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Extended-spectrum β-lactamase *Enterobacteriaceae* from patients in Jeddah, Saudi Arabia: Antibiotic susceptibility and molecular approaches

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Abstract

Objective This study aimed to determine the ***Enterobacteriaceae*** strains among patients who are suffering from diarrhea, phenotypic, and genotypic characterization of extended-spectrum β-lactamase (ESBL)-producing isolates in Jeddah, Saudi Arabia.

Methods Stool samples were collected and cultured to determine the different *Enterobacteriaceae* strains. PCR was done for the isolates to detect the different ESBLs genes and antibiotic susceptibility against different antibiotics.

Results The total number of patients in this study was 200 (114 males [57%] and 86 females [43%]). The patients were categorized to teenagers (21, 10.5%), adults (92, 46%), middle age (25, 12.5%), and elderlies (25, 12.5%) according to age. Five *Enterobacteriaceae* strains were found: *Enterobacter cloaca* (7, 3.5%), *Escherichia coli* (111, 55.5%), *Klebsiella pneumoniae* (75, 37.5%), *Proteus mirabilis* (6, 3.0%), and *Pseudomonas aeruginosa* (1.0, 0.5%). *P. aeruginosa* was absent in all female patients under investigation. The response of the isolated (*E. coli, K. pneumoniae*, and *E. cloaca*) strains to ampicillin, cefotaxime, cefotaxime-clavulanate, ceftriaxone, cephalothin, chloramphenicol, ciprofloxacin, nalidixic acid, streptomycin, tetracycline, and trimethoprim-sulphamethoxazole was highly resistant, while the response was highly susceptible to ampicillin sulbactam, ceftazidime, ceftazidime-clavulanate, and imipenem. The most frequent gene was bla_{CTX-M} (195) followed by bla_{TEM} (149), bla_{SHV} (73), and bla_{OXA} (3), while the highest pair of genes in the same organism was bla_{TEM}+bla_{CTX-M} (134) followed by bla_{SHV}+bla_{CTX-M} (64), bla_{SHV}+bla_{TEM} (52), and the least pairs were bla_{TEM}+bla_{OXA} (3) and bla_{CTX-M}+bla_{OXA} (2). bla_{SHV}+bla_{CTX-M}+bla_{TEM} was found in 44 organisms and bla_{CTX-M}+bla_{TEM}+bla_{OXA} in 2 organisms only. The bla_{SHV}+bla_{OXA}, bla_{SHV}+bla_{CTX-M}+bla_{OXA}, bla_{SHV}+bla_{TEM}+bla_{OXA}, and bla_{CTX-M}+bla_{TEM}+bla_{OXA}+bla_{SHV} were not present in any organism under investigation.

Conclusions In teenager group, there were no organism that contained bla_{OXA} gene, while bla_{OXA} was present in *E. cloaca* and *P. mirabilis* only. bla_{SHV} gene was absent in *E. coli* but present in *E. cloaca* and *K. pneumoniae*. The most susceptible group to infection with *Enterobacteriaceae* was adults' group, while teenage was more resist to infection.

Keywords ESBLs, Antibiotics, Molecular, Virulence genes, *Enterobacteriaceae*

Introduction

The wrong use of antibiotics in medicine has been considered a principal start leading to the emergence of bacteria resist to many antibiotics (multidrug resistance).[1,2] Because of the main sources for transmission of extended-spectrum β-lactamase (ESBL)-producing bacteria to humans are animals, hence some measures were raised recently to reduce antimicrobial agents use in husbandry of animal in Europe.[3] This ESBLs may be transmitted either directly or indirectly by contaminated meat products consumption.[4]

ESBLs bacteria can hydrolyze cephalosporin and penicillin antibiotics by production of β-lactamases enzymes. So, Gram-negative bacteria in the intestine belonging to *Enterobacteriaceae* becomes resistant to these antibiotic classes when they possess an ESBL gene. ESBLs are prevalent globally with more than 1.5 billion people colonized *Enterobacteriaceae* especially with ESBLs.[5] Most of this load falls on the developing countries, but the pervasiveness of organisms producing ESBLs increased in the developed countries.

