RESEARCH ARTICLE

Fecal Carriage of ES β L types TEM, SHV, CTX Producing Genera *Proteus*, *Morganella*, *Providencia* in Patients of Iran

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Abstract

Diseases like urinary tract infection, wound infections, bacteremia and other infections are mainly caused by the members of the genus *Proteus*, *Morganella* and *Providencia* which are mainly either found freely in the environment or in the gastrointestinal tract of humans. We studied Fecal carriage of ES β L producing species in carrier patients. Stool samples obtained from outpatients and inpatients not suffering from diarrhea and were cultured in CTX-MC-Conkey agar. Lactose negative and cefotaxime resistant bacteria were identified by biochemical tests and ES β L-producing isolates were detected using Combined Test. TEM, SHV and CTX genes were investigated by PCR. Total 15 (7.35%) isolates of 204 stool samples were identified as ESBL producing *Proteus* spp. (n=4, 1.96%), *Morganella* spp. (n=5, 2.45%) and *Providencia* spp. (n=6, 2.94%). Further, amongst or of the 15 ESBL producing strains, blaTEM was the commonest genotype (86.66%), followed by blaSHV (26.66%) and blaCTX-M (20%). All isolates were resistant to ampicillin, and cefotaxime whereas all *Providencia* and *Morganella* spp. were found to resist ceftazidime. Although the number of ESBL-producing *Proteus*, *Morganella* and *Providencia* isolates from fecal carriers were low, but still, they can be considered as a reservoir of TEM, SHV and CTX genes and capable to transfer these resistant bacteria to hospitals.

Introduction:

Species of the *Proteus*, *Morganella* and *Providencia* genus are facultative anaerobic gram-negative bacilli that belong to the Enterobacteriaceae family and are found in the open environment, waste water and gastrointestinal tract of mammals, humans and animals (O'Hara *et al.*, 2000). These organisms are one of the main causes of infections such as UTI (urinary tract infection), respiratory tract, wounds, bacteremia and other opportunistic infections and perhaps act as infection sources for extended-spectrum beta-lactamases (ESBLs) productions in both society and hospitals. Some studies have shown that the ESBL and AmpC producing isolates of *P. mirabilis* can be a cause of clonal spread in the hospital, regional and continent-wide outbreak (Nakano, *et al.*, 2012). *Providencia stuartii* has been reported to contain ESBL enzymes such as TEM, SHV or CTX-M (Aubert *et al.*, 2005; Franceschini *et al.*, 1998). Based on Ambler

classification, the class A includes three important genes of SHV, TEM, CTX-M; which are commonly found in the Enterobacteriaceae family (Lagacé-Wiens *et al.*, 2007). The first plasmid beta-lactamase in Gram-negative bacteria was TEM-1 that identified in the early 1960s (Bradford, 2001). CTX-M and TEM are common ESBLs that are found in isolates of *Proteus*, *Morganella* and *Providencia* (Tumbarello *et al.*, 2004) and in recent years, SHV has been reported in Iran (Malekjamshidi *et al.*, 2010). All the three bacteria are normal flora of the gastrointestinal and transferred through endogenous or spreading from person to person, especially in hospitalized patients. The epidemiological analysis suggests that ESBL-producing Enterobacteriaceae species such as *Proteus*, *Morganella* and *Providencia* could be isolated in different environments of hospitals, human feces, infectious and healthy carriers, uncooked foods, and human sewage (Mesa *et al.*, 2006). Fecal carriage of ESBL-

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producing strains has not been studied enough in most of the Asian countries, including Iran. The aim of this study was to isolate and determine the types of ESBL (TEM, SHV, and CTX) produced by *Proteus*, *Morganella*, *Providencia* isolated from patients fecal carriers of teaching and treatment hospital of Tabriz.

Methods and Materials:

Bacterial isolates: in between November 2014 to February 2015, we collected 204 stool samples from non-hospitalized (n=100) and hospitalized patients (after 48 hours of admission; n=104). Patients suffering from gastrointestinal illness and diarrhea were excluded from the study. Stool samples obtained were cultured in MacConkey agar contain 2mg/L cefotaxime (CTX-MacConkey) and were incubated at 37°C for 24 hours. Lactose-negative isolates were collected and were identified by routine biochemical tests such as motility, urea hydrolysis, citrate utilization, phenylalanine deaminase, arginine decarboxylase and other necessary tests. Identified isolates were stored at -20°C in trypticase soy broth containing 12% glycerol (Luvsansharav *et al.*, 2012).

