazidime than cefotaxime. CTX-M-15 belongs to the CTX- M-1 cluster and is derived from CTX-M-3 by one amino acid substitution at position 240 (Asp→Gly); however, the flanking sequences of the β-lactamases can be very different (Peirano and pitout,2010).

Materials and Methods

1-Kit :-

In the present study used DNA Extraction kit (Geneaid / Korea) and KAPA2G Robust hot start Ready Mix PCR kit (Kapa /USA.)

2-Molecular Weight Marker

The molecular weight marker used in this study for DNA has been from Kapa (U.S.A). The range of the marker was 100bp-1 Kp.

3-Primers

The primers used in this study listed in Table(1)bellow :

Polymerase Chain Reaction Assay

Preparation of Buffers and Stains (Sambrook *et al* ., 1989).

1- **Tris- Borate-EDTA (TBE) 5X**

Prepared by dissolving of Tris-acid (54.0) gm. , Boric acid (27.5) gm., and EDTA (3.72) gm. in 800 ml of D.D.W. pH 8, and the volume completed to 1L with D. D.W.

2- TBE (1X)

This buffer was prepared by mixing 5 ml of stock TBE -5X with 45 ml of D.D.W., and stored at 4C° until used in.

3-DNA loading buffer

Bromophenol blue	25 mg
Xylene Xyanol	25 mg
Sucrose	4 g
D.W.	10 ml

4-Ethidium bromide (0.5%)

A stock solution has been prepared by dissolving 0.05 gm. of Ethidium bromide stain in 10 ml of D.D.W, then mixed by vortex for complete dissolving, and stored in sterile dark bottle. It has been used in the electrophoresis as specific DNA stain.

Detection of *bla* – genes by PCR assay

1- DNA extraction and purification

The DNA was extracted and purified according to the instructions of the company (Geneaid / Korea).

2- **DNA Amplification by PCR**

PCR reaction mixture consisted of : 12.5 µl 2X KAPA2G Robust HotStart ReadyMix[2] contains KAPA2G Robust HotStart DNA polymerase (1 U per 25 µl reaction)in a proprietary reaction buffer containing dNTPs(0.2mM of each dNTPsat 1X), $MgCl_2$ (2 mM at 1X) , reaction buffer pH 8.5, 1.25 µl of (100 pmole) of each primer (upstream and downstream) , 2 µl of DNA template (<100 ng) and 8 µl of nuclease free water in a total volume of 25 µl . The amplification program of *bla*-genes was done by simplex PCR (Table 2-7) and (Table 2-8) . The resulting PCR products has been run on 1.4% agarose gels.

3-Agarose gel electrophoresis

The agarose gel was prepared according to the method of Sam-brook *et al.*, (1989).Two concentrations of agarose gel were prepared (1.4% and 2%). The concentration of 1.4% agarose was used in the electrophoresis after DNA extraction process, while 2 % agarose was used for *bla* gene detection by PCR. A 25ml of 1X TBE buffer and 0.2 µl ethidium bromide were added into a beaker, 0.25 g agarose was added to the buffer. The mixture was heated for boiling by hot plate until all gel particles were dissolved and allowed to cool down to 50-60ºC.

4-Casting of the agarose gel

.The gel was assembled to a casting tray and the comb was positioned at one end of the tray.
. The agarose solution was poured into the gel tray and allowed to cool at room temperature for 30 min.
. The comb was carefully removed and the gel replaced electrophoresis chamber. The chamber then filled with TBE – electrophoresis buffer until it reaches 3–5 mm over the surface of the gel.

5-Loading and running DNA in agarose gel

. DNA (6 µl) was mixed with (3 µl) bromophenol blue (loading buffer) and loaded in the wells of the 1.4% agarose gel.
.The cathode was connected to the well side of the unit and the anode to the other side.
.The gel was run at 60 V for 90 min until the bromophenol blue tracking dye migrated to the end of the gel.
.The DNA was observed and viewed under UV transilluminator.

Results

Table (1): Primers sequences used for genes amplification.