There are many ESBLs groups with the same attitude but different in evolution. The largest ones are TEM and SHV β-lactamases mutants. Some critical amino acids were affected by the mutation resulting in enlargement in active sites and enable it to shield the ring of β-lactam by deflecting the oxyimino substitutes.[6] CTX-M enzymes is the second largest group. These are divided into five subgroups based on sequence homology. *Enterobacteriaceae* (especially *E. coli*) that produce the CTX-M enzymes have been identified to cause urinary tract causing infections.[7,8] Many studies reported the ESBLs CTX-M-type is the most frequent ESBL type worldwide.[6]

ESBLs are enzymes leading to increase resistance to aztreonam, cefotaxime, ceftazidime, cephalosporins, and penicillin, while clavulanic acid inhibits them. The CTX-M, TEM, and SHV are the three major types of ESBLs. The CTX-M is more prevalent than TEM and SHV has a distribution among a broad range of clinically important bacteria.[7,8]

The critical patients are susceptible to infection particularly, and the nature of causative agents and epidemiology can very extremely. The pathogens that are drug-resistant are considered as a source of concern as they carry a higher mortality and morbidity, and are difficult to be routinely identified by laboratory assays. This leads to delay in diagnosis and finding the appropriate therapy by antimicrobial. There is also a rising worry regarding the new antibiotics deficiency,[9] especially for ESBLs Gram-negative bacteria.

Drug-resistant strains of *Enterobacteriaceae* are important[10]; thus, the aim of the present study was to determine the *Enterobacteriaceae* strains among patients who are suffering from diarrhea, phenotypic, and genotypic characterization of ESBL-producing isolates in Jeddah, Saudi Arabia.

Materials and Methods

Sample collection and isolation

Stool samples were collected from 200 diarrheal patients at different hospitals in Jeddah, Saudi Arabia. The samples were transferred to the laboratory within 2 h for isolation of bacteria. The samples were streaked on MacConkey agar with crystal violet plates (Difco, Detroit, MI, USA). After incubation at 37°C overnight, the colonies were picked up and transferred to new plates. The purified isolates were identified biochemically by the API 20E (Biomerieux, France).

Antibiotic susceptibility and detection of ESBLs

The susceptibility of isolated bacteria to antibiotics was tested by the Kirby-Bauer agar disc diffusion method. Susceptibility to ampicillin (AMP, 10 µg), amoxicillin/clavulanate (AMC, 30 µg), ampicillin sulbactam (SAM, 20 µg), cefotaxime (CTX, 30 µg), cefotaxime/clavulanate (CTL, 30 µg/10 µg), ceftazidime (CAZ, 30 µg), ceftazidime/clavulanate (CAL, 30/10 µg), ceftriaxone (CRO, 30 µg), cephalothin (CEF, 30 µg), chloramphenicol (CHL, 30 µg), ciprofloxacin (CIP, 5 µg), imipenem (IPM, 10 µg), nalidixic acid (NAL, 10 µg), streptomycin (STR, 10 µg), tetracycline (TET, 30 µg), and trimethoprim-sulphamethoxazole (SXT, 25 µg) was determined according to the criteria of the Clinical Laboratory Standards Institute.[11] All antibiotics were purchased from Oxoid (Italy). The double-synergy test was used to screen the ESBL activity of the isolated bacteria.[12]

