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FAECAL CARRIAGE OF ESBL AND PLASMID-MEDIATED AMPC B-LACTAMASE GENES IN *KLEBSIELLA* SPP. AND *SHIGELLA* SPP. ISOLATED FROM INPATIENT AND OUTPATIENT CARRIERS IN TABRIZ, IRAN

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ABSTRACT

The growing incidence of community acquired infection due to Extended-Spectrum Beta-Lactamase (ESBL) - and plasmid-mediated AmpC β-lactamase prducing bacteria represent a great concern because there are few therapeutic choices. This study was aimed to determine the prevalence of faecal carriage of ESBL producing *Klebsiella* spp. and *Shigella* spp. among inpatient and outpatient and their phenotype-genotype resistance correlation that conveyed ESBL with or without AmpC genes at a university hospital in Tabriz, Iran. During March to May 2015, 200 faecal samples from hospitalized and non-hospitalized patients without gastrointestinal illness and diarrhea were cultured on MacConkey agar plate supplemented with 2 µg/mL of cefotaxime.

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Bacterial identification, antimicrobial susceptibility testing and ESBLs confirmatory tests were performed according to the standard guidelines. Also, polymerase chain reaction (PCR) was used to identify the genetic determinants responsible for ESBL production (TEM, SHV, and CTX-M) and AmpC β-lactamase genes. Of 200 faecal samples analysed, 23 (11.5%) *Klebsiella pneumonia*, and 4 (2%) *Shigella* spp. were isolated**.** The co-resistance frequencies in isolates recovered were as follows: Ciprofloxacin, 4.3%; Gentamicin, 4.3%; Ceftazidime, 13%; Cefepime, 17.4%; amoxicillin-clavulanic acid, 47.8%; and Ampicillin, 87%. Six (26.08%) of the *Klebsiella* isolates had phenotypic evidence of ESBL production, while bla_{SHV} sequences were detected in 18 (66.6%), and bla_{TEM} in 11 (40.7%) isolates. Also, bla_{CTX-M} and bla_{CMY} sequences were found in 4 (17.39%) and 5 (21.7%) *Klebsiella* isolates, respectively. This study revealed that ESBL production was relatively prevalent in faecal carriage of Klebsiella and Shigella isolates in Tabriz. In addition, Persons attending hospital could be a reservoir of such bacteria and enzymes.

Keywords: *Shigella*; *Klebsiella;* ESBL; Faecal carriage; Iran.

1. INTRODUCTION

Resistance to broad spectrum β-lactams, mediated by extended spectrum β-lactamase (ESBL), and AmpC beta-lactamases (AmpC) are an increasing concern around the world (1). These enzymes often cause improper treatments with increased infections and mortality, medical expenses and prolonged hospital stays (2). Strains that have such genes are typically resistant to several antimicrobial agents and can challenge treatment, as therapeutic options are few.

ESBLs found in Gram-negative bacilli have emerged as a critical mechanism of resistance to oxyimino-cephalosporin antibiotics (3). They are effective against β-lactam antibiotics like ceftriaxone, ceftazidime, cefotaxime and oxyiminomonobactam, but not cephamycins or carbapenems (4). ESBL genes frequently code resistance determinants to different classes of antimicrobial drugs and are also liable for the dissemination of the resistance gene to other Gram-negative bacteria within the hospitals and the community (5, 6). However, AmpCs don't seem to be suppressed via clavulanic acid, are susceptible to fourth-generation cephalosporins. Failure to detect these resistance phenotypes might lead to inappropriate utilization of antimicrobial agents, uncontrolled spread of resistant bacteria and unfavourd clinical outcomes (7).

Faecal colonization via multidrug resistant bacteria is related to future clinical infection. The significance of detection of antimicrobial resistant bacteria carriers has recently been highlighted not only in patient group, but also in healthy people (8). An increase within the proportion of carriers among the community will increase the possibility that other individuals will also become carriers through human-to-human transmission of resistant bacteria or via the environment, enriching the resistance gene pool and therefore facilitating the acquisition of resistance mechanisms by susceptible bacteria (9). In addition, the admission into clinic of patients harbouring resistant bacteria will increase the risk of other hospitalized patient contracting an infection (10).

