Characterization Of Extended Spectrum β- Lactamases In Enterobacteriaceae

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ABSTRACT

Antibiotic microbial resistance is a major health problem worldwide in both humans and animals, specifically, *E. coli* producing extended-spectrum β-lactamases (ESBL). In the present study, *E. coli* was isolated from human; broiler; and calves samples, and then the isolates were biotyped. To determine *E. coli* antibiotic sensitivity pattern, isolates were subjected to antibiogram. Consequently, seven of the multi-drug resistant isolate were randomly selected and subjected to polymerase chain reaction to detect ESBL. The isolates showed high degree of resistance to beta-lactame antibiotics. Among the seven isolates, four isolates were TEM enzyme producer; 3 isolates were SHV producers; and another 3isolates were CTX-M producers. Point mutation was detected at different positions in TEM, SHV, and CTX genes as detected by DNA sequencing which releved point mutation in TEM gens at position number (89,90,114,115,116,135,485,1077 and1078) while SHVgens at position number (114,237.643and 635) and CTX-M gens at position number (364,382,383,707,708,736,737,286 and287), in isolates of human, claves and broilers. It could be conclude that the major reason behind E. coli antibiotic resistance is the production of ESBL and that genes undergo mutation at different sites in different species.

INTRODUCTION

Extended-spectrum β-lactamase (ESBL)-producing members of the family Enterobacteriaceae are resistant to penicillins, narrow-and extended-spectrum cephalosporins, and Aztreonam *(1)*. ESBL-producing organisms are also frequently resistant to aminoglycosides, trimethoprim-sulfamethoxazole, and quinolones. Until recently, most infections caused by ESBL-producing *Escherichia coli* (ESBLEC) or *Klebsiella pneumoniae* had mostly been described as nosocomially acquired *(1)* or nursing home related *(2)*. (ESBLs) have been observed in virtually all species of the family *Enterobacteriaceae*. These enzymes are predominantly plasmid mediated and are derived from broad-spectrum lactamase TEM-1, TEM-2, or SHV-1 by a limited number of mutations *(3)*. Studies on laboratory mutants, obtained by in-*vitro* mutagenesis or via directed evolution, contribute to our understanding of the evolutionary past, and allow future predictions *(4)*.

Several groups of acquired β-lactamases have hydrolytic profiles similar to those of the TEM and SHV mutants but have quite different evolutionary histories, although they also belong to molecular class A. Major examples include: the CTX-M family, which is rapidly spreading worldwide *(5)*. SHV enzymes have frequently been found in the widespread pathogens, such as Klebsiella, Escherichia, and Salmonella *(6)*. The enzyme was originally named CTX-1 because of its enhanced activity against cefotaxime and now termed TEM-3, which is differed from TEM-2 by two amino acid substitutions *(7)*.

β-lactamase variants can be detected by protein or DNA sequence comparisons, the previous studies revealed only the evolutionary trends within β-lactamase families, precisely reconstruct the history of particular enzyme lineages and silent mutations and genetic context.

Because *E. coli* causes serious problems in both humans and animals, and developed resistant genes to antibiotics, we focused efforts to determine the most common ESBL enzymes. In this study, investigation of the ESBL in *E. coli* isolated from different species and determination ofmutation(s) in each ESABL genes were done.

MATERIAL AND METHOD

Examined samples

Eighty samples (49 human ,15broiler and 16calves)were collected from different locations(farms and private laboratories) in Sharkia governorate. These samples were urine, feacal samples from newly born calf at age from 1 to 10 days and internal organ of broiler.

Isolation and biotyping

Nutrient agar *(8)* and MacConkey's agar with bileSalt *(9)* were used for isolation of *E. coli*. Indole test medium *(9)*, Methyl red-Voges Preskauer broth MR-VP (glucose phosphate broth) *(10)*, Simmon's citrate agar, Urea agar medium and Sugar media were used for E. coli biotyping.

