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Comparative study on prevalence and association of some virulence factors with extended spectrum beta-lactamases and AmpC producing *Escherichia coli*

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The aim of this study was to gain further insight into the interplay between both extended spectrum β-lactamase (ESBL) and AmpC enzymes and different virulence factors (VFs) among *Escherichia coli* (*E. coli)* isolated from Mansoura University Hospitals, Egypt. For this purpose 100 *E. coli* isolates were collected from different clinical sources. All isolates were investigated for production of ESBL and AmpC enzymes. The prevalence of VF encoding genes including: *KPsMII* (group2 capsule synthesis), *FyuA* (ferric yersiniabactin uptake), *Afa/dra* (Dr-binding a fimbrial adhesins), *PapA* (P fimbriae), *PapC* (formation of digalactoside-binding Pap pili), *iutA* (aerobactin receptor) and *BssS* (biofilm formation) and associations of these genes with both enzyme types were analyzed by polymerase chain reaction. ESBL was produced by 37% of the isolates while AmpC enzyme was produced by 29%. Virulence genes prevalence among ESBL phenotypes were 43.2% *KPsMII*, 51.35% *FyuA*, 62.1% *Afa/dra*, 43.2% *PapA*, 16.2% *PapC*, 67.56% *iutA* and 78.4% *BssS*. Regarding AmpC phenotypes, the prevalence of virulence genes were: 51.7% *KPsMII*, 86.2% *FyuA*, 68.96% *Afa/dra*, 55.17% *PapA*, 24.1% *PapC*, 82.7% *iutA* and 100% *BssS*. Of the tested virulence factor encoding genes, *BssS*, *FyuA* and *iutA* were significantly more prevalent among AmpC producers. In addition, AmpC producers exhibited a statistically significant higher prevalence of multivirulence (MV) (MV≥4) than ESBL producers. Furthermore, AmpC phenotypes showed very significantly higher expression (P<0.01) of *BssS* gene than ESBLs phenotypes. Our results suggest a correlation between AmpC phenotypes and production of some factors that are reported to be involved in the virulence of *E. coli*.

Key words: extended spectrum β-lactamase, AmpC enzymes, virulence factors, *Escherichia coli.*

INTRODUCTION

Great attention has been paid to antimicrobial resistance due to morbidity and mortality from diseases caused by resistant bacteria. β-lactams are the most predominant antimicrobials for the treatment of infectious diseases. Bacterial resistance to this class of antibiotics is mediated by the production of β-lactamase enzymes (Paterson and Bonomo, 2005), sometimes in association with other mechanisms that might lead to either diminished permeability or active efflux (Poole, 2004).

Numerous β-lactamases are known and more continue

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to be described. Of particular clinical and epidemiological importance are extended-spectrum β-lactamases (ESBLs) and AmpC β-lactamases, which are capable of inactivating the effects of broad –spectrum cephalosporins and penicillins. ESBLs are enzymes that hydrolyze penicillins, cephalosporins, aztreonam and are susceptible to clavulanic acid (Paterson and Bonomo, 2005). Resistance mediated by these enzymes is now observed in all species of Enterobacteriaceae and currently disseminated worldwide (El Naggar et al., 2010; Bourjilat et al., 2011). AmpC are clinically important because they confer resistance to cephamycins such as cefoxitin and cefotetan, in addition to narrow, expanded and broad spectrum cephalosporins and aztereonam, moreover they resist inhibition by clavulanic acid (Jacoby, 2009) but they are inhibited by cloxacillin and 3-aminophenylboronic (Tan et al., 2009). In Gram-negative bacteria, AmpC β-lactamases production is either chromosome or plasmid mediated. Plasmid-mediated AmpC β-lactamases have been detected in some isolates of *Klebsiella* species, *Salmonella* species, *Citrobacter freundii*, *Proteus mirabilis*, *Enterobacter aerogenes* and *Escherichia coli* (*E. coli*) and are usually associated with multidrug resistance. Accurate prevalence data of plasmid-mediated AmpC β-lactamases are limited due to lack of testing, but generally they are less common than ESBLs (Jacoby, 2009).

E. coli is one of the commensal bacteria in human intestinal tract. However, the high plasticity of the genome of this species via gene loss or gain, through lateral gene transfer gives it an enormous capability to evolve, leading to the evolution of pathogenic strains from the commensal cohorts (Kaper et al., 2004).

The virulence factors (VFs) help in the survival of *E. coli* under adverse conditions, which allows *E. coli* to cause extraintestinal infections (Johnson and Russo, 2002). *E. coli* possess a broad range of virulence-associated factors including toxins, adhesions, lipopolysaccharides, polysaccharide capsules, proteases and invasions (Mokady et al., 2005).

VFs comprise mechanisms that allow pathogenic bacteria to cause infections. Genomics offers a good tool for defining virulence factors as it can be used to recognize genes encoding for specific factors that contributes to virulence of pathogens. However, the presence of one factor rarely makes an organism virulent; a combination of different factors will determine if an organism can cause infection (Dobrindt, 2005).

