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Class D OXA-48 Carbapenemase in *Escherichia coli* and *Klebsiella pneumoniae*: An Emerging Threat to Burn Patients

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Carbapenem resistance in *Escherichia coli* and *Klebsiella pneumonia* is a main clinical problem. The Class D OXA-48 carbapenemase are emerging as an important menace mostly in the Mediterranean area. This investigation focused on the prevalence of Carbapenem resistant *Escherichia coli* and *Klebsiella pneumonia* using disk diffusion method and modified Hodge test (confirmatory test), as well as PCR for detection of OXA-48 gene. Between December 2012 and June 2013, a total of 138 wound's swab samples was collected from burn units in Sina Hospital, Tabriz, Iran. All isolates were Cultured and diagnosed by standard microbiological and biochemical tests. Subsequently, the isolates were screened by disk diffusion method and confirmatory test (modified Hodge test)for Carbapenemase production. Resistant isolates were evaluated by PCR for molecular assessing. 22 among of 63, *Klebsiella pneumoniae* and 7 among of 38, *E. coli* isolates showed resistance to ertapenem. The results of the modified Hodge test with ertapenem disk showed that carbapenemase was produced by 20 *Klebsiella pneumoniae* and 3 *E. coli* isolates.We report the first isolation of *Escherichia coli* and *Klebsiella pneumonia* strains harboring blaOXA-48 gene in burn units in Sina Hospital, Tabriz, Iran. Our data show that OXA-48 enzymes are the predominant carbapenemase in Tabriz, Iran. The OXA-48 producers were significantly shown more susceptible to colistin and ciprofloxacin. The transmission of OXA-48 producers have occurred among patients as medical tourism. Because patients have travelled from Turkey and Azerbaijan to Tabriz for treatment likely transferred OXA-48 isolates to Tabriz. OXA-48 producer also probably could be colonized by the patient hospital staff and should be screened for these isolates on admission to hospital. The infection control measures should be considered after emerging *OXA-48 producer, in order to* including more attention for hand hygiene, wearing gloves and gowns for the period of patient care activities, and isolation of patients in private rooms and restrict the use of carbapenems as an antimicrobial stewardship program seem to prevent spread of these isolates in Iranian hospitals.

Key words: OXA-48, carbapenemase, *Klebsiella pneumoniae*, *Escherichia coli*, Burn patients.

Burns provide an ideal site for bacterial multiplication and are major potential sources of infection. *Escherichia coli* and *Klebsiella pneumoniae* have become a major etiologic agent of burns infection and has been prominent in Asian countries, including Iran[1]. Carbapenems are considered first-line therapy for treatment of severe infections such as burn wound infection due to extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae*[2]. In recent years, carbapenem resistance has emerged among *Escherichia coli* and *Klebsiella pneumoniae* in health care settings and is mainly attributed to the production of

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carbapenemase. Carbapenemase producing *Escherichia coli* and *Klebsiella pneumoniae* have become more commonly worldwide. Carbapenemases have a rapid international spread, predominantly by *Escherichia coli* and *Klebsiella pneumoniae*[3,4]. β-lactamases hydrolyzing carbapenems belonging to Ambler classes A, B, and D have been found among *Escherichia coli* and *Klebsiella pneumoniae*. The extensive spread of Ambler class D carbapenemases of the OXA-48 type highlights that, carbapenemases may rapidly become threatening[5]. The efficacy of carbapenems for treating severe infections due to OXA-48 producers remains debatable since imipenem-containing therapy failed to treat burns infections. OXA-48 β-lactamases possessing carbapenemase properties have been reported in Enterobacteriaceae[6,7]. OXA-48 is a class D carbapenemase that is primarily found in a *Klebsiella pneumoniae* isolate from Turkey. Since then, several other OXA-48-producing isolates of various enterobacterial species such as *Citrobacter freundii* and *Escherichia coli* have been reported, mainly from Turkey, but also from other countries in Europe. OXA-48 producers are also frequently emerging as a serious threat in the Mediterranean area[8,9] . The purpose of this study was to investigate the prevalence of OXA-48 producers in *Escherichia coli* and *Klebsiella pneumoniae* in Tabriz-Iran.