Characterization of ESBL producing bacteria

Genomic DNA was extracted from the ESBL producing bacteria according to the manufacturer instructions using ***mericon*** DNA Bacteria (Plus) Kit (Qiagen, Valencia, CA, USA). The encoding genes of β-lactamase enzymes was amplified using the primers as follows: bla_{CTX-M} F-ATGTGCAGYACCAGTAARGTKATGGC, R-TGGGTRAARTARGTS ACCAGAAYCAGCGG (593 bp); bla_{OXA} F-ACACAATACATATCAACTTCGC, R- AGTG TGTTTAGAATGGTGATC (813 bp); bla_{SHV} F-CTTTATCGGCCCTCACTCAA, R-AGGTG CTCATCATGGGAAAG (327 bp); bla_{TEM} F - C G C C G C A T A C A C T A T T C T C A G A A T G A , R-ACGCTCACCGGCTCCAGATTTAT (445 bp). PCR was then performed in total volume (50 µl) reaction mixture: DNA templet (50 ng), dNTPs (0.25mM), $MgCl_2$ (1.5 mM), ***Pfu*** DNA polymerase (0.2 U), primers (50 pmol) and complete to 50 µl with distilled H_2O. The temperature profile included an initial denaturation step at 95°C for 10 min, followed by 35 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 1 min and a final extension step at 72°C for 7 min.

Results

In modern medicine, one of the greatest challenges is antimicrobial resistance.[13,14] The most used classes in the infection treatment caused by Gram-negative pathogenic bacteria are the combination of β-lactam/β-lactamase, cephalosporins, and fluoroquinolones inhibitor due to their safety, available in both oral forms and parenteral, and efficacy. The resistance to these agents would restrict the empiric treatment efficacy of Gram-negative infections and also, limit the options of their treatment.

In this study, the total patient's number was 200 (114 males [57%] and 86 females [43%]) (Fig. 1). The patients were categorized to teenagers 13–19 years (21, 10.5%), adults 20–40 years (92, 46%), middle age 41–59 years (25, 12.5%) and elderlies 60 < years (25, 12.5%). It was found that the number of females was greater than males in teenager category only (15 *vs* 6), while the number of males was greater than females in the rest three categories (Table 1, Fig. 2).

Five *Enterobacteriaceae* strains was found in the samples collected where *Enterobacter cloaca* (7, 3.5%), *Escherichia coli* (111, 55.5%), *Klebsiella pneumoniae* (75, 37.5%), *Proteus mirabilis* (6, 3.0%), and *Pseudomonas aeruginosa* (1.0, 0.5%) (Table 2). *E. coli* and *K. pneumoniae* were the most dominant *Enterobacteriaceae* strains in the patients under investigation. The prevalence of *E. coli* was noticed among males of adults, middle age, and elderlies categories in comparing to the other four strains. *P. aeruginosa* was absent in all female patients under investigation, also it is noticed that the high prevalence of *E. cloaca* in both female and male in middle age category (Table 3, Fig. 3).

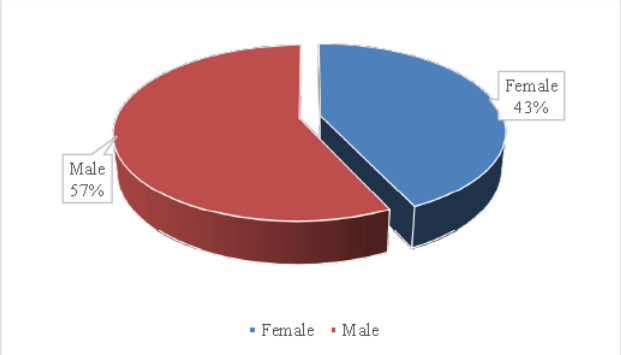

Fig. 1 **Percentage of male and female in the collected samples. (n=8665).**

Table 1. **Age grouping of the patients suffering from diarrhea.**

Category	Age group	Total		Female		Male	
		Number	%	Number	%	Number	%
Teenager	13-19	21	10.5	15	71.4	6.0	28.6
Adults	20-40	92	46.0	38	41.3	54	58.7
Middle Age	41-59	62	31.0	25	40.3	37	59.7
Elderlies	60 <	25	12.5	8.0	32.0	17	68.0
Total	-	200	100	86	43.0	114	57.0