Gene name	primers Sequences (5'-3')		Product Size bp	Reference
CTX-M	F	TTT GCG ATG TGC AGT ACC AGT AA	544 bp	(Edelstein,pimkin *et al*.,2003)
	R	CGA TAT CGT TGG TGG TGC CAT A		
CTX-M1	A	CTT CCA GAA TAA GGA ATC	840 bp	(Dutour,Bonnet *et al*.,2002)
	B	CCG TTT CCG CTA TTA CAA		

F : forward, R : reverse, A. adenine, C. cytosine, G. guanine, T. thymine

Table (2): PCR condition of CTX-M1 (β-Lactamase genes) (Tabbouche *et al*.,2011)

St. No.	Step	Temperature (˚C)	Time	Number of Cycles
I	Initial Denaturation	94	10 min	1
II	Denaturation	94	1 min.	30
	Annealing	48	1 min.	
	Extension	72	1 min.	
III	Final Extension	72	7 min.	1

Table (3): PCR condition of CTX-M (β-Lactamase genes) (Arunagiri *et al.*,2013)

St. No.	Step	Temperature (˚C)	Time	Number of Cycles
I	Initial Denaturation	94	2 min	1
II	Denaturation	95	1 min.	35
	Annealing	51	1 min.	
	Extension	72	1 min.	
III	Final Extension	72	3 min.	1

Simplex PCR assay has been used to detect the presence of Extended-β-lactamases genes (*bla* $_{CTX-M1}$, *bla* $_{CTX-M}$) in *K.pneumonia* subspp. *pneumonia* and *K.oxytoca* isolated in the present study.

A total of 27, isolates of *Klebsiella pneumoniae* subspp. *pneumoniae* showed carry bla_{CTX-M1} gene (clinical isolates), and one isolate of *Klebsiella oxytoca* was carry bla_{CTX-M1} gene (clinical isolate). (table-4 and fig-1).

Also the result showed, 25 isolates of *klebsiella pneumoniae* subspp. *pneumoniae* were carry bla_{CTX-M} gene (24 clinical isolates +1 environmental isolate) , and 3 isolates of *Klebsiella oxytoca* were carry bla_{CTX-M} gene (2 clinical isolates + 1 environmental isolate) (table-5 and fig-2).

Molecular weight of the CTX-M1 gene (544bp),CTX-M gene (840bp)

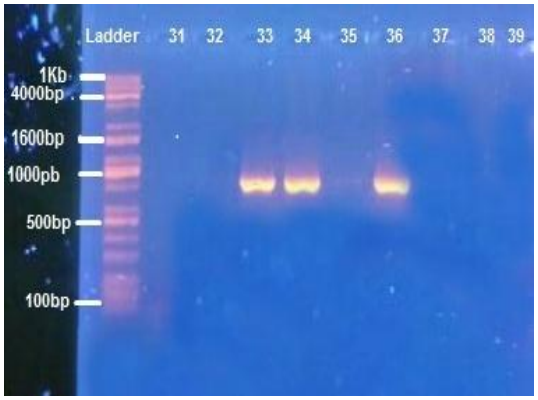
These genes are encoding for enzymes confer higher level resistance to cefotaxime, ceftriaxone and aztreonam than to ceftazidime.

Table (4):show the result of CTX-M1 gene in *Klebsiella* isolates

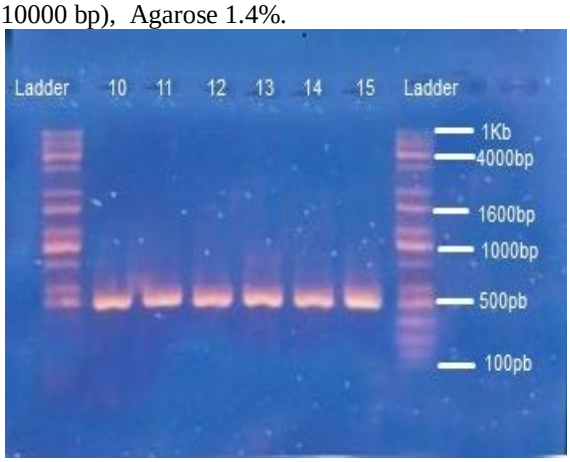
Bacterial type	CTX-M1 gene
Klebsiella pneumoinae subspp. *pneumoinae* (Clinical isolate)	27
Klebsiella oxytoca(Clinical isolate)	1
Total	28

Table (5):show the result of CTX-M1 gene in *Klebsiella* isolates

Bacterial type	CTX-M gene
Klebsiella pneumoinae subspp. *pneumoinae* (Clinical isolate)	24
Klebsiella pneumoinae subspp. *pneumoinae* (environmental isolate)	1
Klebsiella oxytoca (Clinical isolate)	2
Klebsiella oxytoca(environmental isolate)	1
Total	28



Figure(1): PCR amplification ***bla*$_{\text{CTX-M1}}$**(840bp) of *Klebsiella pneumonia*e sub spp. *Pneumonia*e (lane 31-38) and *Klebsiella oxytoca* (lane 39) Ladder (100-10000 bp), Agarose 1.4%.