The ESBLs are generally found on plasmids and most are members of the SHV, TEM and CTX families. Plasmids containing genes encoding for ESBLs frequently incorporate resistance determinants for different classes of antimicrobial agents by having different resistance mechanisms and are easily transmissible from strain to strain and between distinctive species of enteric Gram-negative bacilli (11). ESBL-producing bacteria may be more common in a few parts of Asia, Europe, and South America than in the United States (12).

Despite the high prevalence of ESBL-producing organisms in several parts of the world, information on the prevalence of the extreme infections as a result of such organisms in faecal carriage remain sparse. Subsequently, the point of this research was to look at the prevalence of faecal carriage of drug-resistant *Klebsiella* spp. and *Shigella* spp. in Tabriz, Iran.

2. MATERIAL AND METHODS

2.1. Bacterial Isolation

The current cross-sectional study was conducted from March to May 2015 at Imam Reza Hospital in Tabriz, Iran. A total of 200 fecal samples were studied: 100 from inpatient and 100 from outpatient of hospital, in except Gastroenterology wards, which more than 48 hours have passed from the time of their admission. Those with diarrhea and gastrointestinal illness were excluded from the research. This research was approved by the ethical committee of regional Medical Research of Tabriz University of Medical Science and all patients provided written informed consent for this study (Number: 5/4/12129 -3 Mar.2015). Also, medical and demographic information such as age, gender, occupation, underlying diseases, consumption of

vegetables, previous antibiotic therapy, and recent hospitalization of the patients was gathered utilizing a questionnaire.

Stool samples were spread onto MacConkey agar plate (Merck, Darmstadt, Germany) supplemented with 2 µg/ml of cefotaxime and incubated in ambient air at 35 °C at the least 24 h earlier than initial examination (13). Plates demonstrating no growth in an early examination were incubated for an additional 24 h. The suspected colonies were identified by conventional methods, like Triple Sugar Iron Agar (TSI), Sulfide Indole Motility (SIM), urea, citrate etc. (Merck, Darmstadt, Germany) (14). All isolates other than Shigella and Klebsiella spp. were discarded.

2.2. Antimicrobial Susceptibility testing

The antimicrobial susceptibility test was done by Kirby-Bauer disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI) protocols (15). Antibiogram was done for Ampicillin (10µg), Gentamicin (10µg), Ceftazidime (30µg), Cefepime (30 µg), amoxicillin-clavulanic acid (20/10µg) and Ciprofloxacin (5 µg) antibiotics (MAST Diagnostics, Merseyside, UK).

2.3. **ESBL confirmatory test**

Samples yielding bacteria that grew on Cefotaxime-containing MacConkey agar were initially identified as suspicious for ESBL producing bacteria. The standard CLSI combined disk method involving cefotaxime (CTX) and ceftazidime (CAZ) with and without the inhibitor clavulanic acid (30 µg) (Mast Diagnostics, Merseyside, UK) was used to verify the presence of ESBL (15). ESBL production was indicated by a rise in zone size of more than 5 mm with and without clavulanic acid.

2.4. DNA extraction

DNA extraction was accomplished according to Tissue buffer boiling technique. First, 20µl of Tissue Buffer (0.25% SDS + 0.05M NaoH) was mixed with one colony of bacterial isolate and therefore the combination become incubated for ten minutes in 95°C, after incubation mixture centrifuged for one minute in 13000g and eventually 180µl of MilliQ water was added and extracted DNA freezed in -20°C for durable storage (16).

2.5. Genotypic detection of TEM, SHV and CTX-M ESBLs

To investigate the phenotypic and genotypic correlation, isolates with and without an ESBL phenotype have been selected for detection of β-lactamase encoding genes of the family SHV, TEM, and CTX-M. PCR was done by CINNAGEN MasterMix (Cinnaclon, Tehran, Iran), which performed with the primers that are shown in Table 1, as described previously (17). Amplification was executed in a Gradient MyCycler (Bio-Rad) as follows: 35 cycles of 30 s for denaturation at 95 °C, 30 s for annealing (55°C for TEM, 60°C for SHV and CTX), and 120 s for primer extension at 72°C, followed by a final extension at 72°C for 10 min. Preliminary denaturation at 95°C for 30 s was used for all amplifications. Electrophoresis of PCR products have been accomplished on 1% agarose gel, at 50 volts for 2.5 hours. The gels staining were performed in ethidium bromide for 20 minutes and visualized in gel documentation system (Biorad, UK).