Table 2. **ESBLs producing organisms detected in the collected samples.**

Isolate	Total		Female		Male	
	Number	%	Number	%	Number	%
Enterobacter cloacae	7.0	3.5	3.0	42.9	4.0	57.1
Escherichia coli	111.0	55.5	46.0	41.4	65.0	58.6
Klebsiella pneumoniae	75.0	37.5	33.0	44.0	42.0	56.0
Proteus mirabilis	6.0	3.0	2.0	33.3	4.0	66.7
Pseudomonas aeruginosa	1.0	0.5	0.0	0.0	1.0	100
Total	200	100	84	42.0	116	58.0

Table 3. **Distribution of ESBLs organisms among the group categories.**

Category	*E. coli*		*K. pneumoniae*		*E. cloacae*		*P. aeruginosa*		*P. mirabilis*	
	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male
Teenager	8.0	3.0	7.0	3.0	0.0	0.0	0.0	0.0	0.0	0.0
Adults	19.0	34.0	15.0	16.0	1.0	1.0	0.0	1.0	2.0	2.0
Middle Age	14.0	18.0	8.0	18.0	2.0	2.0	0.0	0.0	0.0	1.0
Elderlies	5.0	10.0	3.0	5.0	0.0	1.0	0.0	0.0	0.0	1.0
Total	46.0	65.0	33.0	42.0	3.0	4.0	0.0	1.0	2.0	4.0
Total	111.0		75.0		7.0		1.0		6.0	

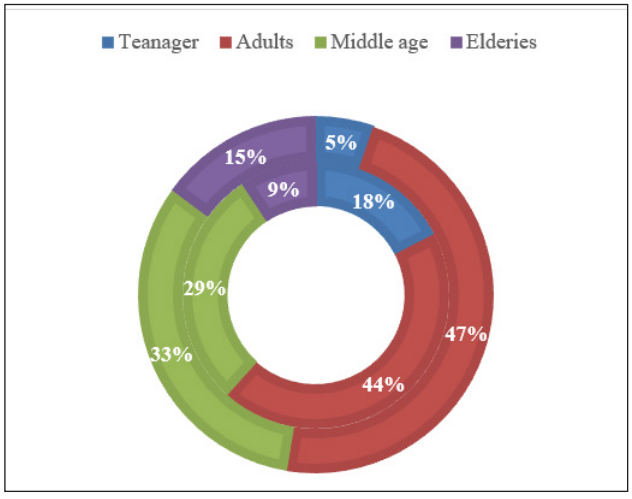

Fig. 2 **Percentage of male and female in the different category. Male: outer circle and female inner circle.**

Discussion

E. coli (65%), *Klebsiella* spp. (25%) *Pseudomonas* (5%), *Enterobacter spp.* (4%), and *Acinetobacter* spp. (2%) were detected in three tertiary care hospitals samples in Lahore, Pakistan.[15]

A few *Enterobacteriaceae* species including *Enterobacter aerogenes, E. cloacae, E. coli, K. pneumoniae, P mirabilis,* and *Serratia marcescens* are responsible for most infections produced by this family.[16,17] The crucial factor in increasing antimicrobial resistance was the production of β-Lactamase in Gram-negative bacteria, like ESBLs CTX-M and SHV and *K. pneumoniae* carbapenemase.[14,18] The broad spreading of the ESBLs has left patients and clinicians with very limited options in the infections treatment caused by multidrug resistant (MDR) *Enterobacteriaceae*.[19–21]