MATERIALS AND METHODS

Setting

This study was conducted in the burn Units of Sina hospital, East Azarbaijan, Iran; this unit serves all the East Azarbaijan region. The teaching Sina hospital of Tabriz consists of different wards such as: emergency and polyclinic, Burn, Surgery, dermatology and infection. The Burn ward is divided into three sections. Intensive care burns unit is located on the ground floor and the children, men and women burn units are located on the second floor. These units are very close together, and each unit has its staff nurse, with the possibility to transfer a patient from one unit to another.

Specimen collection

All the wounds of burn patients were daily decontaminated with normal saline, Antiseptics, mupirocin ointment and silver sulphadiazine cream and were covered by dressings applied in layers. All Specimens were collected with swabs at the beginning of dress changing. Each sampling procedure performed after the removal of dressings scrapping away of topical antibacterial agents by sterile swab, and the wounds before applying new topical antimicrobial agents.

Bacterial isolates

Between December 2012 and June 2013, a total of 138 wound's swab samples was collected from burn units in Sina Hospital, Tabriz, in the North West of Iran. Speciemens were cultured on blood, chocolate and McConkey agars and were incubated for at least 48 hours at 37 °C. The obtained bacteria were identified by conventional biochemical tests such as oxidase, TSI, SIM, urea, etc. In the next step the Microgen™ GN-ID kit (Microgen Bioproducts, England) was used for final confirmation.

Antimicrobial agents susceptibility testing and Screening for detection of carbapenemase production

Susceptibility to antimicrobial agents was determined by disc diffusion method on Muller–Hinton agar (Oxoid, England) according to the guidelines of Clinical and Laboratory Standards Institute (CLSI)[10]. The antibiotics (Mast, UK) used for antibiogram determination of the collected strains were: cefotaxime (CTX; 30 µg), aztreonam (ATM; 30 µg), piperacillin/ tazobactam (PTZ; 100/ 10 µg), colistin (Co; 10µg), chloramphenicol (C; 30 µg), tetracycline (T; 30 µg), ciprofloxacin (CIP; 5 µg), ertapenem (ETM; 10 µg), imipenem (IPM; 10 µg), Doripenem (DOR; 10 µg), meropenem (MEM; 10 µg), gentamicin (GM; 10 µg).

Modified Hodge test (MHT)

An overnight culture of the control organism (*E. coli* ATCC 25922) was adjusted to a turbidity equivalent to that of a McFarland No. 0.5 and diluted 1:10 in saline or broth. Then, these were used to inoculate the surface of the Mueller Hinton Agar and allow the plate to dry three to ten minutes. A 10 µg ertapenem disk was placed in the centre of the test area. Test organism was heavily streaked from the centre (edge of the ertapenem disc) to the periphery of the plates, and then the plate was incubated at 35±2°C in ambient air for

16–24 hours. The Hodge test is interpreted as positive by the presence of distortion of the inhibition zone.

DNA Extraction

The DNA of carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* strains was extracted by using the CTAB method and stored at – 20 °C. In brief, a loop full of bacteria was suspended in 1.5 ml sterile distilled water, vortexed well and then centrifuged in 12000 g for 10 min. The supernatant was discarded and 270 µl T/E buffer plus 30 µl SDS 10% plus 5 µl proteinase K was added to the pellet and then incubated at 50 °C overnight. Then 100 µl of 5 M NaCl solution was added to and mixed well. 80 µl of prewarmed CTAB/NaCl (65°C) solution was added and vortexed well, then the Microtube was incubated at 65 ºC for 10 minutes. Seven hundred µl of chloroform-isoamyl alcohol (24:1) solution was added and vortexed for 20 second. The suspension was centrifuged at 12000 g for 10 minutes and the aqueous phase was transferred to a fresh test tube. Then, 300 µl isopropanol was added and mixed gently, and incubated at -20°C for 30 minutes and finally centrifuged at 12000 g for 1 min. The supernatant was discarded and the pellet was resuspended in 1 ml of 70% cold ethanol, and then centrifuged at 12000g for 5 min. The supernatant was discarded and after air drying, the DNA pellet was dissolved in 50 µl TE buffer and stored at -20° C. Plasmid DNA of the isolates was also extracted using the plasmid extraction kit (Qiagen), according to the manufacturer'sinstructions.

