Novel β-Lactamases in the Clinical Isolates of *Enterobacter* spp. and *Klebsiella pneumoniae* in Ramadi General Hospital: A Pharmacodynamics Study

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Abstract

Background: *Enterobacter* spp. and *Klebsiella pneumoniae* are among the most common gram-negative pathogens associated with hospital infections, they have a distinctive ability to develop antimicrobial resistance during therapy due largely to their unusual β-lactamases, AmpC and ESBLs.

Aim of Study: To investigate the occurrence of novel β-lactamases (AmpC and ESBLs) in clinical isolates of *Enterobacter* spp. and *K pneumoniae* based on Pharmacodynamics approaches.

Materials and Methods: A total of 165 samples collected from AL-Ramadi General Hospital represented by 15 burn swabs, 95 wound swabs and 55 urine samples from in and outpatients from October 2003 to April 2004, by API 20 E system and motility test 13 isolates have been identified to be *Enterobacter* spp. and 33 isolates to be *K pneumoniae* from a total 150 isolates in addition to four isolates of *Enterobacter* spp. identified from 10 isolates taken from a previous study on *Klebsiella* spp. The susceptibility of the isolates to β-lactam antibiotics were determined by the standard disk diffusion method as described in the guidelines of the National Committee for Clinical Laboratory standards (NCCLs). Appropriate tests were performed for detecting AmpC β-lactamase, confirming this detection, inducibility of AmpC β-lactamase, and ESBL production.

Results: The interpretive reading of the antibiogram for Enterobacter spp. 1. Classical AmpC inducible enzyme: 2/17(11.8%) 2. ESBLs enzyme: 10/17(58.8%) 2. The number of derepressed or partially derepressed mutants were 7/17 (41.2%) that representing 7/14 (50%) of *E. cloacae.* While, for *K pneumoniae* infers the following: Classical low SHV-1 β-lactamase 2/33 (6.1%), All *Enterobacter* spp. isolates were resistant to cefoxitin but 15/17 were positive in the AmpC β-lactamase inhibition and the 3DM tests. Out of the 33 isolates of *K pneumoniae* one isolate was resistant to cefoxitin and showed positive result in the AmpC β-lactamase inhibition and the 3DM tests. Seven isolates of *Enterobacter* spp. showed blunting effect in the zone diameter of used β-lactams by cefoxitin or imipenem. According to the double disk synergy test for detecting the ESBLs enzyme, 8/17 (27.1%) of our *Enterobacter* isolates and 19/33 (57.6%) of *K pneumoniae* positive in giving phantom phenomenon.

Conclusion: It is concluded that the high occurrence of the novel β-lactamases in the clinical isolates of *Enterobacter* spp. and *K pneumoniae* infers a poor control of the use of antibiotics and infection in this hospital.

***Key words*:** *Enterobacter* spp*, Klebsiella pneumoniae*, AmpC, ESBLs

Introduction

Enterobacter spp. are among the most common gram-negative pathogens associated with hospital infections [1], they have a distinctive ability to develop broad spectrum antimicrobial resistance **during therapy**, owing largely to their AmpC β-lactamases[2].

The expression of AmpC enzyme is typically inducible in *Enterobacter* spp. and facilitates the emergence under antibiotic pressure of highly resistant stably derepressed mutants which produce large quantity of the enzyme that can hydrolyse the expanded spectrum cephalosporins and penicillin but remains susceptible to carbapenems [3,4,5].

The production of extended-spectrum β-lactamases (ESBLs) has been documented in *Enterobacter* spp. Which was first reported in 1983 in *K. pneumoniae* and *E. coli* [1]. Typically, ESBLs are mutant, plasmid-mediated β-lactamases derived from older, broad-spectrum β-lactamases (e.g., TEM-1, TEM-2, SHV-1), which have an extended substrate profile that permits hydrolysis of all cephalosporins, penicillins, and aztreonam except cephamycins such as cefoxitin. These enzymes are most commonly produced by *Klebsiella* spp. and *Escherichia coli* but may also occur in other gram-negative bacteria, including *Enterobacter* [6,7]. Genes for AmpC β-lactamase are notably absent in *K. pneumoniae* [8] and found on plasmids that transfer to *K. pneumoniae* which are believed to be originated from the chromosome of other Enterobacteriaceae[9,8].