The antibiotic susceptibility test was performed, and the results presented in Table 4. The response of the isolated *E. coli* strains to ampicillin (AMP), cefotaxime (CTX), cefotaxime-clavulanate (CTL), ceftriaxone (CRO), cephalothin (CEF), chloramphenicol (CHL), ciprofloxacin (CIP), nalidixic acid (NAL), streptomycin (STR), tetracycline (TET), and trimethoprim-sulphamethoxazole (SXT) was highly resistant while the response of the isolated *E. coli* strains to amoxicillin-clavulanic acid (AMC) was moderately susceptible, while to ampicillin sulbactam (SAM), ceftazidime (CAZ), ceftazidime-clavulanate (CAL), and imipenem (IPM) was highly susceptible. The response of *K. pneumoniae* strains to AMP, CTX, CTL, CRO, CEF, CHL, CIP, STR, TET and SXT was highly resistant while the response to AMC and NAL was moderately susceptible, and to SAM, CAZ, CAL, and IPM was highly susceptible. The response of *E. cloaca* was highly resistant to AMP, AMC, CTX, CRO, CEF, CHL, NAL, STR, TET and SXT, while the response was moderately susceptible to CTL and CIP, and highly susceptible to SAM, CAZ, CAL, and IPM (Table 4).

Enterobacteriaceae isolates resistant to ceftazidime-avibactam were evaluated for the presence of ESBLs encoding genes.[22,23] More than 99.9% of *Enterobacteriaceae* was inhibited by ceftazidime-avibactam. Only 82.2% of MDR *Enterobacteriaceae* and 64.2% of ceftriaxone-non-susceptible