Detection of OXA-48 gene by polymerase chain reaction (PCR)

PCR was performed for all isolates identified as carbapenemase producers, using antibiogram test to ertapenem and phenotypic confirmatory tests (modified Hodge test). PCR amplifications were performed using an Eppendorf Thermal Mastercycler (Gradient) as follows: 10 min at 94°C and 36 cycles of amplification consisting of 30 s at 94°C, 40s at 52°C, and 50s at 72°C, with 5 min at 72°C for the final extension. Reaction mixtures for PCR contained 1.5 mmol/L of $MgCl_2$, 10 µmol/L of each primer, 0.125 mmol/L of each deoxynucleotide triphosphate, 2 U of Taq polymerase, 1× PCR buffer and five microliters of total DNA. Primers such as OXA-F (52 - GCGTGGTTAAGGATGAACAC-32) and OXA-R (52 - CATCAAGTTCAACCCAACCG-32) (438bp) were used for OXA-48 (11) . 7 µL of reaction mixture containing PCR product were analysed by electrophoresis in 1 % (w/v) agarose (Fermentas).

RESULTS

In this study, During 6 months, 138 wound swabs were taken from burn patients. Only one sample was obtained from each patient. *Patients*, 62% *males* and 38% f*emales, age range* 5 months to 91 years with a mean percent total body surface area burned (TBSA) of range 7% to 80%, were included in the study. Flame injuries (52%) were the most common cause of burn , followed by boiling water, boiling soup, benzene , electricity, molten metal. In this study, the mean duration of hospitalization was 17 days. During this study, 63 *K. pneumoniae* and 38 *E. coli* were isolated from wound swabs of burn patients hospitalised in Sina Hospital, Tabriz, Iran. 22 of 63, Klebsiella pneumoniae and 7 of 38, *E. coli* isolates showed resistance to ertapenem. The results of the modified Hodge test with ertapenem disk showed that

Fig. 1. PCR amplification of OXA-48

L; 100 bp DNA ladder, N; negative control, P; positive control, 1, 2, 4, positive clinical isolates for OXA-48 (438 bp), 3, 6, 7, negative clinical isolates for OXA-48

Table 1. Clinical characteristics of patients, the etiology of burn, Modified Hodge test (MHT), Antibiotic Resistance Profile of carbapenem-resistant *K.pneumoniae*

