International Journal of Infectious Diseases 29 (2014) 139–144

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Countrywide spread of OXA-48 carbapenemase in Lebanon: surveillance and genetic characterization of carbapenem-non-susceptible *Enterobacteriaceae* in 10 hospitals over a one-year period

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ARTICLE INFO

Article history:
Received 26 January 2014
Received in revised form 26 May 2014
Accepted 24 July 2014

Corresponding Editor: Ziad Memish, Riyadh, Saudi Arabia

Keywords:
Enterobacteriaceae
OXA-48
Carbapenem resistance
AmpC

SUMMARY

Objectives: To detect, characterize, and assess the genetic clonality of carbapenem-non-susceptible *Enterobacteriaceae* in 10 Lebanese hospitals in 2012.
Methods: Selected *Enterobacteriaceae* isolates with reduced susceptibility to carbapenems were subject to phenotypic study including antibiotic susceptibility, cloxacillin effect, modified Hodge test, and activity of efflux pump inhibitor. Carbapenemase genes were detected using PCR; clonal relatedness was studied by pulsed field gel electrophoresis.
Results: Out of 8717 *Enterobacteriaceae* isolated in 2012, 102 (1.2%) showed reduced susceptibility to carbapenems. Thirty-one (70%) of the 44 studied clinical isolates harbored bla_{OXA-48}, including 15 *Klebsiella pneumoniae*, eight *Escherichia coli*, four *Serratia marcescens*, three *Enterobacter cloacae*, and one *Morganella morganii*. The majority of OXA-48 producers co-secreted an extended-spectrum beta-lactamase, while one had an acquired AmpC of the ACC type. In the non-OXA-48 producers, carbapenem resistance was attributed to the production of acquired AmpC cephalosporinases of MOX or CIT type, outer membrane impermeability, and/or efflux pump overproduction. DNA fingerprints revealed that OXA-48 producers were different, except for clonal relatedness among four *K. pneumoniae*, two *E. coli*, two *E. cloacae*, and three *S. marcescens*.
Conclusions: Nosocomial carbapenem-non-susceptible *Enterobacteriaceae* are moderately spread in Lebanon and the predominant mechanism is OXA-48 production.

1. Introduction

Enterobacteriaceae are Gram-negative rod-shaped bacteria that normally colonize the human intestinal tract and are capable of causing opportunistic infections in both community and hospital settings. They are easily spread among individuals and can acquire

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http://dx.doi.org/10.1016/j.ijid.2014.07.017

genetic material through lateral gene transfer, largely mediated by mobile elements like plasmids and transposons.[1] The dissemination of *Enterobacteriaceae* producing extended-spectrum beta-lactamases (ESBLs) has compromised susceptibility to cephalosporins in many areas of the world, including Lebanon, and has increased the consumption of carbapenems.[2,3] Recently developed antibiotics among the class of beta-lactams, carbapenems (imipenem, meropenem, ertapenem, and doripenem) are not hydrolysable by EBSLs and represent the antimicrobial options of last resort.[2] In 1993, the first incidence of carbapenem resistance in *Enterobacteriaceae* was described in *Enterobacter cloacae* due to production of carbapenemase NmcA.[4] Since then, various carbapenemases have been detected in *Enterobacteriaceae* and the majority belong to three Ambler classes: (1) class A, such as KPC, first described in the USA but now disseminated worldwide; (2) class B, such as IMP, VIM, and NDM metallo-beta-lactamases, encountered in Japan, Taiwan, and Greece, as well as many European countries; and (3) class D oxacillinases, such as OXA-48, originating in Turkey and now detected in Europe and the Mediterranean region.[1] Rare acquired cephalosporinases of Ambler class C may also show low carbapenem-hydrolyzing activity.[5] Carbapenem resistance in *Enterobacteriaceae* may also arise from non-carbapenemase-mediated mechanisms, like permeability defects or the impact of efflux transporters.[6]