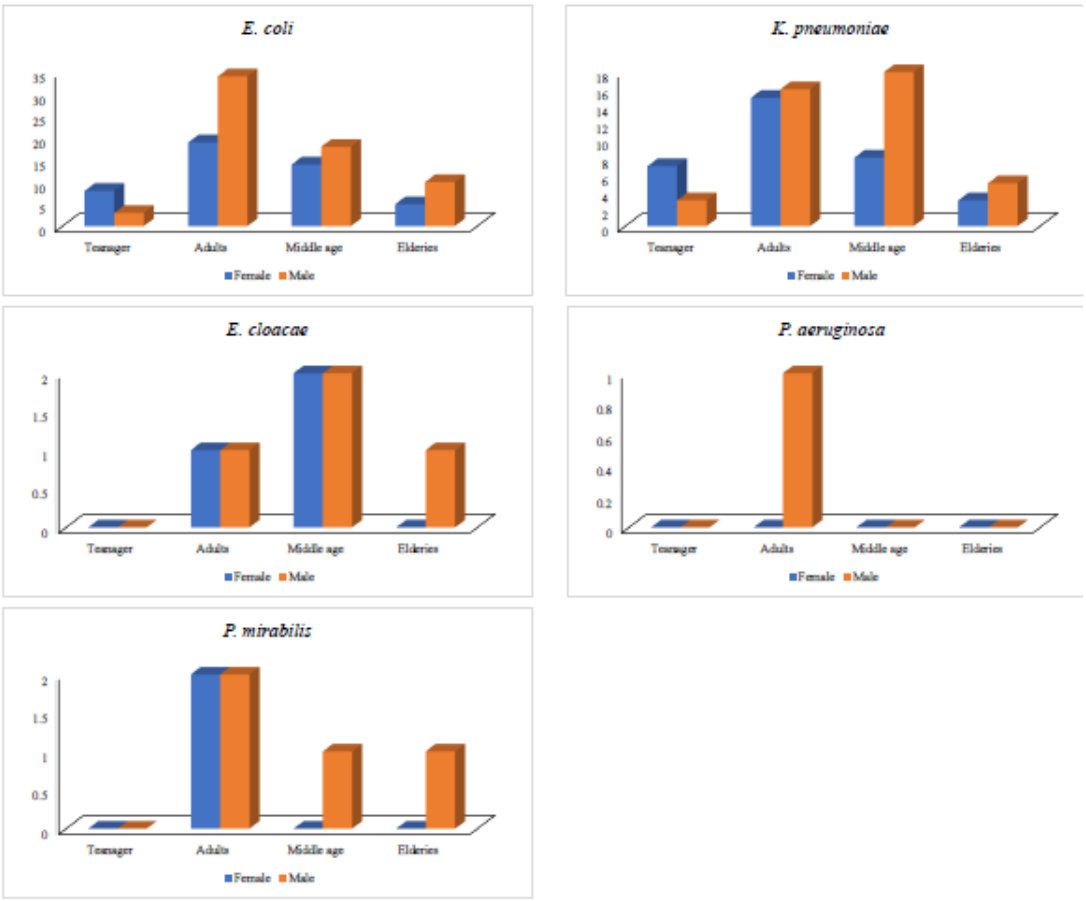

Fig. 3 **The number of the five isolated organisms in different categories.**

Table 4. **The percentage of antimicrobial resistance ESBL producing isolates.**

Antibiotic	Abb.	Conc. (µg)	*E. coli* (n = 111)	*K. pneumoniae* (n = 75)	*E. cloacae* (n = 7)
			n (%)	n (%)	n (%)
Ampicillin	AMP	10	111 (100)	75 (100)	7 (100)
Amoxicillin-clavulanic acid	AMC	30	44 (39.6)	32 (42.7)	7 (100)
Ampicillin sulbactam	SAM	20	11 (10)	11 (14.7)	0 (0.0)
Cefotaxime	CTX	30	100 (90)	64 (85.3)	7 (100)
Cefotaxime-clavulanate	CTL	30/10	88 (79.2)	53 (70.7)	3 (42.9)
Ceftazidime	CAZ	30	11 (10)	11 (14.7)	1 (14.3)
Ceftazidime-clavulanate	CAL	30/10	9 (8.1)	8 (10.7)	0.0 (0.0)
Ceftriaxone	CRO	30	111 (100)	75 (100)	7 (100)
Cephalothin	CEF	30	111 (100)	75 (100)	7 (100)
Chloramphenicol	CHL	30	111 (100)	74 (98.7)	7 (100)
Ciprofloxacin	CIP	5	83 (74.8)	64 (85.3)	4 (57.1)
Imipenem	IPM	10	11 (10)	11 (14.7)	0.0 (0.0)
Nalidixic acid	NAL	10	89 (80.1)	26 (34.7)	6 (85.7)
Streptomycin	STR	10	94 (84.7)	68 (90.7)	7 (100)
Tetracycline	TET	30	100 (90.1)	66 (88)	7 (100)
Trimethoprim-sulphamethoxazole	SXT	25	106 (95.5)	70 (93.3)	7 (100)

Table 5. **The frequency occurrence of ESBL encoding genes in the isolated bacteria.**

Isolate	bla_{CTX-M}		bla_{OXA}		bla_{SHV}		bla_{TEM}	
	Number	%	Number	%	Number	%	Number	%
E. cloacae	9.0	4.5	2.0	66.7	1.0	0.5	11.0	5.5
E. coli	111.0	57.0	0.0	0.0	0.0	0.0	78.0	39.0
K. pneumoniae	74.0	38.0	0.0	0.0	72.0	36.0	59.0	29.5
P. mirabilis	0.0	0.0	1.0	33.3	0.0	0.0	1.0	0.5
P. aeruginosa	1.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0
Total	195	-	3.0	-	73	-	149	-

Table 6. **The prevalence of the coexistence of ESBL encoding genes in the isolated bacteria.**

Genes	*E. cloaca*	*E. coli*	*K. pneumoniae*	*P. mirabilis*	*P. aeruginosa*	Total
bla_{TEM}+bla_{CTX-M}	9	74	51	0	0	134
bla_{SHV}+bla_{CTX-M}	1	0	63	0	0	64
bla_{SHV}+bla_{TEM}	1	0	51	0	0	52
bla_{TEM}+bla_{OXA}	2	0	0	1	0	3
bla_{CTX-M}+bla_{OXA}	2	0	0	0	0	2
bla_{SHV}+bla_{OXA}	0	0	0	0	0	0
bla_{SHV}+bla_{CTX-M}+bla_{TEM}	1	0	43	0	0	44
bla_{CTX-M}+bla_{TEM}+bla_{OXA}	2	0	0	0	0	2
bla_{SHV}+bla_{CTX-M}+bla_{OXA}	0	0	0	0	0	0
bla_{SHV}+bla_{TEM}+bla_{OXA}	0	0	0	0	0	0
bla_{SHV}+bla_{CTX-M}+bla_{TEM}+bla_{OXA}	0	0	0	0	0	0