Strain	Age and Gender	Hospitalized days	Ward	Etiology of Burn	BSA	OXA48-Carbapenemase	Modified Hodge test (MHT)			Antibiotic Resistance Profile	Antibiotic Susceptibility Profile
							IMI Disk	MEM Disk	ETM Disk		
K. pneumoniae 1	7Years M	4	Burn ICU	Flame	3 (55%)	Positive	Positive	Positive	Positive	IMI- MEM- DOR- PTZ- Chl- Tet- ATM- CIP	Co- GM
K. pneumoniae 2	20Years F	41	Burn ICU	Suicide	3 (60%)	Positive	Positive	Positive	Positive	IMI- MEM- DOR- GM- PTZ- CTX- Chl- Tet-ATM- CIP	Co- GM
K. pneumoniae 3	21Years F	11	Burn ICU	Suicide	3 (37%)	Positive	Positive	Positive	Positive	IMI- MEM- DOR- PTZ – CTX- Chl-Tet- ATM- CIP	Co- GM
K. pneumoniae 4	45Years M	6	Burn ICU	Electrical	3 (37%)	Positive	Positive	Positive	Positive	IMI- MEM- DOR- GM-PTZ- CTX- Chl- Tet- ATM	Co- CIP
K. pneumoniae 5	71Years M	12	Men	Flame	1 (7%)	Positive	Positive	Positive	Positive	IMI- MEM- DOR- GM - PTZ CTX- Chl- Tet- ATM	Co- CIP
K. pneumoniae 6	20Years F	7	Burn ICU	Flame	3 (55%)	Positive	Negative	Positive	Positive	MEM- DOR- PTZ-CTX- GM- Chl- Tet- ATM	IMI- Co- CIP
K. pneumoniae 7	30years M	21	Men	Flame	2 (16%)	Positive	Positive	Positive	Positive	IMI- MEM- DOR- GM -PTZ- CTX- Chl- Tet-ATM	Co- CIP
K. pneumoniae 8	17 years F	7	Burn ICU	Suicide	3 (60%)	Positive	Positive	Positive	Positive	IMI- MEM- DOR- GM -PTZ- CTX- Chl- Tet ATM	Co- CIP
K. pneumoniae 9	77years M	10	Women	Flame	1 (7%)	Positive	Negative	Positive	Positive	MEM- DOR- PTZ-CTX- GM- Chl- Tet- ATM	IMI-Co- CIP-
K. pneumonia 10	48years F	32	Burn ICU	Flame	3 (60%)	Positive	Positive	Positive	Positive	IMI- MEM- DOR- GM -PTZ- Chl- Tet- ATM-	Co- CIP
K. pneumonia 11	18 years F	6	Women	Boiling water	3 (33%)	Positive	Positive	Positive	Positive	IMI- MEM- DOR- GM-PTZ- CTX- Chl- Tet-ATM	Co- CIP
K. pneumoniae 12	48years F	18	Women	Flame	3 (60%)	Positive	Negative	Weakly Positive	Positive	MEM- DOR- GM- PTZ-	IMI-Co- CIP

K. pneumoniae 13	35years M	8	Men	Benzine	3 (50%)	Positive	Positive	Positive	Positive	CTX- Chl- Tet- ATM IMI- MEM- DOR- PTZ-CTX- Chl- Tet- ATM-CIP	Co—GM
K. pneumoniae 14	6months M	14	Children	Boiling soup	2 (14%)	Positive	Positive	Positive	Positive	IMI- MEM- DOR- GM-PTZ- CTX- Chl- Tet-ATM	Co- CIP
K. pneumoniae 15	27years M	5	Men	Molten metal	1 (7%)	Positive	Positive	Positive	Positive	IMI- MEM- DOR- GM -PTZ CTX - Chl- Tet- ATM	Co- CIP
K. pneumoniae 16	11months M	60	Children	Boiling soup	3 (40%)	Positive	Positive	Positive	Positive	IMI- MEM- DOR- GM -PTZ- CTX-Chl- Tet-ATM	Co- CIP
K. pneumoniae 17	18years M	10	Men	ûrecrackers	3 (33%)	Positive	Positive	Positive	Positive	IMI- MEM- DOR- GM-PTZ- CTX- Chl- Tet-ATM	Co-CIP
K. pneumoniae 18	82Years M	19	Men	Boiling water		3 (33%)	Positive	Positive	Positive	Positive IMI- MEM- DOR- GM -PTZ- CTX- Chl- Tet-ATM	Co-CIP
K. pneumoniae 19	24 years M	31	Burn ICU	Flame	3 (60%)	Negative	Positive	Weakly Positive	Positive	IMI- MEM- DOR- GM -PTZ- CTX- Chl- Tet-ATM-	Co- CIP
K. pneumoniae 20	79 years M	12	Burn ICU	Flame	3 (70%)	Negative	Positive	Weakly Positive	Positive	MEM- DOR- GM- PTZ-CTX- Chl- Tet- ATM- CIP	IMI-Co- CIP
K. pneumoniae 21	6 months M	7	Children	Boiling soup	2 (16%)	Negative	Negative	Negative	Negative	MEM- DOR- PTZ-CTX- Chl- Tet- GM- ATM-	IMI-Co- CIP
K. pneumoniae 22	51 years F	8	Burn ICU	Flame	3 (70%)	Negative	Negative	Negative	Negative	MEM- DOR- PTZ-CTX- Chl- Tet- GM- ATM	IMI-Co- CIP