It is of therapeutic interest for a clinical laboratory to distinguish between these β-lactamases as **resistance markers** due to increasing number of reports of AmpC or ESBLs producing organisms for which the MICs of expanded-spectrum β-lactams are low and which are associated with therapeutic failures [10,9]. The aim of this study was to investigate the occurrence of novel β-lactamases (AmpC β-lactamase and ESBLs) in clinical isolates of *Enterobacter* spp. and *K pneumoniae* based on Pharmacodynamics approaches. Because of scarce local reports on such clinically important resistance markers and to promote local awareness on such issues, it is felt worthwhile publishing the finding of this study several years after performing it.

Materials and Methods

A total of 165 samples collected from AL-Ramadi General Hospital represented by 15 burn swabs, 95 wound swabs and 55 urine samples from in and outpatients from October 2003 to April 2004, by API 20 E system and motility test 13 isolates have been identified to be *Enterobacter* spp. and 33 isolates to be *K pneumoniae* from a total 150 isolates in addition to four isolates of *Enterobacter* spp. identified from 10 isolates taken from another study on *Klebsiella* spp.

The susceptibility of the isolates to β-lactam antibiotics were determined by the standard disk diffusion method as described in the guidelines of the national committee for clinical laboratory standards [11].

The susceptibility to cefoxitin disk (30 µg) was used as a primary screening for detection of AmpC β-lactamase and the isolate with zone inhibition diameter < 18 mm were selected [9] and then tested for AmpC β-lactamase inhibition by cloxacillin [8]. In our study cloxacillin was used as a pharmacodynamic tool (indicator) for the detection of the production of AmpC β-lactamase in isolates using disk diffusion technique as a synergy effect with cefoxitin as an AmpC inducer (30 µg for each).

The three-dimensional method (3DM) was used as confirmatory test for the detection of AmpC β-lactamase in the positive isolates from the screening tests as described by Coudron *et al* [9] with the exception that bacterial lysis was not performed, i.e. whole cell suspension was used.

The test for inducibility of AmpC β-lactamase by cefoxitin, and imipenem was performed by the disk approximation method [12]. ESBL production was determined using double disk synergy test (DDST) described by Livermore and Brown[13].

Interpretive reading of the antibiogram data was performed to investigate the unusual resistance mechanisms from resistance phenotype [14].

Results:

The results of API 20 E system were as the following: out of 150 isolates 13 (8.7%) have been identified to be *Enterobacter* spp. (positive ornithine decarboylase tests and being motile) distributed as (7,4 and2) and 33(22%) isolates to be *K pneumoniae* (negative ornithine decarboylase test and non motile) distributed as (17, 5, 11) from wound, burn and urine samples respectively. The occurrence of *Enterobacter* spp in urine samples was low compared with that of wound and burn swabs and lowers than that of *K. pneumoniae*, but it was high in burn swabs (26.7%) and slightly less than that of *K. pneumoniae* isolates (33.3%).

The 13 isolates of *Enterobacter* spp. including 11 isolates of *E. cloacae* and 2 isolates of *E. gergoviae.*

Four isolates of *Enterobacter* spp. obtained from 10 isolates taken from a previous study on *Klebsiella* spp.[15]; one of them was identified to be *E. aerogenes* while the remaining 3 isolates were found to be *E. cloacae*. In the primary screening test for AmpC β-lactamase all *Enterobacter* spp. isolates were resistant to cefoxitin but 15/17 were positive in the AmpC β-lactamase inhibition test (figure 1A) and the three dimensional method (3DM) (figure 1B).