Antibiotics susceptibility test

Antibiotic susceptibility test was done according to NCCLS recommended protocol. Briefly, suspensions of 24 h cultures of each isolate, with a concentration equivalent to McFarland tube 1, were spread onto Mueller-Hinton medium. Once the inoculum was dry, disks of Ceftazdime(Az). Ceftiraxone (CRO), Cefouroxime (CXM), Cefoperazone sulabactam (CES), Gentamicin (Gn). Cefotaxime (CTX), Impmen(IPM), Ciprofloxacin (CIP), Doxycycline (DO), Ampicilin (AMP), Amikacin (AK) Amoxicillin/ clavulanic acid (AMC) were placed onto the medium surface at a distance of 2 cm between each other. The plates were incubated at 37°C for 20 hs and the inhibitionzone were measured. ESBLs production were performed by double disk diffusion method with Cefotaxime and Ceftazdime alone and incubation with clavulnic acid as recorded by NCCLS *(11)*.

Polymerase chain reaction (PCR)

Primers in Table 1 were used for ESBL genes amplification. The reaction mixture (total volume of 50µl) was 5µl of 10x reaction buffer (Applied Biosystem), 1.5 µl of 25 mM MgC 2,1µl of nucleotides mix (10 Mm),5µl DNA was added (containing 50 ng) 2 µl primer (containing 400 ng of forward and reverse primers)and, 5µl (2U) of DNA*Taq* polymerase (Applied Biosystem) was added and mixture was completed by ultra pure distilled water to 50µl. PCR was performed on progone "programmable thermal controller" (UK). Amplification was performed by heating the sample for 3mintues at 94°C for initial denaturation, then forty cycles were performed as follows: Dentauration for 20 sec at 94°C, annealing for 40 sec at 54, 43, 55°for TEM,SHVandCTX-M genes respectively followed by extension for 1minute at 72°C then final extension step was held for 5 minutes at 72°C the analysis of PCR amplified products was done by using 8µl of amplified PCR products, mixed with 2µlloading buffer and electrophoresed through 1% agarose gel and DNA was visualized by UV fluorescence after ethidium bromide staining and then photographed.

DNA sequencing: The production of bla TEM, bla SHV and bla CTX-M amplification were used to determine the nucleotide sequence with fluorescence of Taq Fs Dye terminator cycles sequencing and same primers. DNA Sequencing analysis was performed with genetic computer software PCR primers *(12, 13)*.

Table 1. Primers used for ESBL genes amplification

Primers	Sequences	Size
PANCTX-M.F	5'-TTTGCGATGTGCAGTACCAGTAA-3'	540
PANCTX-M.R	5'-CGATATCGTTGGTGGTGCCATA-3'	
SHV F	F (5_ CACTCAAGGATGTATTGTG-3_)	885
SHVR	R (5_-TTAGCGTTGCCAGTGCTCG-3_)	
TEM F	F (5_TTCTTGAAGACGAAAGGGC-3_)	1150
TEM R	R (5_ACGCTCAGTGGAACGAAAAC-3_)	
CTXM.groupl.F3	5'-GACGATGTCACTGGCTGAGC-3'	840
CTXM.groupl.R2	5'-AGCCGCCGACGCTAATACA-3'	

RESULTS

Out of 80 examined samples were recored with incindence of76.2%of isolates were E.coli

Antibiotic susceptibility testing: As shown in Table 2, all isolates from human, broilers and calves were resistant to ampicilin and amoxicillin/clavulanic acid and most of the isolates were resistant to cephalospornes. Interestingly, all human isolates were sensitive to imipenem and most of isolates showed high susceptibility to amikacin with percentage of 87.5%.