2.6. Genotypic detection of AmpC

Strains hyperproducing AmpC β-lactamases were suspected once resistance to cefotaxime plus clavulanic acid combination disk, and consequently a negative ESBL test were found. PCR was utilized as a molecular test for detection of AmpC plasmid mediate (*cmy*) gene. The related primer is shown in Table 1. The polymerase chain reaction for *cmy* gene was conducted in following conditions: The early denaturation at 94°C for 5 min and 30 cycles of 94°C for 60 s, annealing at 58°C for 60 s, extension at 72°C for 60 s and at last the terminal extension at 72°C for 5 min. Electrophoresis of PCR products and gels staining were performed according to above.

2.7. Statistical analysis:

All statistics have been measured by means of SPSS software version 18.0 (SPSS Inc, Chicago, IL, USA). Comparative information was calculated using the two-tailed $\chi 2$ test and Fisher's exact test, once appropriate. A p-value of ≤ 0.05 was thought to be significant.

3. RESULTS

Of the 200 faecal samples analysed, 204 Enterobacteriaceae isolates were identified that some samples included more than one strain. Of which, 23 (11.5%) isolates were *K. pneumonia* and 4 (2%) isolates were *Shigella* species. Among *Klebsiella* isolates, 14(60.8%) belonging to inpatient and 9 (39.1%) belonging to outpatient. Also, among *Shigella* isolates, 1(25%) belonging to inpatient and 3 (75%) belonging to outpatient. Of all samples, only 26 (13%) did not grow on

MacConkey screening medium. Patient's age ranged from 18 to 85 years old and the ratio between genders was 1:0.62 (males/females). In the case of vegetable consumption, taking one time per week announced by nearly 50 percent of patients. About 35% of patients had a history of cephalosporins use in last 3 months. Also, 33% of them were suffered from underlying diseases such as cancer, asthma, etc. The results of antibiotic susceptibility testing are summarized in Table 2. In general, third- and fourth-generation cephalosporins showed moderate activity on isolates. Also, the resistance rates of isolates to ampicillin and amoxicillin/clavulanic acid were high (87% and 47.8 %, respectively). Six (26.08%) of the *Klebsiella* isolates had phenotypic evidence of ESBL production that of which four patients were hospitalized and two were outpatient. All isolates were subjected to PCR experiments to detect ESBL genes, including bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$ and bla_{CMY}. Regarding PCR results showed that bla_{SHV} and bla_{TEM} genes were the most present in studying bacterial isolates from than $bla_{\text{CTX-M}}$ and bla_{CMY} genes (Figure 1). bla_{SHV} sequences were detected in 18 (66.6%) strains (17 *Klebsiella* and 1 *Shigella*), and bla_{TEM} in 11 (40.7%) isolates (9 *Klebsiella* and 2 *Shigella*). Also, $bla_{\text{CTX-M}}$ and bla_{CMY} sequences were found in 4 (17.39%) and 5 (21.7%) *Klebsiella* isolates, respectively. In our study, the co-expression of CTX-M or CMY together with other enzymes was found in 17.3% of the *Klebsiella* isolates. Conversely, the co-expression of these genes was not determind in *Shigella* isolates. Of all the isolates, 7 (25.9%) isolates had only one gene, which 5 (18.5%) were bla_{SHV} and 2 (7.4%) were bla_{TEM}.

4. DISCUSSION

This study demonstrates the presence of ESBLs and AmpC in faecal strains of *K.pneumonia* and *Shigella* spp. from both inpatient and outpatient of hospital. In the current study, about 11% of cefotaxime resistant isolates were *K. pneumonia,* which demonstrated a relatively high prevalence of ESBL production among carriers in our study population. It frequency were due to the immoderate use of broad-spectrum antibiotics inside the health facility and to a higher stage in our community placing, together with a lack of attention to laboratory screening of ESBL production by clinical isolates.

Despite the fact that carriers of ESBL producers are anticipated to be present in general practice, their occurrence has rarely been reported and there are few research conducted within the community or in health center settings during nonoutbreak situations (18, 19). However, many

reports have addressed faecal carriage of these organisms throughout nosocomial outbreaks (20, 21). Similarly, higher prevalence of ESBL-producing Enterobacteriaceae faecal carriage has been pronounced in the nosocomial setting than in the community (8).