Out of the 33 isolates of *K pneumoniae* one isolate was resistant to cefoxitin and showed positive result in the AmpC β-lactamase inhibition test and the three dimensional method . The interpretive reading of the antibiogram data described in tables (1) for *Enterobacter* spp. and (2) for *K. pneumoniae*. Seven isolates of *Enterobacter* spp. showed blunting effect (antagonism effect) in the zone diameter of used β-lactam by cefoxitin or imipenem induced enzyme (figure 1C). According to the double disk synergy test for detecting the ESBLs enzyme 8/17(27.1%) of our *Enterobacter* isolates and 19/33 (57.6%) of *K. pneumoniae* positive in giving phantom phenomenon (figure 1D).

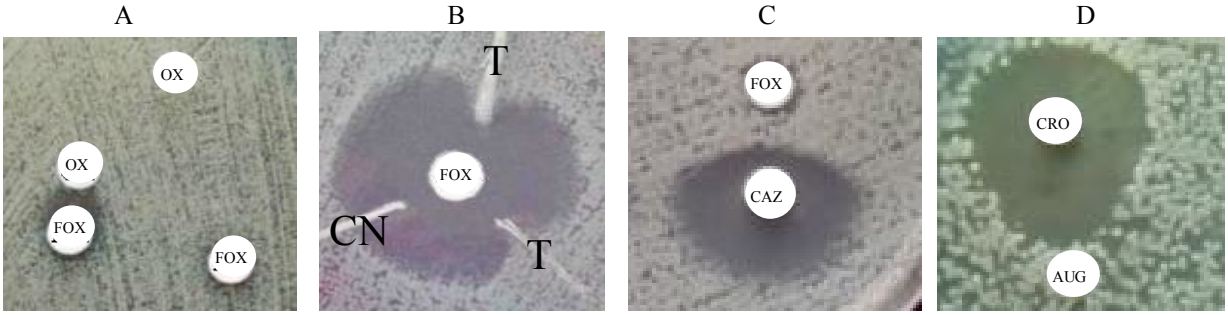

Figure.1. ***A****. showing the extension of the inhibition zone around the cefoxitin disk (FOX) towards the cloxacillin disk (OX) indicating the inhibition of AmpC enzyme.* ***B****. enhanced growth of the surface organism, E. coli ATCC 25922 at the point where the slit, containing Enterobacter spp., intersected the zone of inhibition is considered as positive 3DM indicating the presence of AmpC enzyme; CN: control negative isolate, T: AmpC enzyme producing isolates.* ***C****. detection of an inducible AmpC enzyme in E. cloacae, the disk with large zone is (CAZ) with 30 µg and the disk with very small zone is cefoxitin 30 µg. Note the blunting of the inhibition zones by the cefoxitin-induced enzyme.* ***D****. DDST. K. pneumoniae shows the phantom phenomenon between Co-amoxiclav (Augmentin, AUG) with ceftriaxone (CRO).*

Table (1): phenotypes: interpretation of mechanisms of antibiogram: β-lactam versus *Enterobacter* spp.

Isolate no.	AMP	AMX/CLAV	PIP	CEF	FOX	CXM	CAZ	CTX	CRO	FEP	ATM	IMP	ESBLs test	Induction test	Inferred β-lactamase type
E. cloacae B 3 *E. cloacae* U 10	R	R	S	R	R	S/r	S	S	S	S	S	S	-	+	Typical classical AmpC inducible Enzyme
E. cloacae B 4	R	R	R	R	R	r	R	r	r	r	R	S	+	+	AmpC enzyme and ESBL– Ceftazidimase [17]
E. cloacae B 5													+	+	
E. cloacae W 6													+	-	
E. cloacae B 7													+	+	
E. cloacae W 8													+	+	
E. aerogenes B 15													+	-	
E. cloacae W 9	R	R	R	R	R	R	R	R	R	R	R	S	+	-	AmpC enzyme and ESBL-Broad
E. cloacae W 11													+	-	
E. cloacae W 1	R	R	R	R	R	R	R	R	R	S	R	S	-	-	Hyperproduction of AmpC derepressed enzyme
E. cloacae W 2	R	R	R	R	R	R	R	R	R	S	R	S	-	-	
E. cloacae B 13	R	R	R	R	R	R	R	R	R	S	R	S	-	-	
E. cloacae B 14	R	R	R	R	R	R	R	R	R	S	R	S	-	-	
E. cloacae B 12	R	R	R	R	R	R	R	R	R	S	R	S	-	+	Partially derepressed enzyme [18]
E. gergoviae W 16 *E. gergoviae* U 17	R	R	R	R	R	R	R	R	R	R	R	S	- -	- -	ESBL? [6]