Table 2a. Antibiogram of *E. coli* isolates from human

	Isolate code number	CAZ	CXM	CRO	CTX	CIP	AMC	CES	GN	AK	DO
Human	H1	R	R	R	R	R	R	R	S	S	S
	H2	R	R	R	R	R	R	S	R	S	R
	H3	R	R	R	R	R	R	R	R	S	R
	H4	R	R	R	R	S	R	R	S	S	R
	H5	R	R	S	R	R	R	R	R	S	R
	H6	R	R	IM	R	R	R	R	S	S	R
	H7	R	R	R	R	S	R	R	R	S	R
	H8	R	R	R	R	R	R	R	S	S	R
	H9	R	R	IM	R	IM	R	R	S	S	R
	H10	R	R	R	R	S	R	R	R	S	R
	H11	R	R	R-	R	R	R	IM	IM	S	R
	H12	R	R	S	S	R	R	R	S	S	R
	H13	R	R	R	R	S	R	R	R	S	R
	H14	R	R	R	R	R	R	R	S	S	R
	H15	R	R	R	R	S	R	R	R	S	R
	H16	R	R	S	R	R	IM	R	S	R	R
	H17	R	S	R	R	R	R	S	R	S	R
	H18	IM	R	R	R	IM	R	R	S	S	R
	H19	R	R	S	R	R	R	R	S	R	R
	H20	R	R	S	R	S	R	R	R	S	R
	H21	R	S	R	R	R	R	R	R	S	R
	H22	R	R	R	R	R	R	R	S	S	R
	H23	R	R	R	R	R	R	R	R	S	R
	H24	S	R	R	R	S	R	R	R	IM	R

H= human C= calf B= broiler

S= Susceptible R= resistant IM= intermediate

CTX =Cefotaxme AMP=Ampicillin GN=Gentamicin AK = amikacin DO= doxycycline
CAz= ceftazdime AMP= ampicilin IPM= Imipenem CIP = ciprofloxacin RO=Ceftiraxone
CXM = cefouroxime AMC = amoxicillin/clavulanic acid

Table 2b. Antibiogram of *E. coli* isolates from calves

	Isolate code number	CAZ	CXM	CRO	CTX	CIP	AMC	CES	GN	AK	DO
Calves	C1	R	R	R	R	R	R	R	S	R	R
	C2	R	R	S	R	R	R	R	R	S	R
	C3	R	R	R	S	IM	R	R	S	13	R
	C4	R	R	R	S	S	R	S	R	S	R
	C5	R	R	S	S	S	R	R	R	S	R
	C6	R	S	R	R	S	R	R	R	S	R
	C7	R	R	S	R	R	R	R	R	S	R
	C8	R	R	R	R	S	R	R	R	S	R
	C9	R	R	S	R	R	R	R	S	R	R

Table 2c. Antibiogram of *E. coli* isolates from broilers

	Isolate code number	CAZ	CXM	CRO	CTX	CIP	AMC	CES	GN	AK	DO
Broiler	B1	R	R	S	R	R	R	S	R	S	R
	B2	R	S	S	R	R	R	S	R	S	R
	B3	S	R	R	S	S	R	R	S	S	R
	B4	R	S	R	R	S	R	R	S	S	R
	B5	R	R	R	R	S	R	R	S	S	R
	B6	R	S	R	R	R	R	R	S	S	R
	B7	R	R	S	S	S	R	R	R	R	R
	B8	R	R	R	R	R	R	R	R	S	R

ESBL Production. Out of 41 *E. coli* isolates 24human,9 calves and8 broiler were subjected to double disk diffusion method. There were 15 (52.5%), 4 (44.4%) and 4 (50%) of *E. coli* isolates were obtained from human,calvesand broiler were positive to extended spectrum beta lactamases, respectively.

PCR amplification of blaTEM, blaSHV and blaCTX-M

Seven isolates were subject to polymerase chain reaction Most common type of ESBL was TEM genes, where it was detected in four isolates (H1, H2, C2 and B2). SHV and CTX-M genes in three isolates of the *E. coli* (H1, C1and B1) and (H2, C1and B2), were detected respectively. TEM gene (Figure 1), SHV gene (Figure 2) and CTX-M (figure 3) were detected in isolates from human, calves and broilers samples.

Sequencing of ESBL-encoding genes

DNA sequencing was done to determine the ESBL genes sequence and to determine if there is any mutation. There were

mutations in the different ESBL genes. In calves, TEM gene occurred at ambler positions 89, 90, 114, 116, 116, 135, 485, 1077, and 1078. Meanwhile, in SHV gene, the mutation occurred at ambler positions,114, 237, 643 and 635. In CTX-M, point mutation were determined in isolates from both human at ambler positions 364, 382, 383, 707, 708, 736 and 737 and in calves at ambler positions 86 and 287, respectively.