CTX, cefotaxime; ATM, aztreonam ; PTZ, piperacillin/ tazobactam; Co, colistin; Chl , chloramphenicol; Tet, tetracycline; CIP, ciproûoxacin; IPM, imipenem; DOR, Doripenem; MEM, meropenem;ETM, ertapenem; GM, gentamicin. * Intermediate-resistance; BSA (burn surface area)

Table 2. Clinical characteristics of patients, the etiology of burn, Modiûed Hodge test (MHT), Antibiotic Resistance Profile of carbapenem-resistant *Escherichia coli*

Strain	Age and Gender	Hospitalized days	Ward	Etiology of Burn	BSA	OXA48-Carbapenemase	Modified Hodge test (MHT)			Antibiotic Resistance Profile	Antibiotic Susceptibility Profile
							IMI Disk	MEM Disk	ETM Disk		
E. coli 1	8 Years M	19	Burn ICU	Flame	3 (50%)	Positive	Negative	Positive	Positive	MEM- DOR- PTZ - CTX – GM- Chl – Tet –ATM	IMI- Co- CIP
E. coli 2	5 Years M	6	Burn ICU	Boiling water	3 (55%)	Positive	Positive	Positive	Positive	IMI- MEM- DOR- PTZ- GM- Chl- Tet- ATM	Co - CIP-
E. coli 3	9 months M	26	Children	Boiling soup	1 (7%)	Positive	Positive	Positive	Positive	IMI- MEM- DOR- GM- PTZ CTX- Chl- Tet- ATM-	Co – CIP
E. coli 4	34 years M	32	Men	Flame	3 (33%)	Negative	Negative	Negative	Negative	MEMm- DORm-PTZ - CTX -Chl- Tet -ATM- - GM	IMI- Co- CIP
E. coli 5	57 years M	14	Men	Flame	1 (7%)	Negative	Negative	Negative	Negative	MEMm- DORm- PTZ –Ch l-Tet- CTX - GM ATM	IMI- Co- CIP
E. coli 6	5 years M	6	Children	Boiling soup	3 (33%)	Negative	Negative	Negative	Negative	MEMm- DORm- PTZ - CTX- Chl- Tet- ATM- GM	IMI- Co- CIP
E. coli 7	29 years F	10	Burn ICU	Flame	3 (60%)	Negative	Negative	Weakly Positive	Weakly Positive	MEMm- DORm- PTZ - CTX- - GM- Chl-Tet-ATM	IMI- Co- CIP

CTX, cefotaxime; ATM, aztreonam ; PTZ, piperacillin/ tazobactam; Co, colistin; Chl , chloramphenicol; Tet, tetracycline; CIP, ciproûoxacin; IPM, imipenem; DOR, Doripenem; MEM, meropenem;ETM, ertapenem; GM, gentamicin; * Intermediate-resistance; BSA (burn surface area)

carbapenemase was produced by 20 *Klebsiella pneumoniae* and 3 *E. coli* isolates. Fig. 1 shows PCR amplification of OXA-48 with 438 bp PCR product. The carbapenemase OXA-48 gene was detected in 21 (20.8%) of all isolates. Table-1and 2 show the clinical characteristics of patients, the etiology of burn, modiûed Hodge test (MHT) (with imipenem, meropenem and ertapenem disk) and antibiotic resistance profile of carbapenem-resistant *Klebsiella pneumonia* and E.coli respectively

