THE DETERMINATION OF CARBAPENEM RESISTANCE IN *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE* ISOLATES RELATED TO NOSOCOMIAL INFECTIONS AND THE EVALUATION OF RISK FACTORS

S Budak[1], O Oncul[1], Z Aktas[2], A Acar[1], M Ozyurt[3], V Turhan[1], H Erdem[1] and L Gorenek[1]

[1]Department of Infectious Diseases and Clinical Microbiology, [3]Department of Clinical Microbiology, Gulhane Military Medical Academy, Haydarpasa Training Hospital; [2]Department of Clinical Microbiology, Istanbul Medical Faculty, Istanbul University, Istanbul, Turkey

Abstract. We aimed to investigate carbapenem resistance, resistance mechanisms, risk factors and epidemiological features of *Escherichia coli* and *Klebsiella pneumoniae* strains isolated from related infections in intensive care unit (ICU) patients. Carbapenemase activity was determined by MHT, MBL Etest and enzyme extraction methods. Presence of extended-spectrum β-lactamase (ESBL) and carbapenemase-encoding genes were investigated by PCR and sequencing. Clonal relationship of the strains was investigated by pulse field gel-electrophoresis. Acquired *AmpC* and *Qnr* were investigated by PCR. Throughout this study, 1,657 patients, and 11,483 hospitalization days were followed by active surveillance in the ICU of our 1,000-bed training hospital. Out of 108 of 196 patients, 130 *E. coli*- and *K. pneumoniae*-related nosocomial infections were determined. Minimum inhibitory concentration (MIC) levels of ertapenem were ≥1 mg/l in 14 *K. pneumoniae* and 2 *E. coli* strains. The highest MIC level of carbapenem was found in *K. pneumoniae* and *E. coli* strains of ≥128 mg/l and 8 mg/l, respectively. In the carbapenem resistant strains, KPC and MBL activity were not found. On the other hand, 14 strains of *K. pneumoniae* and one strain of *E. coli* exhibited OXA-48 β-lactamase activity. Fifty-seven percent of *K. pneumoniae* isolates produced OXA-48 orginating from two clones and remaining isolates originated from different clones. Thus carbapenem resistance was determined as 22% and 3% in *K. pneumoniae* and *E. coli* strains, respectively. Invasive devices, duration of total parenteral nutrition, duration of hospitalization, presence of transfusions, ESBL and multiple drug resistance were found to be risk factors for carbapenem resistance.

Keywords: *Escherichia coli, Klebsiella pneumoniae*, carbapenem resistance, OXA-48 beta-lactamase, ICU patients, risk factors.

Correspondence: Assoc Prof Zerrin Aktas, Department of Clinical Microbiology, Istanbul Medical Faculty, Istanbul University, 34390 Istanbul, Turkey.
Tel: 00 90 212 414 20 00 32279; Fax: 00 90 212 4142037
E-mail: aktaszerrin@yahoo.com

INTRODUCTION

Plasmid-mediated resistance against the carbapenem class of antibiotics has been increasing gradually (Endimiani *et al*, 2008; Nordmann and Poirel, 2013). OXA-48 type carbapenemases have been

identified mostly in Mediterranean and European countries and in India (Walsh, 2010; Castanheira *et al*, 2011; Cuzon *et al*, 2011). *Klebsiella pneumoniae* carbapenemases have been reported especially in the United States, Greece and Israel (Walsh, 2010). Metallo-beta-lactamases have been reported with a higher prevalence in southern Asia and Europe (Anderson *et al*, 2007; Endimiani *et al*, 2010; Hsuesh, 2010; Walsh, 2010).

Due to increase in the utilization of carbapenems in recent years, particularly for nosocomial *K. pneumoniae* and *E. coli* infections in our 1,000-bed training hospital, we investigated extended-spectrum β-lactamases (ESBLs) production, carbapenem resistance rates, potential risk factors that may lead to the development of resistance and the other types of resistance mechanisms.