K. pneumoniae isolates were susceptible to meropenem. Among *E. cloacae* strains (99.8%) were susceptible to ceftazidime-avibactam. Only 0.06% *Enterobacteriaceae* isolates were non-susceptible to ceftazidime-avibactam.[24] A potent activity of ceftazidime-avibactam was shown against *P. aeruginosa, where* 97.1% isolates was susceptible, and 71.8% was inhibited of non-susceptible isolates to ceftazidime, meropenem, and piperacillin-tazobactam.[22,23] Most of the *E. coli* (87.5%) *and K. pneumoniae* (75%) isolates were MDR and showed also MDR phenotypes.[17,21–23]

The most frequent gene was bla_{CTX-M} (195) followed by bla_{TEM} (149), bla_{SHV} (73), and bla_{OXA} (3). The bla_{CTX-M} was the most widespread gene among the *E. coli* strains and in all age categories, while the rarest gene was bla_{OXA} (Table 5). Only *E. coli* and *K. pneumoniae* were detected in teenagers. The presence of these genes in pairs in the same strain was detected, where the highest pair of genes in the same organism was bla_{TEM} + bla_{CTX-M} (134) (*E. coli* (74), *K. pneumoniae* (51) and *E. cloaca* (9)), followed by bla_{SHV} + bla_{CTX-M} (64) (*K. pneumoniae* (63) and *E. cloaca* (1)), bla_{SHV} + bla_{TEM} (52) (*K. pneumoniae* (51) and *E. cloaca* (1)) and the least pairs were bla_{TEM} + bla_{OXA} (3) (*E. cloaca* (2) and *P. mirabilis* (1)) and bla_{CTX-M} + bla_{OXA} (2) in *E. cloaca* only. The bla_{SHV} + bla_{CTX-M} + bla_{TEM} was found in 44 organisms and bla_{CTX-M} + bla_{TEM} + bla_{OXA} in two organisms only. The bla_{SHV} + bla_{OXA}, bla_{SHV} + bla_{CTX-M} + bla_{OXA}, bla_{SHV} + bla_{TEM} + bla_{OXA} and bla_{SHV} + bla_{CTX-M} + bla_{TEM} + bla_{OXA} was not found in any organism under investigation (Table 6).

E. coli strains had 32.4% bla_{CTX-M}, *81%* bla_{TEM}, and 16.2% bla_{SHV} genes, while in *K. pneumoniae strains had* 41.1% bla_{CTX-M}, 64.7% bla_{TEM}, and 35.2% bla_{SHV} genes.[24]

PCR showed that bla_{CTX-M} gene represented by 76% followed by 52% bla_{OXA}, 28% bla_{TEM} and 21% bla_{SHV} were most predominant detected by CDST among ESBLs. The 78% of bla_{OXA}, 65% of $bla_{CTX-M-1}$, and 57% of bla_{TEM} genes were found on plasmids. Amplicon sequencing demonstrated that $bla_{CTX-M-15}$ (75%), bla_{OXA-1} (49%) and bla_{TEM-1B} (34%) and 21 isolates carried three genes in them.[15,25]

Conclusion

In conclusion, in teenager group, there were no organism that contained OXA gene, while OXA present in *E. cloaca* and *P. mirabilis* only. SHV gene was absent in *E. coli* but present in *E. cloaca* and *K. pneumoniae* in this study. The most susceptible group to infection with *Enterobacteriaceae* was adults' group, while teenage group was more resist to infection.

Conflict of Interest

None

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