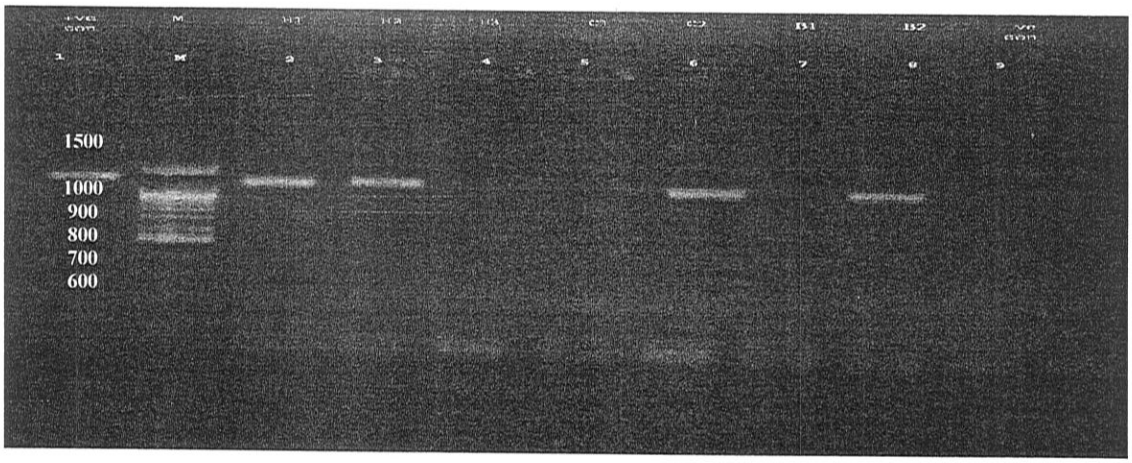

Fig.1. bla$_{TEM}$ gene in *E. coli* isolates from human, calves and broilers.

M:marker
Lanes :2,3,6,8are +ve for blaTEM gene
Lane1:control –ve
Lanes:4,5,7,are –ve for blaTEM gene

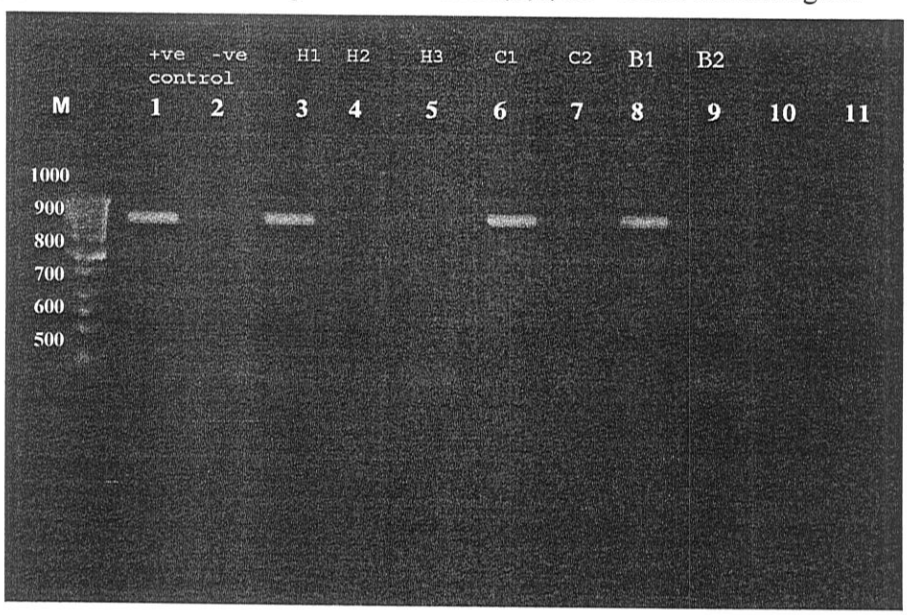

Fig. 2. bla$_{SHV}$ gene in *E. coli* isolates from human, calves and broilers.