MATERIALS AND METHODS

Subjects

This was a prospective study involving patients admitted to 6 intensive care units (ICUs) (anesthesiology, neuro surgery, general surgery, burn unit, internal medicine and neurology) of GATA Haydarpasa Training Hospital, Istanbul, Turkey between June 2009 and January 2011. Demographic data and medical conditions of patients were recorded to form a database. All patients were visited by members of the Infection Control Commitee at least once a day. Diagnosis of nosocomial infection (NI) was based on Centers for Disease Control (CDC) criteria (PHT, 2010). For rational antibiotic use, culture specimens were collected preceding treatment. Empirical treatments were designed taking into consideration the hospital flora and antibiotic susceptibility patterns. In cases of a reinfection, patients were reevaluated. Ethical approval was given by Gulhane Military Medical Academy Ethical Board, No: 90-25.05.2010.

Determination of risk factors

Age over 65 years old was assumed to be an advanced age. Patients were evaluated for chronic obstructive lung disease (COPD), diabetes mellitus (DM), chronic renal failure (CRF), malignities and other comorbid conditions. Apache II scores of patients were recorded. Prior to antibiotic use was defined as the use of antibiotics for 4 weeks prior to hospital infection related to *E. coli* or *K. pneumoniae* (Lee *et al*, 2004). Use of broad spectrum beta-lactam and beta-lactamase inhibitors, third generation cephalosporins, glycopeptides, aminoglycosides, parenteral quinolones and carbapenems was recorded (Lee *et al*, 2004). History of hospitalization in the last 3 months was recorded. In addition, the use of chemotherapy and steroids in the last 4 weeks was recorded as being immunosuppression.

Strain identification

Specimens collected from patients with NI were inoculated onto appropriate media. API ID BBL Crystal (Becton Dickinson, Franklin Lakes, NJ) identification kit was used to identify the genus of colonies.

Antimicrobial susceptibility test

Antimicrobial susceptibility test was performed using disk diffusion method (CLSI, 2010). The tested antimicrobials were ampicillin, cefazolin, cefoxitin, ceftriaxone, cefotaxime cefepime, ceftazidime, aztreonam, amoxicilin/clavulanic acid, imipenem, ertapenem, meropenem, trimethoprim/sulfamethoxazole (TMP-SMX), amikacin, tobramycine and ciprofloxacin. Minimum inhibiory concentrations (MICs) of imipenem, meropenem, ertapenem and doripenem were determined using agar dilution method according to

the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2010). ESBL production was determined using Etest ESBL cefotaxime, cefotaxime/clavulanic acid (CTX/CTX-CA) (AB Biodisk, Solna, Sweden) and results of the relevant tests were interpreted according to CLSI (2010). Quality control testing was performed employing *Pseudomonas aeruginosa* ATCC 27853, *E. coli* ATCC25922 and *K. pneumoniae* ATCC 700603.

Investigation of carbapenemase activity

Carbapenemases were phenotypically investigated by the modified Hodge test (MHT), MBL (IMP/IPI) Etest, a combined disc test (phenyl boronic acid) and a disc enzymatic assay (Lee *et al*, 2004; Doi *et al*, 2008; Tsakris *et al*, 2009, 2010; CLSI, 2010; Endimiani *et al*, 2010; Giske *et al*, 2011). The presence of carbapenemase-encoding genes was investigated by polymerase chain reaction (PCR) and sequencing. Genes encoding production of serine carbapenemases (bla_{KPC}), Class B (bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{SPM}, bla_{SIM}), OXA beta-lactamases genes ($bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA24\text{-}like}$, $bla_{OXA51\text{-}like}$, $bla_{OXA\text{-}58\text{-}like}$ and $bla_{OXA\text{-}48}$) were identified using a set of primers that amplify specific segments of the individual beta-lactamase genes (Fiett *et al*, 2006; Woodford *et al*, 2006; Ellington *et al*, 2007; Aktas and Kayacan, 2008; Aktas *et al*, 2008; Endimiani *et al*, 2008; Tzouvelekis *et al*, 2012). In addition, several extended-spectrum ESBLs (PER-1, TEM, SHV and CTX-M) and acquired *AmpC* and *Qnr* (*qnr*A, *qnr*B, *qnr*C and *qnr*S)*aac(6')-Ib-cr* and *qep*A) were investigated by PCR (Gniadkowski *et al*, 1998; Poirel *et al*, 2000; Karim *et al*, 2001; Cattoir *et al*, 2007; Pérez-Pérez and Hanson, 2010; Nordmann *et al*, 2012).

Plasmid analysis

Plasmid DNA extraction was performed as described by Kado and Liu (1981).