Antibiogram and detection of ESBL producing isolates: disk diffusion tests on isolates were carried out using Mueller-Hinton agar plates and antibiotic discs including ampicillin (10 µg), cefotaxime (30 µg), ciprofloxacin (5 µg), gentamycin (10 µg), ceftazidime (30 µg), ceftazidime (30 µg) and cefepime (30 µg). All the plates were incubated at 37°C for 24 hours. Zones of inhibition around the disc were recorded using Clinical Laboratory Standards Institute instruction (CLSI, 2014). All isolates resistant to ampicillin, ceftazidime and cefotaxime were evaluated to confirm ESBL production by combined disc test (CDT). Ceftazidime (30 µg), ceftazidime+ clavulanic acid (30+10 µg), were placed on Muller medium and after 24 hours of incubation at 37°C, increasing more than 5mm inhibition zone around the disk containing clavulanic acid compared to without clavulanic acid confirmed as ESBL producing isolates (CLSI, 2014) (Figure 1).

Figure 1: Phenotypic detection of ESBL-producing isolates; increasing more than 5mm inhibition zone around the disc containing cefotaxime + clavulanic acid (CEC, 30/10µg) compared to cefotaxime (CTX, 30µg) are confirmed as ESBL producing isolates.

Detection of TEM, SHV, CTX gene all bacterial isolates were grown for 24 hours at 37°C in Lauria-Bertani (LB) broth. DNA of isolates were extracted by sodium dodecyl sulphate-proteinase K modified with N, N, N-trimethyl ammonium bromide (Ranjbar *et al.*, 2007). The PCR reacted with specific primers for amplification of 569bp and 293 bp and 403 bp fragments (Bali *et al.*, 2010):

blaCTX-M F: 5' - CGCTGTTGTAGGAAGTGTG - 3'
R: 5' - GGCTGGGTGAAGTAAGTGAC - 3' (569 bp)
blaSHV F: 5' - CGCCTGTGTATTATCTCCCT - 3'
R: 5' - CGAGTAGTCCACCAGATCCT - 3' (293 bp)
blaTEM F: 5' - TTTCGTGTCGCCCTTATCC - 3'
R: 5' - ATCGTTGTCAGAAGTAAGTTGG - 3' (403 bp)

The amplification was done in a DNA thermal cycler (Eppendorf master cycler gradient, Germany), programmed for a primary denaturation, (95°C, for 3 minutes), followed by 35 cycles of denaturation (94°C, 45s), 30 seconds for annealing (60°C for SHV and CTX-M, 55°C for TEM), elongation (72°C, one minute), and then extension (72°C, 10 minutes). A negative control without template was included in each PCR run. The amplified products were visualized by electrophoresis on 1.2% agarose gel in 1x TBE buffer (1 M Tris, 0.9 M boric acid, 0.01 M EDTA, pH = 8.4), at 80 V, for two hours. A 100-bp DNA ladder was used as a molecular mass marker. The gels were stained with ethidium bromide (0.5 µg mL⁻¹) and photographed on a gel documentation system (UVP, USA) for the analysis of the bands (all the PCR materials including primers were provided by CinnaGen; Nedayeh Fan Co., Iran). The total volume of PCR mix was 25 µl, including sterile redistilled H₂O 17.05µl, 10X PCR buffer 2.5µl, dNTP mix (10mM) 0.5µl, MgCl₂ (50mM) 0.75µl, forward primer (25µM) 0.5µl and reverse primer (25µM) 0.5µl for each gene, Taq DNA polymerase (5U/µl) 0.2µl, template DNA 3µl. Negative controls contained all components except template DNA. Primers and other reagents were prepared according to the manufacturer's recommendation (Akhi *et al.*, 2015; Sharma *et al.*, 2013). (Figure 2).

Figure 2: Genotypic detection of ESBL producing isolates. Ladder, positive control for three genes, representative of SHV (383 bp), TEM (495 bp), CTX (560 bp) positive bacteria, negative control 1, negative control 2 and ladder left to right are shown respectively.