In this study, by analyzing more important resistant phenotypes, we found faecal carriage of resistant strains of *K. pneumonia* was relatively higher than a study conducted in 2012 (22). Also, the prevalence of ESBLs producing *K. pneumoniae* isolates from patients with respiratory tract infections in some major Iran hospitals were investigated in a study by Ghafourian et al. (23). In their study, in Ilam hospitals, 43.6%, in Tehran Milad hospital, 74% and in Tehran Emam Reza hospital, 54.2% were ESBLs producer and among all isolates 94% were positive for bla_{SHV}, 16.4% contained bla_{TEM} and 23.9% harbored $bla_{\text{CTX-M}}$. The statistical difference with our study may be due to differences in sample isolated and hospitalized patients in different parts of the country. In a study from Egypt, the prevalence of ESBLs among *K. pneumonia* isolates separated from patients with suspected nosocomial infections was 21%, which among them, 10% and 53.3% were positive for bla_{SHV} and $bla_{\text{CTX-M}}$ genes respectively (24). Also, in another Egyptian study among healthcare laborers, 29.5% of *E.coli* isolates were resistant to third-generation cephalosporins (25). In their study, investigations confirmed 3% (6/200) were colonized by AmpC-producing *E. coli* and 21% (42/200) were colonized via ESBL-producing *E. coli*. The bla_{SHV} gene was the predominant ESBL gene, identified in 81.8% of the isolates. According to the patient's informations, we can estimate that, usage of cephalosporins prior admission, longer hospital stay and usage of invasive device can be some factors influencing this high rate in hospitalized patients and repeated usage of cephalosporins prior admission, food chain, trade and human migration can be one of the factors influencing this high rate in outpatient. This data is similar to a recent study conducted by Altinkum et al. (26).

Evaluation of clinically important resistance phenotypes among isolates of those bacteria from the patients with nosocomial infections and non-hospitalized patients,assist us to assess distribution of resistant strains among the community (22). However, in our study there was no significant differences between the rate of faecal carriers of ESBL producers among hospitalized patients and outpatients (p-value > 0.05)**.** In addition, though there was no history of recent antibiotic use or hospitalization among the community-dwelling study subjects, many were likely to were exposed to multiple courses of antibiotics because of the unlimited over-the-counter accessibility of these drugs in developing countries.

Presence of resistance factors among the non-pathogenic bacteria in the environment and their transition to the pathogenic bacteria within the community are considered as one of the most imperative procedures for emerging new resistance phenotypes in these bacteria. Despite normally living harmlessly in the intestine, this organisms will cause numerous types of infections. Additionally, community-acquired strains possessing ESBLs might be chosen from the existing gastrointestinal flora, once they are exposed to broad-spectrum antimicrobial agents (19). So, healthy member of a community can act as most important reservoirs for these resistance genes. As well as, considering the extensive use of β-lactam antibiotics as well as third-generation cephalosporins for the treatment of each community- and hospital-acquired infections in Iran, the prevalence of ESBL positive clinical isolates is extremely probable. Therefore, hospitalization of carriers will increase the risk of infection for other hospitalized patients via cross infection (27). Recently, a pharmaceutical medications regimen including colistin introduced, which temporarily suppresses ESBL-Enterobacteriaceae carriage, but had no long-term impact (28). What's more, the long-term impact of decolonization regimens on the gut microbiota may lead to the emergence of colistin resistant strains (29).

Our research has some limitation. Firstly, it should better that sample size used in this study became more. Furthermore, using cefotaxime could have somewhat biased the selection against preferred ceftazidimase activity. Finally, plasmid analysis and sequencing was not conducted.

In conclusion, our study showed that ESBL production was relatively prevalent in faecal carriage of *Klebsiella* and *Shigella* isolates. Therefore, the use of surveillance cultures may be beneficial for monitoring and tracking the spread of ESBL-producing Enterobacteriaceae among the community settings. In addition, healthy carriers will be a reservoir of such bacteria and enzymes. Further drug resistance surveillance in our hospitals and molecular characteristics of ESBL isolates in the country is necessary. This study is important for strict antibiotic policy implementation in hospitals to estimate the effect of higher drug resistance in bacteria and to take steps in reducing this resistance.

5. CONFLICT OF INTEREST

We declare no conflict of interest for the Authors of the present study.