The mechanism of resistance was interpreted according to the interpretative reading [6, 14] and otherwise stated. B: burn, W: wound, U: urine

Table (2): phenotypes: interpretation of mechanisms of antibiogram: β-lactams *versus K. pneumoniae*

AMP	AMX/CLAV	PIP	CEF	FOX	CXM	CAZ	CTX	CRO	FEP	ATM	IPM	ESBLs test	Inferred β- lactamase type
R	S	r	S	S	S	S	S	S	S	S	S	-ve 2/2	Classical low SHV-1
R	r/R	R	R	S	S	S	S	S	S	S	S	-ve 8/8	Penicillinase-high level
R	R	R	R	S	R	R	R	R	R	R	S	+ve 6/10	ESBL-Broad
												-ve 4/10	
R	R	R	R	S	r	R	r	r	r	r	S	+ve 10/10	ESBL-Ceftazidimase
R	R	R	R	R	R	R	R	S	R	R	S	+ve 1/1	Acquired AmpC enzyme & ESBLs

The mechanism of resistance was interpreted according to the interpretative reading [6, 14].

Discussion:

Species of *Enterobacter* are becoming increasingly important nosocomial pathogens although they were much less commonly encountered than *Escherichia coli* and *Klebsiella* strains [1]. Most nosocomial *Enterobacter* infections appear to arise endogenously from a previously colonized site in the involved patient [3].

The results of this study demonstrated the occurrence of *Enterobacter* spp. was high in burn swabs (26.7%) and slightly less than that of *K. pneumoniae* isolates (33.3%) due to the selective environment with burn patients like loss of fluids and immunoglobulins with low tissue viability and low drug delivery so the high use of antibiotic therapy which suppresses the other normal flora and provides an excellent opportunity for colonization by

Enterobacter spp.[3], but in the case of urine sample where the drug is concentrated in the site of infection and can eliminate the organism[14].

The phenotypic tests for AmpC detection are not well defined [6] and the NCCLs documents do not indicate the screening and confirmatory tests that should be used for the detection of the AmpC β-lactamase in *K. pneumoniae* [9].

In our study all *Enterobacter* spp. showed resistance to cefoxitin indicating primarily the presence of AmpC β-lactamase. Of the cephamycins, cefoxitin was used as screening agent for AmpC β-lactamase because this antibiotic is stable against the activity of multiple β-lactamase like TEM-1, -2, SHV-1 and ESBLs but hydrolysed by AmpC enzyme [16]. Therefore, the resistance to cefoxitin may be due to the presence of AmpC enzyme or reduction in outer membrane permeability [6].

Out of 17 of our *Enterobacter* isolates, 15 showed extension in the zone of inhibition around the disk of cefoxitin towards the cloxacillin disk (30 μg) and these isolates yielded positive results in the 3DM indicating the presence of AmpC β-lactamase. *Enterobacter gergoviae* known to produce very low non-inducible level of AmpC β-lactamase [4] and that our two isolates showed negative results in the cloxacillin inhibition test and 3DM and the resistance to cefoxitin may be due to the reduction in outer membrane permeability [9].

Out of 33 isolates of *K. pneumoniae*, one isolate (3%) was resistant to cefoxitin and gave positive results in the cloxacillin inhibition test and 3DM, thus, inferring the presence of acquired plasmid mediated AmpC β-lactamase, and not reduction in the outer membrane permeability, to be responsible for the resistance to cefoxitin[19].