M:marker
Lanes:3,6,8are +ve for bla SHVgene
Lane:1,2+ve and –ve control
Lanes:4,5,7,9are-ve for bla SHVgene

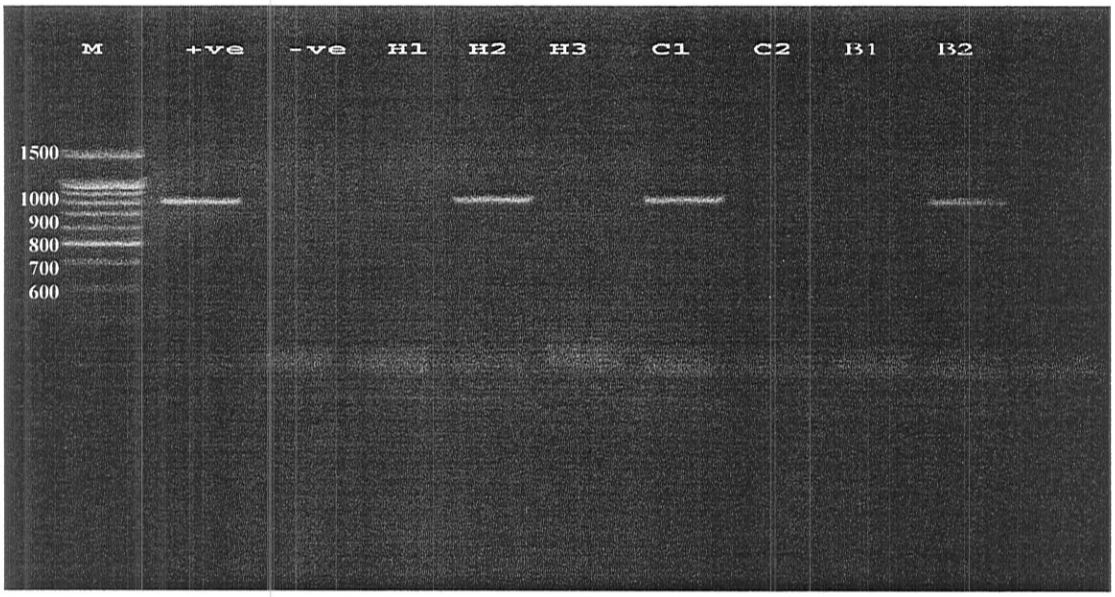

Fig. 3. ge bla$_{CTX-M}$ ne in *E. coli* isolates
M:marker
Lanes:4,6,9are +ve for blaCTX-M gene
Lanes:1,2+ve and –ve control
Lanes:3,5,7,8 –ve for blaCTX-M gene

Table 3. Mutation occurance in TEM, SHVandCTX-Mgenes

M.O	antibiotic susceptibility pattern	DDST	Gene mutation		
			TEM	SHV	CTX-M
H1	AMP^r,AMC^r,STXr, CAZ^r, CES^r,CRO^randCXM^r	+	NM	NM	-ve
H2	AMP^r,AMC^r,CTX^r,CAZ^r, CES^S,CRORandCXM^S	+	NM	-ve	364(G→C),382(A→G), 383(A→G),707(A→C), 708(C→A),737and(G→A)
H3	AMP^r,AMC^r,CTX^r,CAZ^r, CES^r,CRO^randCXM^r	+	-ve	-ve	-ve
C1	$AMP^r$$AMC^r$,$CTX^r$,$CAZ^r$, $CES^r$$CRO^r$,andCXM^r	+	-ve	114(T→A),237,(G→C), 643(C→A)and635(C→A)	286(C→G),287(C→G)
C2	AMP^r,$AMC^r$$CTX^r$,$CAZ^r$ CES^r,CRO^SandCXM^r	+	89(A→T),90(A→T), 114(G→C)115(G→C), 135(C→G)485(G→C), 1077(T→G)and1078(C→G)	-ve	-ve
B1	$AMP^r$$AMC^r$$CTX^r$$CAZ^r$ $CES^S$$CRO^S$andCXM^r	+	-ve	NM	-ve
B2	AMP^r,AMC^r,$CTX^r$$CAZ^r$, $CES^S$$CRO^S$andCXM^S	+	NM	-ve	NM