The treatment of infection caused by *E. coli* has been complicated by the emergence of antimicrobial resistance (Da Silva and Mendonça, 2012). β-Lactam antibiotics, especially the third generation cephalosporins, are essential drug class used to treat severe community-onset or hospital-acquired infections caused by *E. coli*. Among *E. coli*, production of β-lactamase enzymes still represents the most important mediator of β-lactam resistance (Livermore and Woodford, 2006). Most importantly is the increasing numbers of *E. coli* isolates producing “newer β-lactamases” which includes the extended- spectrum β-lactamases and plasmid-mediated AmpC β-lactamases (Jacoby and Munoz-Price, 2005). The dissemination of such resistance is associated with genetic mobile elements, such as plasmids, that may also carry virulence determinants and transmit them (Da Silva and Mendonça, 2012). This will necessitate the studying the interplay between antimicrobial resistance and bacterial virulence determinants.

The present study has been undertaken to gain further insight into the interplay between both ESBL and AmpC enzymes and different VFs among *E. coli* isolated from Mansoura University Hospitals, Egypt. For this purpose the prevalence and associations of VFs among ESBLs and AmpC producing *E. coli* was investigated.

MATERIALS AND METHODS

Isolation and identification of bacterial isolates, media and growth requirements

A total of 180 clinical samples were collected from Mansoura University Hospitals Dakhlia governorate, Egypt over a period of 6 months from January 2013 until June 2013. These isolates were obtained from different clinical sources including pus, urine and sputum. Hundred isolates were identified biochemically as *E. coli* based on Gram staining, colony morphology on MacConkey's agar and laboratory biochemical standards (Crichton, 1996). The experimental protocol conducted in the study complies with the ethical guidelines and the principals of care, use and handling of human subjects in medical research adopted by "The research Ethics Committee, Faculty of Pharmacy, Mansoura University, Egypt which is in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki involving use and handling of human subjects).

Phenotypic detection of ESBL enzymes

E. coli isolates were tested for ESBL production using modified double disc synergy test (DDST) by using a disc of amoxicillin-clavulanate along with three cephalosporins discs; cefotaxime (30 μg), ceftazidime (30 μg) and ceftriaxone (30 μg). A disc which contained amoxicillin-clavulanate was placed in the centre of the plate. The discs of cefotaxime, ceftazidime and ceftriaxone were placed around amoxicillin-clavulanate disc keeping the distance of 16-20 mm from it (centre to centre) on Mueller-Hinton agar plate inoculated with suspension equivalent to 0.5 McFarland standards. The plate was incubated at 37ºC for 24 h. When the zone of inhibition around any of these cephalosporins discs showed a clear-cut increase towards the amoxicillin-clavulanate disc, the organism was considered as positive for ESBL production (Paterson and Bonomo, 2005).

Phenotypic detection of AmpC enzymes

The isolates were tested by three dimensional extract method for the production of AmpC enzymes. The surface of the Mueller-Hinton agar plate was inoculated with *E. coli* DH5α. Cefoxitin disc (30 μg) was placed on the plate. Crude enzyme was extracted from nutrient agar slope culture of the tested isolate as described previously (Livermore et al., 1984). Linear slits were cut 3 mm away

Table 1. Oligonucleotide primers used to amplify the tested genes.

Gene name	Type	Sequence
PapA	Fw	ATGGCAGTGGTGTCTTTTGGT
	Rv	GATAAGTCAGGTTGAAATTCGCAA
PapC	Fw	TGGATTGTCAGCCTCAAGGTCTA
	Rv	CACTGACGCCGAAAGACGTA
iutA	Fw	ATCAGAGGGACCAGCACGC
	Rv	TTCAGAGTCAGTTTCATGCCGT
Afa/dra	Fw	ACCCGACGCCGTTTTACATCAACCTG
	Rv	CCCTTCCCGCCACCTTTCAGCA
KpsMTII	Fw	GCGCATTTGCTGATACTGTTG
	Rv	CATCCAGACGATAAGCATGAGC
fyuA	Fw	ATACCACCGCTGAAACGCTG
	Rv	CGCAGTAGGCACGATGTTGTA
BssS	Fw	GATTCAATTTTGGCGATTCCTGC
	Rv	TAATGAAGTCATTCAGACTCATCC
V1to V6 region of 16S-rRNA	Fw	AGAGTTTGATCMTGGCTCAG
	Rv	ACGAGCTGACGACARCCATG

from the edge of cefoxitin disc, small circular wells were made inside the outer edge of the slit. The well was loaded approximately with 30-40 µl of enzyme extract. The plate was kept upright for 5-10 min until the solution dried and was then incubated at 37 ºC for 24 h. The isolates which showed clear distortion of inhibition zone around cefoxitin disc were reported as AmpC β-lactamase producers (Manchanda and Singh, 2003).