According to the DDST in our study 8/17 (47.1%) of *Enterobacter* spp. and 19/33 (57.6%) of *K. pneumoniae* were found to be ESBLs producers. In the study of Canton *et al* (2003) in Spain showed that (35%) of *Enterobacter* spp. were ESBLs producers by DDST. AL-Jumaily [15] reported that (52%) of clinical *Klebsiella* isolates were ESBLs producers and Al-Kubaisy [20] reported that 19/35 (54.28 %) of *Klebsiella* isolates collected from Ramadi General Hospital were ESBLs producers in DDST. All ESBLs producing isolates should be reported as resistant to all pencillins, cephalosporins and aztreonam because these isolates have a dramatic inoculum size effect with these agents [6].

The detection of ESBLs in AmpC producing organisms are more difficult [21] that high level expression of AmpC may prevent recognition of an ESBLs and in addition the AmpC enzyme may be induced by clavulanate and then attack the indicator cephalosporin, thus masking any synergy arising from inhibition of an ESBLs and producing false-negative result [13].

The high level of AmpC expression has minimal effect on the activity of cefepime making this drug a more reliable detecting agent for ESBLs in the presence of an AmpC β-lactamase[6], therefore, the *K. pneumoniae* isolate that was resistant to cefoxitin, gave the phantom phenomenon with cefepime only.

Upon the induction test, seven isolates of our *Enterobacter* spp. showed a positive result by flattening (blunting) of the antibiotic inhibition zone on the side facing the cefoxitin or imipenem disks while the remainder isolates considered to be derepressed mutant and/or ESBLs producing isolates[22].

Enterobacter spp. and *Klebsiella* spp. are important clinical pathogens that frequently exhibit resistance to third-generation cephalosporins. In *Enterobacter* spp. strains, resistance is usually due to derepression of the AmpC locus, whereas plasmid-encoded ESBLs are primarily responsible for resistance in *Klebsiella* spp. [22].

The result of sensitivity testing showed that *Enterobacter* spp. were non-sensitive to a wide range of β-lactam antibiotics used but they showed good sensitivity to imipenem (100%). Ampicillin and amoxicillin, first and second generation cephalosprins are strong AmpC β-lactamase inducers in wild type of *Enterobacter* spp. and rapidly inactivated by these β-lactamases while third generation cephalosprins, extended spectrum penicillins and aztreonam are weak inducers and the resistance is expressed in stably derepressed bacteria (mutant hyperproducers of β-lactamase) [23]. Hence, the high resistance in our study isolates may infer the presence of AmpC derepressed mutants.

The problem is that AmpC inducible organisms may be isolated from a patient and found to be susceptible to third generation cephalosporins when, however, these drugs are used clinically, there is in-therapy selection of derepressed resistant mutants, with higher possibility of therapeutic failure. This risk is as high as 20% of *Enterobacter* bacteraemia, it is therefore recommended that all *Enterobacter* spp. should be reported resistant to all third generation cephalosprins, except where these isolates are from urinary tract where very high cephaloporins concentrations can be attained [13].

While in *K. pneumoniae* we showed also high resistance to the extended spectrum cephalosporins and aztreonam but (97%) of them showed sensitivity to cefoxitin and (100%) to imipenem, that may refer to the presence of ESBLs producing isolates, ESBLs enzymes can hydrolyze all β-lactam antibiotics but remain non active against cephamycins and are most prevalent in Klebsiellae especially in intensive care and other specialized units[13].

Imipenem are strong AmpC β-lactamase inducer but remain very stable to the action of these enzymes[1].

The apparent absence of resistance mechanism to imipenem may be attributed to being effectively resistant to all the common plasmid encoded class A, C and D enzymes including extended spectrum TEM and SHV derivatives, and AmpC types [16].