Clonal relatedness

Clonal relatedness of the isolates was assessed by pulsed-field gel electrophoresis (PFGE) (Aktas *et al*, 2007).

Data analysis

SPSS 15 was used for statistical analysis. Differentiation between the groups was investigated using Mann-Whitney *U* test. The relation between variables performed in the study was investigated by logistic regression analysis. Data were calculated as mean ± standard error. In all tests, Alpha, the degree of freedom, is accepted as 0.05. Hence, the calculated p-value < 0.05 is considered significant.

RESULTS

In this study, a total of 1,657 patients for 11,483 hospitalization days were followed by active surveillance in ICUs of GATA Haydarpasa Training Hospital, Istanbul, Turkey between June 2009 and January 2011. During the study period, 265 NIs in 196 patients were observed (the rate of hospital infection being 15.9%, the incidence density of infection 23.07 for 1,000 days). In total, 130 NIs developed in 108 followed patients [68 (52%) with *E. coli*, 62 (48%) *Klebsiella* spp]. The average age of cases with *E. coli* and *K. pneumoniae* NIs was 69.6 years and the average hospitalization period was 34.2 days. In all NIs, *E. coli* and *K. pneumoniae* was the most frequent infection in urinary tract (48%) and blood stream (25%), respectively.

During the study period, aminoglycosides (73.9%) and TMP-SMX (59.5%) for *E. coli*, aminoglycosides (65%) and quinolones (47.7%) for *Klebsiella* spp were the most effective antimicrobials following carbapenems. The highest resistance

Table 1
MIC_{50} and MIC_{90} breakpoints of ESBL-negative, ESBL-positive and OXA-48 positive/ ESBL-positive *E. coli* and *K. pneumoniae* strains.

Antibiotic	*E.coli*			*Klebsiella* spp		
	ESBL (-)	ESBL (+)	OXA+/ESBL(+)	ESBL (-)	ESBL (+)	OXA+/ESBL(+)
Ertapenem (µg/l)						
MIC_{50}	0.008	0.06	1	0.03	0.03	4
MIC_{90}	0.06	0.25	8	0.06	0.25	>128
Imipenem (µg/l)						
MIC_{50}	0.12	0.12	0.25	0.25	0.25	0.5
MIC_{90}	0.25	0.25	2	0.25	0.50	64
Meropenem (µg/l)						
MIC_{50}	0.03	0.03	0.25	0.008	0.06	1
MIC_{90}	0.03	0.12	0.25	0.06	0.25	32
Doripenem (µg/l)						
MIC_{50}	0.016	0.03	0.25	0.06	0.06	0.5
MIC_{90}	0.03	0.06	0.50	0.12	0.12	8

rate was found for third generation cephalosporins (58-60%) in both species. Table 1 shows the comparison of MIC_{50} and MIC_{90} breakpoints of the 4 carbapenems in ESBL-negative, ESBL-positive and OXA-48/ESBL-positive isolates of *K. pneumoniae* and *E. coli*.

All 16 isolates non susceptible to ertapenem (MIC ≥ 1 µg/ml) were defined as carbapenem non-susceptible (CNS) isolates. During the study period, MIC values of ertapenem were found ≥1 mg/l in 16/130 (12%) strains isolated from 13 patients. Among these isolates, 22% (14/62) were *K. pneumoniae* and 3% (2/68) was *E. coli* (Table 2). Carbapenemase activity was found in all 16 CNS isolates by ertapenem MHT, 15 by meropenem MHT, and 11 by enzyme extraction method. The CNS isolates were negative for combined DDT using phenyl boronic acid and MBL Etest. Among these CNS isolates, 14/16 were found positive for ESBL production by E-test (CTX/CTX-CA). The *bla* (OXA-48) genes were located on a 70 kb plasmid.

OXA-48 gene was detected in 94% (15/16) of CNS isolates and these results were verified by sequence analysis. CTX-M-15, TEM and SHV gene was detected in 69% (11/16), 50% (8/16) and 25% (4/16) of the CNS isolates, respectively. The *qnrA* and *qnrB* was detected in 19% (3/16) and 6% (1/16) of the CNS *K. pneumoniae* isolates. Four isolate co-expressed $bla_{CTX\text{-}M\text{-}15}$ and bla_{TEM}, 2 isolate $bla_{CTX\text{-}M\text{-}15}$ and bla_{SHV}, 1 $bla_{CTX\text{-}M\text{-}15}$, bla_{TEM} and $bla_{CTX\text{-}M\text{-}15}$, and 1 bla_{TEM} and bla_{SHV} (Table 2). None of the CNS strains was found to encode genes of metallo-beta-lactamase, KPC carbapenemase and plasmidic Amp-C enzymes.