-ve = Negative NM = No mutation + = Positive

DISCUSSION

ESBLS most commonly produced by Enterobacteriacae are conferring resistance to B- lactam, monobactam antibiotic. In the present, Impmen was of most effective antimicrobial agents inhuman. while all the isolates were resistant toAmpicillin and Amoxicillin clavulanic. Amkacin, Ciprofloxacine, Gentamycin, Ceftaraxone, were effective against *E. coli* isolates. Carbapenem was reported as the drug of choice for the treatment of severe infections due to ESBL- producing organisms *(14)* while Amoxicillin/Clavulanic acid and first-generation Cephalosporin show high levels of resistance in *E. coli* isolates *(15)*. In a study *(16)*, 24.5% *E. coli* isolates from both calf and human source were resistant to three or more antimicrobial agents so considered as multi drug resistant isolates.

In the present investigation, β-lactamases genes production by *E. coli* was studied by PCR in seven isolates. Out ofa total seven *E. coli* isolates, four *E. coli* were encoded bla_{tem} gene, three *E. coli* isolates were encoded bla_{SHV}, and three *E. coli* isolates were encoded bla_{CTX-M}. These findings were probably share of resistance to Amoxicilin, Amoxicillin Clavulanic and Ampicillin- sulbactam as reported in another study *(17)*. On other hand, TEM derivatives reduced affinity for β-lactamase inhibitors as reviewed elsewhere *(18)*. Meanwhile, CTX reflects the potent hydrolytic activity of these β-lactamases against cefotaxime. Organisms producing CTX-M-type-lactamases. However, some CTX-M-type ESBLs may actually hydrolyze Ceftazidime and confer resistance to this Cephalosporin similar study reported previously *(19-21)*. And Kinetic studies have shown that the CTX-M-type lactamases hydrolyze Cephalothin or Cephaloridine better than Benzylpenicillin and they preferentially hydrolyze Cefotaxime over Ceftazidime *(22, 23)*.

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الملخص العربى

خواص انزيمات بيتا لاكتاماز ذات الطيف الممتد فى الميكروبات المعوية الممرضة

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اجريت هذة الدراسة على ٨٠ عينة جمعت من مزارع مختلفة من محافظة الشرقية ومعامل خاصة من الأنسان والأبقار والداوجن . تم عزل وتصنيف ٦١ معزول من الأشيرشيا القولونية عن طريق بعض التجارب الكيميائية الحيوية التشخيصية مثل تخمر اللاكتوز وانتاج الاندول والأستفادة من الخلات فى تجربة الميثيل الاحمر وغيرها. تم اجراء اختبار حساسية المعزولات للمضادات الحيوية والتى نتج عنها ان جميع العينات كانت مقاومة للأمبسيلين وكانت هناك نسبة مقاومة عالية لمجموعة السيفالوسبورنيز. تم اجراء تفاعل اختبار الحساسية باستخدام القرص لمعرفة وجود انزيمات البيتالاكتاماز فى هذة المعزولات وكانت النسبة ٦١% ، ٤٥%، ٥٠% من كل من الأنسان والأبقار والدواجن على التوالى . ثم تم اجراء اختبار تفاعل أنزيم البلمرة المتسلسل الخاص بجينات TEM, SHV, CTX-M على سبع عينات وجد ان اربعة عينات تحتوى على TEM جين وثلاث عينات تحتوى على SHV جين وثلاث عينات بها CTX-M جين تم اجراء اختبار تتابع النيوكليتدى على ثلاث عينات من الأنسان والأبقار والدواجن ومقارنتها بالعترة المرجعية حيث اعطت حزمة مميزة عند TEM جين ٣٦٤، ٣٨٢، ٣٨٣، ٧٠٧، ٧٠٨، ٧٣٧وكانت وبالنسبة الى SHV جين كانت فى الأبقار عند و١١٤، ٢٣٧، ٦٤٣، ٦٣٥ وبالنسبة الى CTX-M كانت فى كل من الأنسان والابقار عند ٣٦٤، ٣٨٢، ٣٨٣، ٧٠٧، ٧٠٨، ٧٣٧ و٢٨٦، ٢٨٧ على الترتيب.