Therefore, this level of sensitivity indicates the absence of class B enzymes (Metallo β-lactamase) and carbapenamase which encoded chromosomally and belong to class A β-lactamase [16,14]. AL-Kubaisy[20] reported that 7/25 (28%) of *K. pneumoniae* isolates were resistant to imipenem and 1/25 (4%) were Metallo β-lactamase producer that showed resistance to ceftazidime, cefotaxime, ceftriaxone and imipenem despite the fact that, to the date of conducting the study, imipenem has not been used clinically in AL-Ramadi General Hospital, and there is no cross-resistance conferred by the usage of cephalosporins to imipenem. In addition the detection of metallo β–lactamase needed a test for inhibition by EDTA [8] and this **very high** occurrence of resistance to imipenem is difficult to reconcile considering that the available body of literature states that resistance to imipenem is unusual to encounter in Enterobactericae (except with *Proteus* spp.). It has been advised that when such result is encountered the species identification and antibiogram data should be re-checked [14].

An approach has been introduced to read and utilize antibiogram data by (Interpretative reading) that aims to analyze the resistance pattern. Not just the results for individual antibiotics, and so to predict and recognize the unusual and inferring resistance mechanisms from resistance phenotypes to achieve better therapeutic outcome[14].

The interpretive reading of our data in table (1) showed that 2/17 (11.8%) of our *Enterobacter* spp. were designated as classical inducible AmpC β-lactamase. the problem with inducible AmpC producing organism that may be isolated from a patient and found to be susceptible to the third generation cephalosporins, when, however these drugs are used clinically, there is selection of derepressed resistant mutants with contingent clinical failure especially in severely-ill patients, therefore, should be reported as resistant to all third generation cephalosporins[14].

Out of 17 isolates, 10 (58.8%) were ESBLs producing (60%) of them were ESBLs ceftazidimase and (40%) producing ESBLs broad enzyme. The two isolates of *E. gergoviae* failed to produce positive ESBLs–confirmatory test; it has been suggested that hyperproduction of TEM and SHV β-lactamase or reduction in outer membrane permeability in organisms with ESBLs may cause false-negative phenotypic confirmatory test results[6].

AmpC derepressed enzyme producing isolates were found to represent 4/17(23%) which showed high resistance to all β-lactams except imipenem and considered to be as derepressed mutants [24]. One out of 17 (5%) isolate gave positive result in induction test with some β-lactams inferring to be partially derepressed mutant [18] and showed negative result in ESBLs detection.

It is suggested that the number of derepressed or partailly derepressed mutants were 7/17 including two isolates showed positive ESBLs with cefepime only and the seven isolates were *E cloacae* 7/14 (50%). Pai *et al* [18] in his study in Korea reported that (50%) of *Enterobacter* spp. were fully or partially derepressed mutant and (6.9%) were classical inducible β-lactamase (wild-type).

The Interpretative reading of *K. pneumoniae* antibiogram pattern (table-2) showed the high percentage of ESBLs producing isolates 23/33 (69.7%) and only one of them (3%) produced both ESBLs and acquired AmpC enzyme that showed phantom phenomenon with cefepime only and resistant to cefoxitin [14]. *K. pneumoniae* acquired plasmids encoding AmpC enzyme that have escaped from other Enterobacteriaceae [25, 8].

AL-Jumaily [15] reported that (8%) of *Klebsiella* spp. in his study produced plasmid mediated AmpC enzyme, and Coudron *et al* [9] reported that (1.1%) of *K. pneumoniae* in his study produced this enzyme. The high percentage stated by the former [15] was probably due to misidentification of *Enterobacter* spp as *Klebsiella* spp. and this was evident after passing ten of his study isolates to be re-checked during the course of this study, four of the ten isolates were found to be *Enterobacter* spp. Further, it has been suggested that plasmid-mediated AmpC enzymes present the greatest threat clinically by seriously limiting therapeutic choices, even more so than ESBLs[26].

It is concluded that the high occurrence of the novel β-lactamases in the clinical isolates of *Enterobacter* spp. and *K pneumoniae* infers a poor control of the use of antibiotics and infection in this hospital. Further, although clinically the usual occurrence of *Enterobacter* spp. is less than that of *K pneumoniae*, in certain clinical settings like burn, the ability of the former to express unusual multi-resistance mechanisms favouring selection and emergence of resistant clones makes them more difficult to control. Our clinical laboratories should catch up with new bacterial developments; otherwise, new pathogens will spread, resulting in increasing treatment outcome problems and costs for patients and institutions.

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