Based on PFGE results, the 14 *K. pneumoniae* isolates revealed 8 clonal clusters separated by similarity values < 70% and two pulsotypes were detected in *E. coli* isolates. The majority of isolates (n = 8) belonged to pulse type 1 (4 isolates) and 2 (4 isolates) in *K. pneumoniae* strains (Table 2). Four *K. pneumoniae* isolates

Table 2
MIC values of carbapenems and moleculer characteristics of carbapenem non-susceptible or reduced susceptible *K. pneumoniae* and *E. coli* isolates.

Number of strains	Ward/ sample	Definition of infection	Ertapenem MIC (µg/l)	Meropenem MIC (µg/l)	Imipenem MIC (µg/l)	Doripenem MIC (µg/l)	PFGE pattern	Class D OXA-48	Class A TEM	Class A SHV	Class A CTX-M-15	Plasmidic quinolone gene (*qnr*)
1 *K. pneumoniae*	Int.Med/Blood	BSI	4	0.5	0.5	0.25	3	+	+	-	+	*QnrA*
2 *K. pneumoniae*	N.Sur/Blood	BSI	4	0.25	0.25	0.25	1	+	+	-	+	*QnrA*
3 *E. coli*	Int.Med/Urine	USI	8	0.5	0.25	0.25	A	-	+	-	+	-
4 *K. pneumoniae*	Int.Med/Urine	USI	8	0.5	0.5	0.50	1	+	-	-	+	*QnrB*
5 *K. pneumoniae*	G.Sur/Blood	BSI	4	0.5	0.25	0.25	4	+	+	-	+	-
6 *K. pneumoniae*	G.Sur/Urine	USI	6	4	4	0.50	2	+	-	+	+	-
7 *K. pneumoniae*	G.Sur/Blood	BSI	>128	32	64	8	5	+	-	-	+	-
8 *K. pneumoniae*	G.Sur/Urine	USI	8	0.5	1	0.50	1	+	-	-	_	-
9 *K. pneumoniae*	Anesthesia/TA	VAP	2	0.25	0.25	0.25	1	+	+	-	+	-
10 *K. pneumoniae*	Anesthesia/Blood	BSI	4	0.5	0.25	0.25	6	+	-	+	+	*QnrA*
11 *K. pneumoniae*	Int.Med/Urine	USI	4	0.5	0.5	1	2	+	+	-	_	-
12 *K. pneumoniae*	Int.Med/TA	VAP	4	0.5	0.5	0.25	2	+	+	+	+	-
13 *E. coli*	Neurology/Urine	USI	1	0.25	0.25	0.12	B	+	-	-	+	-
14 *K. pneumoniae*	Int.Med/Blood	BSI	4	0.5	0.25	0.25	2	+	+	+	_	-
15 *K. pneumoniae*	Int.Med/Blood	BSI	16	1	1	0.50	7	+	-	-	_	-
16 *K. pneumoniae*	Int.Med/Blood	BSI	>128	64	>128	32	8	+	-	-	_	-

Int. Med, Internal medicine; N. sur, neuro surgery; G. Sur, general surgery; TA, tracheal aspirate; BSI, blood stream infection; USI, urinary system infection; VAP, ventilator associated infection.