Molecular screening of some virulence genes among ESBL and AmpC producers

ESBL and AmpC producers were analyzed by PCR for the presence of seven virulence factors associated genes: *KPsMII* (group2 capsule synthesis), *FyuA* (ferric yersiniabactin uptake), *Afa/dra* (Dr-binding a fimbrial adhesins), *PapA* (P fimbriae), *PapC* (formation of digalactoside-binding Pap pili), *iutA* (aerobactin receptor) and *BssS* (biofilm formation). PCR analysis of tested genes was performed using specific primers listed in Table 1. Genomic DNA was prepared by picking up one – two colonies were grown overnight on nutrient agar medium then they were resuspended in 100µl of nuclease free water and heated to 95 ºC for 10 min. A reaction mixture containing 0.5 µM of each primer, 1.5 Mm $Mgcl_2$, 0.2 Mm dNTPs, 1 U Taq polymerase (Thermoscientific Dream Taq Green DNA polymerase), 5 µl of DNA and nuclease free water was added for a total volume of 25 µl per reaction.

The PCR program consisted of an initial denaturation step at 94 ºC for 3 min, followed by 40 cycles of DNA denaturation at 94 ºC for 30 s, annealing at 68 ºC for *Afa/dra*, at 48 ºC for *BssS* gene and at 60 ºC for other gene, then extension at 72 ºC for 1 min. This is followed by a final extension step at 72 ºC for 7 min.

An aliquot of each reaction was analyzed on a 1% agarose gel stained with ethidium bromide and compared with 100 bp DNA molecular weight ladder to verify the success of the PCR reaction after visualization under UV illumination.

Quantitative real time-PCR

Total RNA was extracted from the tested cultures using Trizol reagent (Sigma, USA) according to the manufacturer's instructions. The concentration and the purity for each RNA sample were determined spectrophotometrically at 260 and 260/280 nm ratio respectively, using NanoDrop (ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, Delaware, USA). One microgram of RNA was then reverse transcribed into complementary DNA (cDNA) using QuantiTect Reverse Transcription kit, QIAGEN, USA (according to the manufacturer's instructions).

The expression level of biofilm in both AmpC and ESBL clinical isolates was estimated using the *BssS* primer listed in Table 1. Amplification and expression were performed using 5x FIREPol EvaGreen, qPCR Mix, ROX Dye; Solis BioDyne (according to the manufacturer's instructions). The reaction mixture was prepared as following: (4 µl of FIREPol ® EvaGreen ®qPCR Mix, 100 nM of forward and reverse primers, and 12 µl of RNase-free water). The prepared master mix was distributed as 18 µl in each tube. Control tubes were set as no template control (NTC). To each tube, 2 µl (100 ng) of the template cDNA (sample tubes) or 2 µl of RNase-free water (NTC tube) were added to the master mix and mixed well to give a final volume of 20 µl.

RT-PCR was performed using Rotor Gene Q thermocycler (QIAGEN, Hilden, Germany) programmed as follow: initial denaturation at 95°C for 15 min, followed by denaturation at 95°C for 15 s, annealing at 48°C for 30 s and extension at 72 °C for 1 min for 40 cycles. The expression of the gene in the samples were measured relative to standard sample and were analyzed with NTC sample. Expression of the target genes was normalized to the

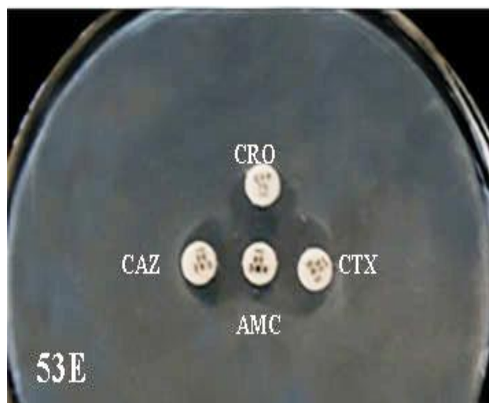

Figure 1. Modified double-disk synergy test on Mueller-Hinton agar plate of isolate 53E. The inhibition zone around all cephalosporins discs showed a clear-cut increase towards the amoxicillin-clavulanate disc (presumptive extended spectrum extended spectrum β-lactamase producer).

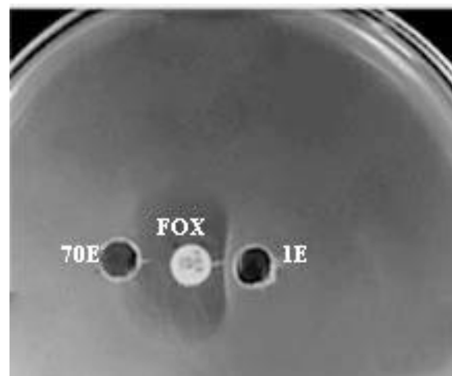

Figure 2. Three-dimensional test on Mueller-Hinton agar plate of isolate 1E and 70 E. Isolate 1E showed clear distortion of inhibition zone around cefoxitin disc (presumptive AmpC producer). No distortion was noted for isolate 70E and it was considered non AmpC producer.

expression of reference gene *16S-rRNA*.