Statistical analysis: the study data was analyzed by using descriptive statistics (frequency - percent) and using the software spss-17. Chi-square test were applied to evaluated the incidence of genes with antibiotic susceptibility, to observe the correlation between the prevalence of ESBL genes and antibiotic susceptibility, Significance of results were calculated at 95% confidence level ($p \leq 0.05$).

Results:

Total 15 (7.35%) isolates from 204 stool samples were identified as *Proteus* (n=4, 1.96%), *Morganella* (n=5, 2.45%) and *Providencia* (n=6, 2.94%). Ratio between women and men were 39.1% and 60.9% respectively. The age ranges of the patients were 15-83 year; distribution of age according to Kolmogorov - Smirnov test was normal ($p \geq 0.05$).

Antibiogram and detection of ESBL producing isolates: all strains of *Proteus*, *Morganella* and *Providencia* were found to be resistant to ampicillin. *Proteus* spp. showed resistance to cefotaxime, ceftazidime and susceptible to other antibiotic. *Morganella* spp. showed resistance to amoxicillin + clavulanic acid, cefotaxime and ceftazidime while *Providencia* spp. was resistance to cefotaxime and ceftazidime. Antibiogram result of each genus has been shown in the table-1 separately.

Table 1: The frequency of resistance strains of species (Spp.) 1.- <i>Proteus</i> , 2.- <i>Morganella</i> and 3.- <i>Providencia</i>											
Spp.	Amp	Amc	Cec	Ctx	Cip	Cac	Gen	Caz	x	Cpm	A/S
1.	100	25	0	100	0	0	0	0	100	25	0
2.	100	100	0	100	0	0	0	100	0	0	0
3.	100	50	50	100	16.7	50	16.7	100	16.7	50	16.7

Amp (Ampicillin, 10 µg), **Amc** (Amoxicillin + clavulanic acid, 30 µg), **Cec** (cefotaxime + clavulanic acid, 40 µg), **Ctx** (cefotaxime, 30 µg), **Cip** (ciprofloxacin, 5 µg), **Cac** (ceftazidime + clavulanic acid, 40 µg), **Gen** (gentamycin, 10 µg), **Caz** (ceftazidime, 30 µg), **Cx** (cefoxitine, 30 µg), **Cpm** (cefepime, 30 µg), **A/S** (ampicillin + sulbactam, 20 µg)

Detection of TEM, SHV, CTX gene: of the 15 (7.35%) ESBL producing strains, blaTEM was the most common genotype (86.66%), followed by blaSHV (26.66%) and blaCTX-M (20%). Most of the *Proteus* spp. were TEM and SHV genes positive whereas all of the *Morganella* spp were TEM gene positive and SHV gene negative and most of the *Providencia* spp. was TEM gene positive. The results of genes detection in each genus has been shown in the table 2 separately.

Table 2: The frequency of genes detection in strains of species (Spp.) 1.- <i>Proteus</i> , 2.- <i>Morganella</i> and 3.- <i>Providencia</i>					
Spp.	TEM	SHV	CTX	TEM + SHV	SHV + CTX
1.	% 75(3)	% 75(3)	0	% 50(2)	0
2.	% 100(5)	0	% 40(2)	0	% 40(2)
3.	% 83.3(5)	% 16.7(1)	% 16.7(1)	0	% 16.7(1)

Discussion:

ESBL production by nosocomial pathogens is a major challenge for infection control committees in hospitals all over the world. ESBL-producing strains and their encoding genes can stay permanently in hospitals, causing colonization and outbreaks. In recent years, antibiotic-resistant ESBL producing bacteria in hospitalized patients has increased all over the world (Bradford, 2001; Denton, 2006; Gupta *et al.*, 2003).

Till date not sufficient reports available about the distribution and source of infectious of these bacteria. In addition, how many healthy people are carrying ESBL-producing bacteria or are transmitted by inpatient or outpatient to the hospital are not known. Asymptomatic colonization of the intestinal compartment with ESBL-producing Enterobacteriaceae isolates has already been reported (Miró *et al.*, 2005; Valverde *et al.*, 2004). Higher prevalence of ESBL-producing *E. coli*, *Klebsiella* and other Enterobacteriaceae of fecal carriage has been reported in the nosocomial setting than in any community (Chong *et al.*, 2013). Nevertheless, little information is available about healthy carrier of *Proteus*, *Morganella* and *Providencia*.