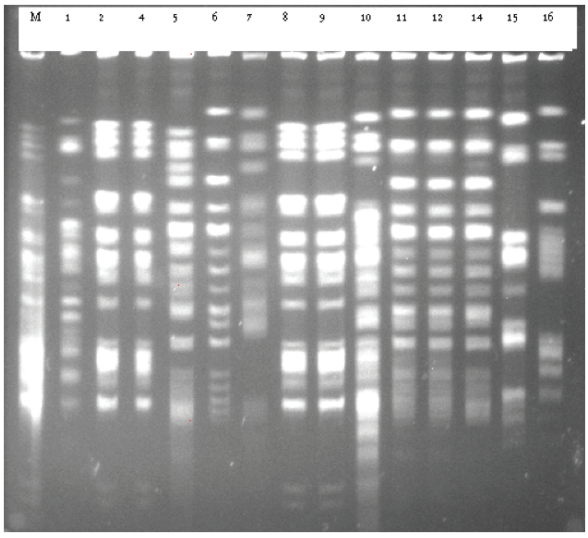

Fig 1–Pulsed-field gel electrophoresis patterns of 14 OXA-48-producing *K. pneumoniae* isolates. Lane 1, molecular size marker; lane 2, Kp1; lane 3, Kp2; lane 4, Kp4; lane 5, Kp5; lane 6, KP6; lane 7, Kp7; lane 8, Kp8; lane9, Kp9; lane 10, Kp10; lane 11, Kp11; lane 12, Kp12; lane 13, Kp 14; lane 14, Kp15; lane 15, Kp16.

(Kp6, Kp11, Kp12 and Kp14) isolated from internal medicine and neurosurgery ICUs were clonally related, and 4 *K. pneumoniae* isolates (Kp2, Kp4, Kp8 and Kp9) isolated from neurosurgery, general surgery, internal medicine and anesthesiolgy ICUs shared identical PFGE patterns. Interestingly, 3 *K. pneumoniae* isolates (Kp6, Kp7 and Kp8), which were recovered from the same patient hospitalized in the general surgery ICU, were not clonally related and isolates Kp15 and Kp16 recovered from another patient hospitalized in the internal medicine ICU were clonally distinct.

Seventy-seven percent of the patients from whom carbapenem-resistant strains were isolated were males ($p = 0.03$). Comparing patients with carbapenem-resistant strains with those of carbapenem-susceptible strains, the mean hospitalization days (57 ± 29), central venous catheter (25 ± 14 days), urinary catheter (30 ± 23 days), total parenteral nutrition and nasogastric tube usage time (16 ± 15 and 43 ± 24 days, respectively), transfusion (93.3%) were more common in carbapenem-resistant group. The differentiation between these 2 groups (carbapenem-sensitive and carbapenem nonsusceptible) was investigated by Mann-Whitney *U* test, which showed that in addition to these risk factors, ESBL and multidrug resistance activity were also risk factors for carbapenem resistance (Table 3). When the risk factors were investigated with logistic regression analysis, none of the risk factors were identified as independent risk factors. When antibiotic usage rate in carbapenem-resistant group was investigated, use of beta-lactams in 53% of the patients, quinolones in 13% and carbapenems in 13% were found.

DISCUSS0ON

This is the first report of our hospital on carbapenem-resistant Enterobacteriaceae isolates. During the two-year period, 22% of *K. pneumoniae* and 3% of *E. coli* strains, isolated from ICU patients, proved to be carbapenem-resistant. Gradually increasing in parallel with the use of carbapenems, the spread of resistance among enteric bacteria was shown in this study through molecular methods, which delineated with the relationship between resistance and clonal distribution. Knowledge of the spread of OXA-carbapenemases in enteric bacteria is important as none of the currently available phenotypic tests (except super carba medium) is suitable for detecting OXA-48/OXA-181 producers (Nordmann and Poirel, 2013). The activity of these enzymes is not inhibited

Table 3
Results of comparison of carbapenem-sensitive and carbapenem non susceptible groups.

Risk factor	Carbapenem-susceptible (n = 95)	Carbapenem non susceptible (n = 13)	p-value
Male	51 (54%)	10 (77%)	0.03
Hospital stay (day)	30 ± 31	57 ± 29	<0.0001
Duration of CL use (day)	11 ± 13	25 ± 14	0.001
Duration of UC use (day)	13 ± 17	30 ± 23	0.001
Duration of TPN use (day)	7 ± 9	16 ± 15	0.004
Duration of NGL use (day)	26 ± 27	43 ± 24	0.01
Transfusion	54 (57%)	12 (93%)	0.015
Duration of Prior antibiotic use (day)	6 ± 4	9 ± 4	0.018
ESBL	42 (44%)	2 (15%)	0.009
MDR	44 (46%)	1 (8%)	0.001

CL, central venous catheter; UC, urinary catheter; TPN, total parenteral nutrition; NGL, nasogastric tube usage.

by clavulanic acid, tazobactam, sulbactam, boronic acid, EDTA or dipicolinic acid (Nordmann *et al*, 2012). Enterobacteriaceae showing reduced susceptibility or resistance to at least one carbapenem may be used as a first indicator towards identifying possible OXA-48 producers (Nordmann *et al*, 2012).