DISCUSSION

The increasing reports on OXA-48 producing Enterobacteriaceae is becoming a major threat to public concern[12]. *Klebsiella pneumoniae* and *Escherichia coli* are pathogens that widely distributed in hospitals, and are inhabitants of the intestinal flora. Antimicrobial agents for treating multidrug-resistant *Klebsiella pneumoniae* and *Escherichia coli* infections are carbapenems, aminoglycosides, and fluoroquinolones. Among these, carbapenems are the most important antibiotics[6,13]. Consequently, the wide resistance to carbapenem antibiotics in *Klebsiella pneumoniae* and *Escherichia coli* has attracted much attention because of its pivotal role in the treatment multidrug-resistant infection. The identification of OXA-48 producer in *K. pneumoniae* and *Escherichia coli* that results in resistance or intermediate resistance to one or more carbapenems have significant implications in hospital infection control practices and is useful for epidemiological investigations[14,15]. The increasing prevalence of isolates of Enterobacteriaceae producing carbapenemases is one of the largest resistance problems of this decade. OXA-48 producer isolate has been identified in various countries predominantly from North African countries, the Middle East, India and Turkey, those areas are considered one of the most important reservoirs[6]. The present study represents an initial insight on the prevalent OXA-48 gene in *K. pneumoniae* and *E. coli* isolated from burns patients in Tabriz, Iran. The results from the current study demonstrated that among *Klebsiella pneumoniae* and *Escherichia coli* isolated from burn wound infection, there were high levels of resistance against carbapenems that are commercially available in Iran. Our data show that OXA-48 enzymes are the predominant Carbapenemase in Tabriz, Iran. This study showed that OXA-48 producers isolated from burns were significantly more susceptible to colistin and ciprofloxacin. The modified Hodge test with ertapenem disk showed higher sensitivity for carbapenemase-production than imipenem or ertapenem disks.

The use of carbapenems as the choice of treatment for multidrug-resistant Enterobacteriaceae at Sina centre may be a possible explanation for increasing carbapenem-resistant isolates. Prolonged hospitalization in burn patients, extensive use of antibiotic chemotherapy during hospital stay, possibly ignoring sanitation and basic infection control practices such as hand washing or usage of disinfection, lack of routine screening for carabapenem-producing Enterobacteriaceae strains and colonisation of environmental multidrug-resistant strains could be constituted the reasons of the increasing incidences of carbapenem-resistant in the *Klebsiella pneumoniae* and *Escherichia coli* isolated in the current study. Several countries recently accepted new guidelines for the screening of patients transferred from high prevalence foreign hospitals or patients returning from travels in foreign countries identified that endemic in multiresistant bacteria. Certainly, the prevention of the spread of OXA-48 producers isolate is dependent on the rapid and precise detection of carriers[6,16,17,18]. The gastrointestinal (GI) tract continues to be a potential reservoir for microorganisms that colonize the burn wound surface. It is likely that endogenous microorganisms continue to be transmitted to burn wound surfaces by feces[19]. The transfer of OXA-48 producer can be controlled by a combination of appropriate infection control measures and physical isolation of these patients. In conclusion, the rates of OXA-48 producing *Klebsiella pneumoniae* and *Escherichia coli* isolates from burn patients were notable, and, unfortunately, only a limited number of antimicrobial agents are effective. Consequently, the management and treatment strategy should be improved and the proper use of infection-control measures and judicious use of currently available antimicrobial agents to prevent the emergence of resistant organisms are needed to reduce the spread of

resistant genes in the clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli*. Preventive measures screening for carbapenem-resistant *Enterobacteriaceae* in patients transferred from high prevalence areas or from any foreign hospital could be imperative to maintain this low incidence of OXA-48 producer. the occurrence of OXA-48 producers was demonstrated to be related to medical tourism. As regards, Adler et al study has shown that transmission of OXA-48 isolates have occurred among patients as medical tourism[6,20, 21]. Because patients have travelled from Turkey and Azerbaijan to Tabriz for treatment likely transferred OXA-48 isolates to Tabriz. OXA-48 producer also probably could be colonized by the patient hospital staff and should be screened for these isolates on admission to hospital. According to the presented data, the OXA-48 producers were common in patients with third-degree burns. The infection control measures should be considered after emerging *OXA-48 producer, in order to* including more attention for hand hygiene, wearing gloves and gowns during patient care activities, and isolation of patients in private rooms and limit the use of carbapenems as an antimicrobial stewardship program seem to prevent spread these isolates in Iranian hospitals.

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