Statistical analysis

Data concerning the presence of various virulence factors associated genes in ESBL and AmpC groups was analyzed using the $\chi 2$ test. Differences were considered significant at $p \leq 0.05$.

Regarding relative expression of *BssS* gene, the Excel data analysis package was used to calculate mean, standard deviation of the mean and standard error. Data was analyzed using t test between ESBL and AmpC groups by the GraphPad Instate software package (version 3.05). Differences were considered significant at p value ≤ 0.05.

RESULTS

A total of 180 clinical isolates were collected from different patients distributed among Mansoura University Hospitals, Dakahlia Governorate, Egypt. One hundred isolates were identified as *E. coli* using standard biotyping methods. Most *E. coli* isolates were obtained from urine (60%) while the remaining isolates were 23 from wound and 17 from sputum.

Phenotypic detection of ESBL and AmpC enzymes

Detection of ESBL activity using modified double disc synergy test method indicated that 37% of the tested isolates were considered positive for ESBL production (Figure 1) and 63 isolates exhibited negative results. On the other hand, detection of AmpC activity using three dimensional extract method have shown that 29% of the tested isolates were considered positive for AmpC production (Figure 2) and 71 isolates were AmpC non producers. None of the tested isolates produced both type of enzymes.

Virulence factors associated genes among ESBLs and AmpC phenotype

The distribution of different virulence factors associated genes among ESBLs and AmpC producing isolates are illustrated in Table 2. PCR detection of *KPsMII* gene reveled that it was harbored by 43.2 and 51.7% of ESBLs and AmpC producing isolates respectively with amplicon size of 269 bp (Figure 3a).

Regarding *FyuA* gene it was detected in 51.35 and 86.2% of ESBLs and AmpC producing isolates respectively with amplicon size of 277bp (Figure 3b).

For *Afa/dra*, it was harbored by 62.1 and 68.96% of ESBLs and AmpC producing isolates respectively with amplicon size of 380 bp (Figure 3c).

Regarding genes for pili it was found that *PapA* is more predominant than *PapC*. For *PapA*, it was harbored by 43.2% and 55.17% of ESBLs and AmpC producing isolates respectively with amplicon size of 519bp (Figure 3d). While, *PapC* was detected only in 16.2% and 24.1% of ESBLs and AmpC producing isolates respectively with amplicon size of 319bp (Figure 3e).

For *iutA*, it was detected in 67.56 and 82.7% of ESBLs and AmpC producing isolates respectively with amplicon size of 253 bp (Figure 3f).

Regarding *BssS* gene for biofilm formation, it is the most predominant among the tested genes. It was harbored by 78.4 and 100% of ESBLs and AmpC producing isolates respectively with amplicon size of 210 bp (Figure 3g).

Among AmpC producing isolates, it was found that four isolates harbored all the tested virulence factors

Table 2. Distribution of different virulence factors associated genes among ESBLs and AmpC producing isolates.

Pattern	MVS	VS	Virulence factor genes							Isolate code	Enzyme type
			BssS	***iutA***	***PapC***	***PapA***	***Afa/dra***	***FyuA***	***KPsMII***		
P1		4	+	+	-	-	+	+	-	1E	
P2		5	+	-	-	+	+	+	+	2E	
P1		4	+	+	-	-	+	+	-	3E	
P3		7	+	+	+	+	+	+	+	4E	
P4		4	+	+	-	+	-	+	-	5E	
P5		6	+	+	+	+	-	+	+	6E	
P1		4	+	+	-	-	+	+	-	7E	
P6		6	+	+	-	+	+	+	+	8E	
P7	4.68	4	+	-	-	-	+	+	+	9E	
P1		4	+	+	-	-	+	+	-	10E	
P8		5	+	+	-	-	+	+	+	11E	
P9		3	+	-	-	+	-	+	-	12E	
P3		7	+	+	+	+	+	+	+	13E	
P3		7	+	+	+	+	+	+	+	14E	
P10		3	+	+	-	-	-	-	+	15E	AmpC
P11		2	+	+	-	-	-	-	-	16E	
P12		5	+	+	-	+	+	+	-	17E	
P8		5	+	+	-	-	+	+	+	18E	
P13		3	+	+	-	-	+	-	-	19E	
P14		4	+	+	-	-	+	-	+	20E	
P15		4	+	+	-	-	-	+	+	21E	
P12		5	+	+	-	+	+	+	-	22E	
P16		3	+	+	-	-	-	+	-	23E	
P17		5	+	-	+	+	-	+	+	24E	
P2		5	+	-	-	+	+	+	+	25E	
P12		5	+	+	-	+	+	+	-	26E	
P3		7	+	+	+	+	+	+	+	27E	
P12		5	+	+	-	+	+	+	-	28E	
P18		5	+	+	+	+	-	+	-	29E	
P19		1	-	-	-	-	+	-	-	30E	
P12		5	+	+	-	+	+	+	-	31E	
P20		3	+	-	-	+	+	-	-	32E	
P21		2	-	-	-	+	-	+	-	33E	
P6		6	+	+	-	+	+	+	+	34E	
P12	3.62	5	+	+	-	+	+	+	-	35E	
P22		5	+	+	-	+	+	-	+	36E	
P23		2	+	-	-	-	+	-	-	37E	
P24		3	-	+	+	-	+	-	-	38E	
P13		3	+	+	-	-	+	-	-	39E	
P25		5	-	+	+	+	+	-	+	40E	Extended spectrum β-lactamae
P26		4	+	+	-	+	+	-	-	41E	
P27		3	+	-	-	+	-	-	+	42E	
P16		3	+	+	-	-	-	+	-	43E	
P28		6	+	+	+	-	+	+	+	44E	
P29		3	+	-	-	-	+	-	+	45E	
P16		3	+	+	-	-	-	+	-	46E	
P18		5	+	+	+	+	-	+	-	47E	
P30		1	+	-	-	-	-	-	-	48E	
P16		3	+	+	-	-	-	+	-	49E	