OXA-carbapenemase activity with an increasing rate of ESBL activities of enteric bacteria in hospital environment results in the development of multidrug resistance activity (Rasmussen and Hoiby, 2006; Walsh, 2010). Carbapenems are supposed to be the last option for treatment and the effectiveness of carbapenems has diminished (Rasmussen and Hoiby, 2006; Erdem *et al*, 2013). Another important point of this study was that the risk factors associated with development of resistance were identified, which included significant information to allow guidance towards the control of resistance.

In this study, all the ertapenem-resistant strains of *K. pneumoniae* were found positive for OXA-48 gene. One *E. coli* strain (number 3) was resistant to ertapenem (8 mg/l). In this strain, although carbapenemase activity gave positive results by MHT with ertapenem and disc enzymatic assay, but no carbapenemase gene was detected. *K. pneumoniae* strains with multiple resistance mechanisms and their transferability potential do not only pose a problem in our hospital, but also in other hospitals (Rasmussen and Hoiby, 2006; Walsh, 2010; Cuzon *et al*, 2011). In the present study, multiple resistant genes were found to be co-expressed in the same CNS isolates. Of note, one patient in general surgery ICU was infected by 3 different clones of OXA-48 -producing *K. pneumoniae* strains while another patient who was hospitalized in the internal medicine ICU was infected by 2 different OXA-48-producing *K. pneumoniae* strains. Clearly, there is also a hospital spread of two clones and the horizontal transmis-

son of bla_{OXA-48} gene in different hosts is presumably by a common plasmid. This genetic exchange is significant and its transferability occured even in the same patient. Additionally, the loss of outer membran protein and the presence of an efflux-pump, which were not screened in the study, also may be responsible for increasing carbapenem resistance and for the highest level of MIC along with other resistance mechanisms.

Studies conducted up to date have shown that hospitalization time, staying in ICU, invasive device usage, immunosuppression, and prior antibacterial usage are independent risk factors for colonization or infection (Carmelli *et al*, 2010). It is very important to determine the cause(s) of resistance in our country where the carbapenem use is widespread. Similar to the other studies (Jeon *et al*, 2008; Carmelli *et al*, 2010), the hospitalization time, invasive device usage, transfusion, and prior antibacterial usage were found as risk factors for carbapenem resistance in our study. However, none of these was determined as independent risk factors for carbapenem resistance.

One of the most important factors for developing resistance in gram-negative bacteria is inappropriate antibacterial usage. For critically ill patients in ICU, inappropriate antibacterial use can cause selective pressure for emergence of resistant strains (Carmelli *et al*, 2010). In this study, prior beta-lactams usage in 53%, quinolone usage in 13% and carbapenem usage in 13% of the patients infected with carbapenem-resistant strains were identified. Although prior carbapenem usage was suggested to be an important risk factor for developing resistance in several studies (Jeon *et al*, 2008; Carmelli *et al*, 2010), this was not determined to be an independent risk factor in this study. However, it was found that the time period of antibiotic intake poses a significant risk factor for the development of carbapenem resistance.

Carbapenem resistance of gram-negative enteric bacteria in ICU is becoming an increasing problem. In the present study, we have identified for the first time Enterobacteriaceae strains identified in our hospital carrying a carbapenem-hydrolyzing oxacillinase. There is no consensus on the cut-off value of MICs of carbapenems that should be applied for research into carbapenemase activity. Intermediate susceptibility and even susceptibility to carbapenems have been observed for OXA-48 producers (Nordmann and Poirel, 2013).

It is worth mentioning that 13 of the 16 isolates were susceptible to imipenem and meropenem according to CLSI guidelines. Ertapenem disk diffusion test may be an effective method especially for the identification of the carbapenem resistance in *K. pneumoniae* and *E. coli* strains. Additionally, more advanced methods should be used to determine carbapenem resistance and MIC values in suspected patients especially in the centers with high ESBL rates, instead of resorting to the use of carbapenem. However, as the problem has become increasingly global, each center needs to determine their resistance profiles, to seek for solutions to prevent the spread of resistance, to develop more appropriate policies governing antibiotic use and to implement more effective infection control measures.

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