6. ACKNOWLEDGMENT

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Table 1. Oligonucleotide primers used in study

Target(s)	Sequence (5`–3`)	Amplicon size (bp)[a]	Reference
TEM-1	F: 5`-GAGACAATAACCCTGGTAAAT R: 5'-AGAAGTAAGTTGGCAGCAGTG	459	[19]
SHV	F: 5`-GTCAGCGAAAAACACCTTGCC R: 5`-GTCTTATCGGCGATAAACCAG	383	(17)
CTX-M	F: 5`-GAAGGTCATCAAGAAGGTGCG R: 5'-GCATTGCCACGCTTTTCATAG	560	(17)
CMY	F: 5`-ATTCCGGGTATGGCCGT R: 5`-GGGTTTACCTCAACGGC	835	(30)

[a]The numbering indicates the 5` nucleotide of each primer and is according to the sequence referenced.

Table 2. The results of antibiotic susceptibility testing*

		Klebsiella spp. (n= 23)						*Shigella* spp. (n = 4)					
Antibiotic	(µg/disc)	S	%	I	%	R	%	S	%	I	%	R	%
AMP	10	0	0	3	13	20	87	0	0	0	0	4	100
GEN	10	19	82.6	3	13	1	4.3	3	75	1	25	0	0
CAZ	30	19	82.6	1	4	3	13	4	100	0	0	0	0
CPM	30	19	82.6	0	0	4	17.4	4	100	0	0	0	0
AMC	20/10	4	17.3	8	34.7	11	47.8	0	0	2	50	2	50
CIP	5	22	95.6	0	0	1	4.3	3	75	1	25	0	0

AMP: Ampicillin, GEN: Gentamicin, CTX: Cefotaxime, CAZ: Ceftazidime, CPM: Cefepime, AMC: amoxicillin-clavulanic acid, CIP: Ciprofloxacin

*The numbers are written with a decimal point.

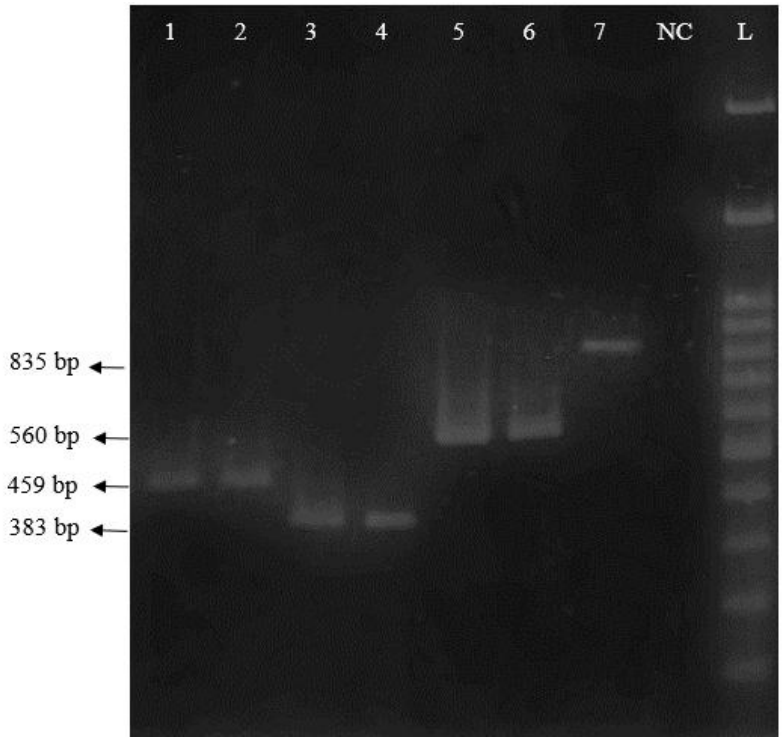

Fig.1. PCR amplification of *bla*$_{SHV}$ (383bp), *bla*$_{TEM}$ (459bp) *bla*$_{CTX-M}$ (560bp) and *bla*$_{CMY}$ (835bp) in *K. pneumoniae* and *Shigella* isolates, 1,2: *bla*$_{TEM}$; 3,4: *bla*$_{SHV}$; 5,6: *bla*$_{CTX-M}$; 7: *bla*$_{CMY}$;NC: negative control; L: 100bp DNA Ladder

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