Table 2. Contd.

Pattern	MVS	VS	Virulence factor genes							Isolate code	Enzyme type
			BssS	*iutA*	*PapC*	*PapA*	*Afa/dra*	*FyuA*	*KPsMII*		
P31		-	-	-	-	-	-	-	-	50E	
P32		1	-	-	-	-	-	-	+	51E	
P16		3	+	+	-	-	-	+	-	52E	
P33		6	+	+	+	+	+	+	-	53E	
P8		5	+	+	-	-	+	+	+	54E	
P6		6	+	+	-	+	+	+	+	55E	
P34		3	+	-	-	-	-	+	+	56E	
P6		6	+	+	-	+	+	+	+	57E	
P6		6	+	+	-	+	+	+	+	58E	
P35		5	-	+	-	+	+	+	+	59E	
P14		4	+	+	-	-	+	-	+	60E	
P36		3	+	-	+	-	+	-	-	61E	
P13		3	+	+	-	-	+	-	-	62E	
P4		4	+	+	-	+	-	+	-	63E	
P11		2	+	+	-	-	-	-	-	64E	
P8		5	+	+	-	-	+	+	+	65E	
P32		1	-	-	-	-	-	-	+	66E	

VS, Virulence score, it was calculated for each isolate as the sum of all virulence-associated genes detected; MVS, Mean virulence score, the sum of all the VS of the isolates was calculated, and this sum was divided by the number of isolates to give the mean virulence score.P, Pattern.

associated genes and 82.7% of the isolates contained four or more virulence factors associated genes. On the other hand, none of ESBLs producers harbored all the tested virulence factors associated genes and there were only 45.92% of the isolates containing four or more virulence factors associated genes.

Statistical analysis revealed that there was extreme significant difference in the presence of *BssS* and *FyuA* gene between AmpC and ESBLs producers as P value was < 0.0001. In addition there was a significant difference in the presence of *iutA* gene between AmpC and ESBLs producers as P value was < 0.05. Regarding other VF associated genes there was no significance difference between AmpC and ESBLs producers.

Based on the distribution of the various VF associated genes, all ESBLs producers exhibit 27 patterns, referred to as P, while AmpC producers exhibit 18 patterns (Table 2). P6 and P16 is the most common pattern among ESBLs producers where each is shared by 4 isolates (10.8%). The majority of patterns (77.7%) were detected by single isolate. Regarding AmpC producers P1, P3 and P12 were the most common patterns (each was exhibited by 4 isolates). The distribution of patterns among all studied isolates revealed that 9 patterns were shared by ESBLs and AmpC producers, 18 patterns were detected only in ESBLs producers and 9 patterns were found only in AmpC producers (Figure 4).

Expression of BssS gene

The relative expression of *BssS* gene in randomly selected 14 ESBL and 15 AmpC producers was performed (Figure 5). Statistical analysis revealed that AmpC producing *E. coli* showed very significantly higher expression (P<0.01) of *BssS* gene than ESBLs producing *E. coli.*

DISCUSSION

E. coli is a serious cause of urinary tract infections, enteric infections and systemic infections in humans (Mandell et al., 2005). Pathogenic *E. coli* clones have acquired specific virulence factors, which confer an increased ability to cause a broad spectrum of diseases (Kaper et al., 2004).

The control of *E. coli* infections is complicated due to the increasing resistance to antibiotics. The greater prevalence of resistance to common antibiotics has been reported by Hassan et al. (2010). The production of β-lactamase remains the most important mediator of β-lactam resistance among *E. coli* as ESBL and AmpC β-latamases are important causes of β-lactam resistance among extraintestinal pathogenic *E. coli* (ExPEC) (Pitout, 2012).

Similar to ESBL-producing bacteria, organisms harbored plasmid- mediated AmpC enzymes have mostly been responsible for nosocomial outbreaks on a worldwide although the risk factors associated with infection are not as well defined as those associated with ESBL- producing bacteria (Philippon et al., 2002). This study was therefore designed to investigate the virulence factors of *E. coli* that produce ESBL and AmpC enzymes.