Figure(2): PCR amplification ***bla*$_{\text{CTX-M}}$** (544bp) of *Klebsiella pneumonia*e sub spp. *Pneumoniae* Ladder (100-10000 bp). Agarose 1.4%

DISSCUSION

Polymerase chain reaction(PCR) is considered the best efficacy method for ESBLs detection because it is faster than phenotypic detection method (Chiangjong,2006) ,and also detect the existence of poorly or non-expressed (silent) genes difficult to determine by phenotype; PCR may also be used to directly test patient samples as an early predictor of infection (Diekema *et al*.,2004). In addition , each plasmid may contain more than one gene that may encode for different types of ESBLs enzyme. Furthermore , the spread of most broad-spectrum β-lactamases is facilitated by transferable and transconjugable plasmids, which frequently carry other resistance genes by means of their integron architecture (Jacoby and Munoz-Price,2005).

In the present study, from the 45 isolates of *Klebsiella* spp. Only 28 (62.22) have been positive for the presence of genes *bla* $_{\text{CTX-M}}$ and *bla* $_{\text{CTX-M1}}$. Result presented in this work is disagreement with Jemima & Susan (2008) from India , they have reported 25 of 62 (40%) *Klebsiella* species for *bla*$_{CTX\text{-}M}$ gene .and with result of study Azar *et al*., (2013) who showed low percentagethan this study.

In the present study, it has been shown that isolates which carry the genes (*bla*$_{\text{CTX-M}}$ and *bla*$_{\text{CTX-M1}}$) were resistance to cefotaxime. and the isolates which carry one gene (*bla*$_{\text{CTX-M}}$ or *bla*$_{\text{CTX-M1}}$) were resistance to cefotaxime .while the isolates that don't carry *bla*$_{\text{CTX-M}}$,*bla*$_{\text{CTX-M1}}$ were sensitive to cefotaxime.

The elevated rate of CTX-M β-lactamases among *E. coli* and *Klebsiella* spp. isolates suggest that the horizontal transfer of *bla*$_{\text{CTX-M}}$ genes, mediated by plasmids and /or mobile elements, contributes to ease with which these enzymes are spreading in E. coli and *K. pneumoinae* isolates and the dissemination of CXT-M enzymes. Moreover, in hospital environment, plasmids could be transferred easily between bacteria through health care workers due to hand carriage and antimicrobial selection pressure,(Al-Hilli,2010).

European studies on Enterobacteriaceae have also confirmed the persistence of strains producing TEM and SHV, and the increasing
prevalence of strains producing CTX-M (Ruppé,2010). The prevalence of ESBL productions revealed a significant geographical differences, ranging from 0% (Iceland) to less than 1% (Estonia) to 41% for *E. coli* and 91% for *K. pneumonia* (Romania) (Coque *et al*.,2008).

The CTX-M gene predominates in Europe, while in other countries, the ESBL genes are more diverse (Livermore *et al*.,2007) . In the United Kingdom, a dramatic increase of the ESBL producing strains was observed both in the hospitals and in the community, and this increase is attributed to CTX-M-15 (Coque *et al*.,2008) . In Norway and Portugal, the CTX-M is the ESBL enzyme most frequently found in *E. coli* (Tofteland *et al*.,2007; Machado *et al* ., 2007).

The major prevalence of ESBLs was attributed first to *K. pneumonia* strains which have produced TEM and SHV, and then followed by the emergence of E. coli strains producers of CTXM which become the prevalent type (Kanj *et al*., 2008; Daoud and Hakime,2003).

Conclusions

PCR is a molecular method used for detection of ESBLs genes. The $bla_{\text{CTX-M}}$ and $bla_{\text{CTX-M1}}$ were showed high prevalence in the tested isolates 28(62.22%)for these genus.

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