ESBL producing *Proteus*, *Morganella* and *Providencia* causes clinically significant hospital associated infections and are also known to cause community-acquired infections due to selective pressure owing to widespread use of third generation cephalosporin (Chong *et al.*, 2013; Mahrouki *et al.*, 2014; Poirel *et al.*, 1999).

Therefore, in this study, we have cultured hospitalized and non-hospitalized normal gastrointestinal patient's stool samples that attended to the hospital and examined ESBL producing, antibiotic susceptibility pattern and the presence of TEM, SHV and CTX genes in *Proteus*, *Morganella* and *Providencia* isolates. The rate of ESBL producing isolates in our study was 7.35%, including *Proteus* (n=4, 1.96%), *Morganella* (n=5, 2.45%) and *Providencia* (n=6, 2.94%) which are higher than studies carried on Enterobacteriaceae in different parts of the world such as Switzerland (5.8%), Sweden (3%), Spain (5.5%), India (10%) and Saudi Arabia (13.2%) (Geser *et al.*, 2011; Tängdén *et al.*, 2010) fortunately no report available on ESBL producing *Proteus*, *Morganella* and *Providencia*. Albeit all of these studies were of different genus and species of Enterobacteriaceae but most of them isolated only ESBL producing *E. coli* and *Klebsiella* spp. Our research was the first report about healthy ESBL carrier of *Proteus*, *Morganella* and *Providencia* in Tabriz. The main reason for a high prevalence rate of ESBL producers in our city could be the lack of strict policy for an antibiotic prescription and also the excessive use of these antibiotics could explain the higher prevalence of fecal carriage of ESBL-producing

organisms in the hospital, compared with the rate in the community.

In contrast to our results Kader *et al.* (2007) reported that the rate of fecal carriage of ESBL-producing organisms among inpatients (26.1%) was higher than that among outpatients (15.4%).

Saharman & Lestari (2013) reported 8.04% of ESBL producing *Proteus mirabilis* isolated from ICU patients which is much higher than our results (1.96%) indicating that there is much difference between isolates of healthy carriers and those isolated from patients.

Our results showed that blaTEM was the commonest genotype (86.66%), followed by blaSHV (26.66%) and blaCTX-M (20%), which corresponds to the results obtained by Bali *et al.* (2010) in Turkey for *E. coli* and *Klebsiella* indicating that *Proteus*, *Morganella* and *Providencia* similar to other members of family Enterobacteriaceae can be one of the major sources for this medically important genes and they can pass the gene to other clinical strains.

In our study, the cefotaxime, ampicillin and cefoxitin resistance rate were high for *Proteus* spp. while *Morganella* spp. showed high resistance to ampicillin, amoxicillin+clavulanic, cefotaxime and ceftazidime. *Providencia* spp. was 100% resistant to cefotaxime, ampicillin and ceftazidime and moderately to other tested antibiotics. These results are nearly similar to the susceptibility findings for clinical isolates (Dropa *et al.*, 2009, indicating that healthy carrier isolates are potentially able to produce serious infections. Although documentation confirming recent exposure to antibiotics along both outpatients and hospitalized patients was not available for us but the unrestricted sale of antibiotics in developing countries is likely to create a general pool of resistant organisms in the population. Oral consuming of amoxicillin-clavulanate and fluoroquinolone (eg, ciprofloxacin) are some of the antibiotics that are frequently obtained and used without prescriptions and also are used for prophylactic purposes in surgery operations.

The existence of ESBL-producing organisms in the gut of outpatients and hospitalized patients could make some clinical problems, as intestinal tract colonization is a necessary precondition for induction of infection by ESBL-producing organisms (Lucet *et al.*, 1996). Hence, infectious diseases physicians and clinical microbiologists should be precisely informed about ESBL-producing organisms that they are not only circulating in hospital environments but in the community as well by the healthy carrier and they have to be under consideration. To control or reduce the high rate of carriage for these organisms, efficient action should be considered to prevent the availability of antibiotics without a prescription and to increase knowledge among the people of the hazards of taking antibiotics without medical consultation.

Although the number of ESBL-producing *Proteus*, *Morganella* and *Providencia* isolates from fecal carriers are low, but still, they can be considered as a reservoir of TEM, SHV and CTX genes; thus carriers are also able to transfer these resistant bacteria to hospitals.

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