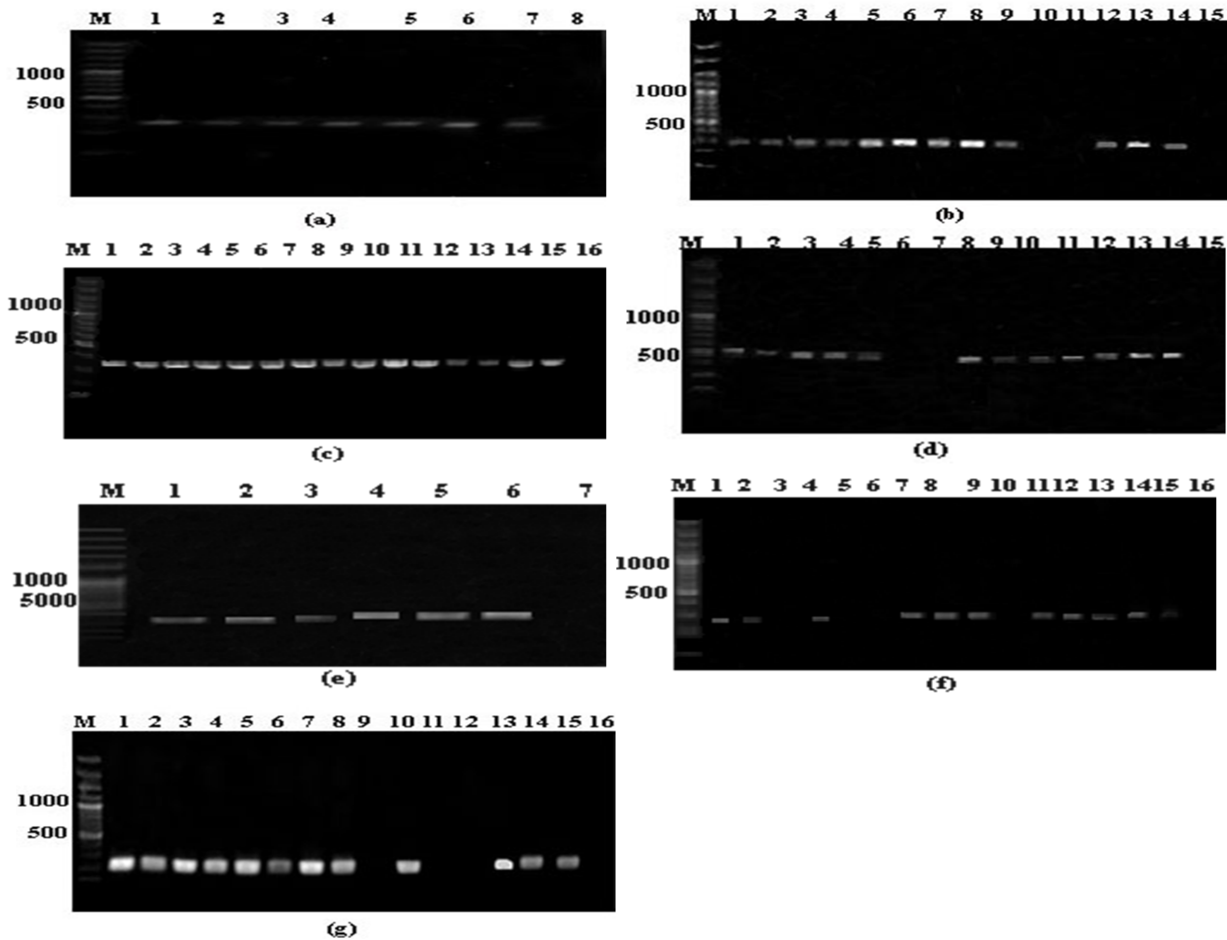

Figure 3. Agarose gel electrophoresis of gene amplicones. a. KpsMT Lane M was 100 bp plus DNA Ladder. Lanes from 1 to 7 were amplicones from *E. coli* isolates No. 18E, 21E, 27E, 40E, 54E, 57E and 60E respectively. Lane 8 was negative control. **b.** fyuA. Lane M was 100 bp plus DNA Ladder. Lanes from 1 to 14 were amplicones from *E. coli* isolates No.1E, 2E, 3E, 4E, 5E, 6E, 7E, 33E, 34E, 41E, 42E, 46E, 47E and 52E respectively. Lane 15 was negative control. **c.** Afa/dra. Lane M was 100 bp plus DNA Ladder. Lanes from 1 to 15 were amplicones from *E. coli* isolates No 8E, 9E, 13E, 14E, 17E, 18E, 19E, 20E, 30E, 31E, 32E, 38E, 39E, 40E and 58E respectively. Lane 16 was negative control. **d.** papA. Lane M was 100 bp plus DNA Ladder. Lanes from 1 to 14 were amplicones from *E. coli* isolates No 4E, 5E, 6E, 14E, 17E, 21E, 23E, 47E, 53E, 55E, 57E, 58E, 59E, and 63E respectively. Lane 15 was negative control. **e.** PapC. Lane M was 100 bp plus DNA Ladder. Lanes from 1 to 6 were amplicones from *E. coli* isolates No.13E, 14E, 29E, 38E, 40E and 61E respectively. Lane 7 was negative control. **f.** iutA. Lane M was 100 bp plus DNA Ladder. Lanes from 1 to 15 were amplicones from *E. coli* isolates No 10E, 11E, 12E, 13E, 24E, 25E, 26E, 27E, 28E, 30E, 49E, 52E, 53E, 54E and 55E respectively. Lane 16 was negative control. **g.** BssS. Lane M was 100 bp plus DNA Ladder. Lanes from 1 to 15 were amplicones from *E. coli* isolates No. 19E, 20E, 21E, 22E, 24E, 25E, 26E, 28E, 30E, 32E, 33E, 50E, 44E, 46E and 53E respectively. Lane 16 was negative control.

Results of our study have shown that ESBL producing *E. coli* was 37% and this result is comparable to those obtained by Chayakulkeeree et al. (2005) who reported that the rate of ESBL producing *E. coli* was 33.3% while a higher level of ESBL producing *E. coli* (60.9%) was reported in Egypt (Al-Agamy et al., 2006). Regarding AmpC enzymes, it was present in lower percentages of isolates (29%) and this result was equivocal with studies carried by El-Hefnawy (2008), Barwa et al. (2012) and Fam et al. (2013) from Egypt where AmpC prevalence was 34, 31.6 and 28.3% respectively while studies from India have shown that 50-75% prevalence of ESBL (Goyal et al., 2009; Chakraborty et al., 2013) and 30-50% prevalence of AmpC production among *E. coli* (Chakraborty et al., 2013). These differences between studies may be assumed to the difference in the study number population, time of collection, types of organisms tested and geographical area (Shah et al., 2004; Thabit et al., 2011).

Extraintestinal pathogenic *E. coli* possess a broad spectrum of specialized VFs responsible for pathogenesis outside the gastrointestinal tract including different adhesions, capsules, toxins, siderophores and invasions. These VFs contribute to fitness, colonization,

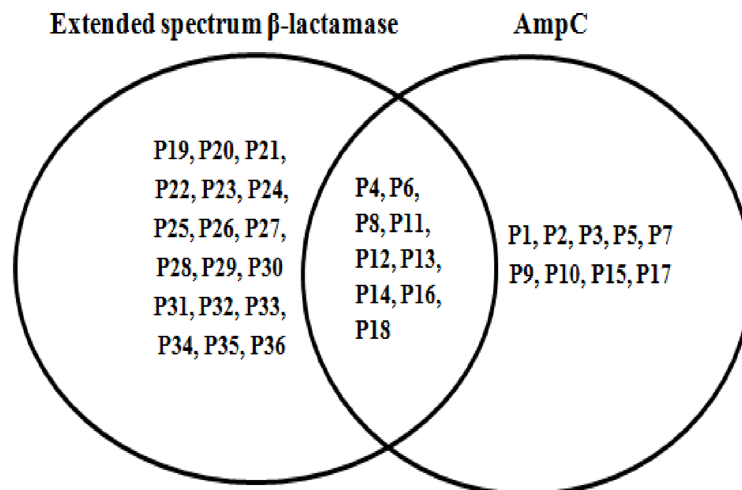

Figure 4. Distribution of virulence gene patterns in relationship with extended spectrum β-lactamase and AmpC phenotypes.

invasion into host tissues, avoidance of immune responses and antimicrobial drugs in addition to acquiring nutrients from the host (Pitout, 2012). Genomics offers an interesting tool for defining virulence factors; it can be used to identify genes encoding for specific factors that are responsible for virulence in pathogens. However, the presence of a single factor rarely makes an organism virulent while combination of factors would determine if a bacterium can cause infection (Dobrindt, 2005). In this study, the detection of several virulence factors associated genes (*KPsMII, FyuA, Afa/dra, PapA, PapC, iutA* and *BssS*) among *E. coli* isolates revealed a higher prevalence in AmpC producers than ESBL producers probably because usually low amounts of AmpC are produced as AmpC is regulated by a weak promoter and strong attenuator (Olsson et al., 1982). Clermont et al. (2008) has suggested that the prevalence of VFs decreased within resistant strains. It was found in this study that biofilm formation gene (*BssS*) and siderophore genes (*fyuA* and *iutA*) were the most prevalent genes in AmpC producers and at the same time Pap pili gene *PapC* was less prevalent as compared to other virulence genes. But among ESBL producers biofilm formation gene (*BssS*) siderophore genes (*iutA*) and adhesion gene (*Afa/dra*) were the most prevalent genes. Similar to AmpC producing isolates Pap pili gene PapC was less prevalent when compared to other virulence genes. In a previous study by Lillo et al. (2014) regarding ESBL producing *E. coli*, *fyuA* and *iuTA* genes were more prevalent, also adhesion gene *afa/dra* was detected in high percentage of strains.

Among the tested VFs in our study, only *BssS*, *fyuA* and *iuTA* genes exhibited a statistically significant higher prevalence among AmpC producers than ESBL producers. Among the investigated isolates, AmpC producers exhibited a statistically significant higher prevalence of multivirulence (MV) (MV≥4) than ESBL producers (Table 2). Our finding suggests that AmpC phenotypes might be more virulent than ESBL phenotypes.

In our study, various combinations of detected genes were designated as virulence patterns. Analysis of virulence patterns distribution in ESBL and AmpC producers did not allow the determination of a clear correlation between a determined genes distribution and the type of β-lactamase enzyme (Figure 4). This is because most of the patterns detected in ESBL and AmpC producers were represented by single isolate. In addition 25% of patterns were shared by both ESBL and AmpC producers.

Lee et al. (2008) had reported that there is a strong relationship between biofilm formation and production of betalactamases. In this study, the relationship between biofilm formation and both ESBLs and AmpC producers was also investigated by detecting the relative expression level of BssS gene in some isolates (Figure 5). It was found that AmpC producers exhibited a statistically very significant higher expression of *BssS* gene than ESBL producers. Swarna et al. (2014) reported that antimicrobial resistance is compounded by their ability to form biofilm that increases their virulence. This could explain our findings where AmpC producers exhibited a statistically significant higher prevalence of multivirulence (MV) (MV≥4) than ESBL producers.

In conclusion, the present results suggest a correlation between AmpC phenotypes and production of some factors that are reported to be involved in the virulence of *E. coli*. In addition *E. coli* isolates producing AmpC are thought to be more virulent than ESBL producers. Investigation of the bacterial pathogenicity associated with ESBL and AmpC phenotypes may contribute to a more clear medical intervention.

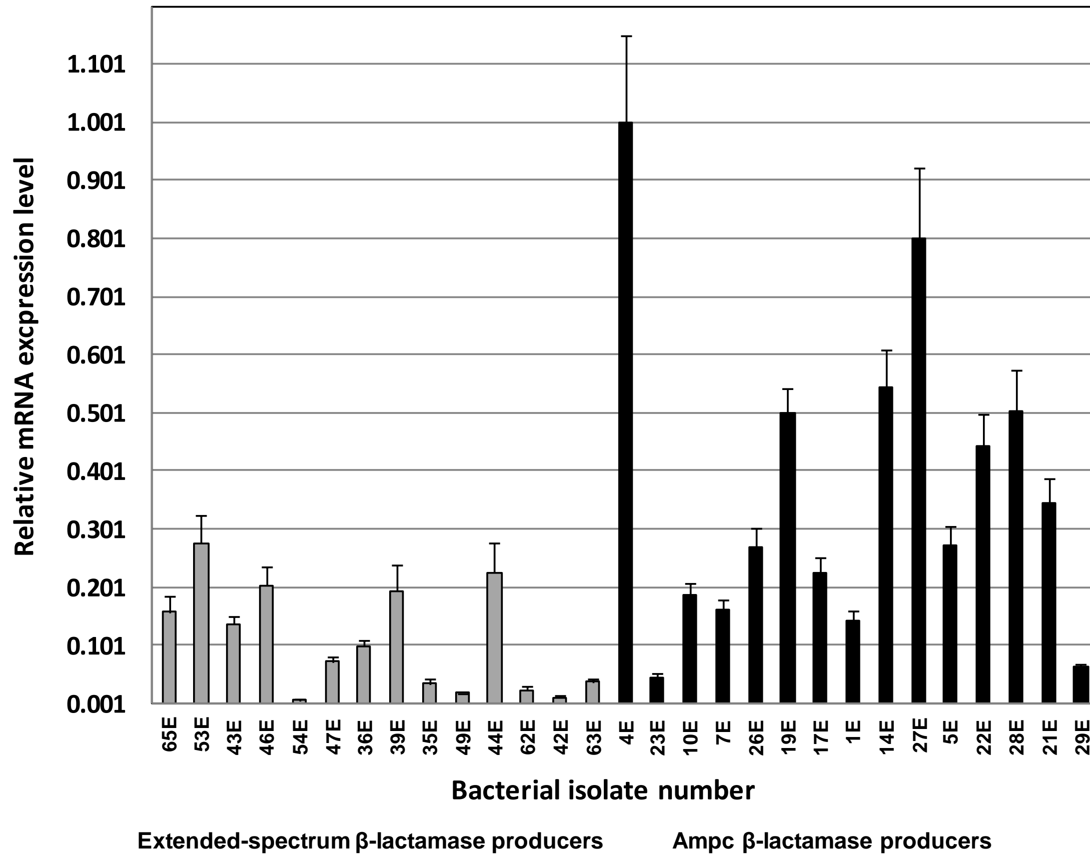

Figure 5. Relative expression of BssS gene in randomly selected 14 extended spectrum β-lactamase and 15 AmpC phenotypes.

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Conflict of interests

The authors did not declare any conflict of interest.

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