In Lebanon, carbapenem-resistant *Enterobacteriaceae* have been detected since 2008, with single case reports of OXA-48, IMP-1, and NDM-1 carbapenemases.[7–10] The aims of the current study were to evaluate the dissemination of carbapenem-resistant *Enterobacteriaceae* in various Lebanese hospitals, to identify the mechanisms of resistance, to examine the types of carbapenemases involved in resistance, and to analyze the clonal relationship of such isolates collected during the year 2012. Part of this work was presented at the RICAI (Réunion Interdisciplinaire de Chimiothérapie Anti-infectieuse) 2012 congress in Paris, France (November, 22–23, 2012; abstract number 300).

2. Materials and methods

2.1. Bacterial isolates

From January 1, 2012 to December 31, 2012, carbapenem-intermediate/resistant isolates of *Enterobacteriaceae* were collected from the bacteriology laboratories of 10 Lebanese hospitals located in diverse geographic areas: Beirut (Hotel Dieu de France, Saint-George Hospital, Clinique de Levant); Mount Lebanon (Bellevue Medical Center, Arz Hospital); North Lebanon (Monla Hospital); South Lebanon (Secours Populaire Libanais, Labib Medical Center), and Bekaa (Farhat Hospital, Chtaura Hospital). Using standard disk diffusion testing, and following the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST),[11] strains with inhibition zone diameters of imipenem smaller than 22 mm, or of ertapenem smaller than 25 mm, were included. These isolates were delivered to the central microbiology laboratory at the Faculty of Pharmacy, Saint-Joseph University, Beirut.

2.2. Phenotypic analysis

2.2.1. Antimicrobial susceptibility testing and detection of ESBLs

Antimicrobial susceptibility testing was performed by disk diffusion on Mueller–Hinton agar plates, according to EUCAST guidelines.[11] Amoxicillin/clavulanic acid (AUG), ceftazidime (CAZ), cefotaxime (CTX), cefepime (CPM), cefoxitin (FOX), aztreonam (ATM), and imipenem (IMP) disks (Mast Diagnostics, Merseyside, UK) were tested. After overnight incubation, an increase in the inhibition zone of the third- and/or fourth-generation cephalosporin disk towards the clavulanate-containing disk by at least 5 mm was considered as indicating synergy and the presence of an ESBL.[12] The minimum inhibitory concentration of ertapenem (MIC_{ERT}) was determined using the Etest (Liofilchem, Roseto degli Abruzzi, Italy).

2.2.2. Cloxacillin test for detection of AmpC cephalosporinases

Antibiotic susceptibility testing was performed with cloxacillin (25–500 mg/l) to inhibit AmpC cephalosporinases. An increase in susceptibility to cephalosporins in the presence of cloxacillin was considered to be due to AmpC production. The cloxacillin test was also used to allow better visualization of synergy between: (1) clavulanic acid and third- and/or fourth-generation cephalosporins, indicating the possibility of ESBL production, and (2) clavulanic acid and imipenem, indicating the production of an ESBL with carbapenem-hydrolyzing activity.[12]

2.2.3. Modified Hodge test

A modification of the Hodge test was applied to screen for carbapenemase production.[13] The indicator organism, *Escherichia coli* ATCC 25922 at a turbidity of 0.5 McFarland, was used to inoculate the surface of Mueller–Hinton agar plates, and a 10 μg meropenem disk (Mast Diagnostics, Merseyside, UK) was placed at the center. The test strain was heavily streaked from the disk to the plate periphery. After overnight incubation, the presence of a cloverleaf-shaped indentation of growth of the test strain versus the indicator strain was interpreted as carbapenemase production, with the highest sensitivity to those belonging to Ambler classes A and D.[14] Heavy streaks of carbapenemase-positive and carbapenemase-negative strains were used as internal controls.

2.2.4. Activity of efflux pump inhibitor

Mueller–Hinton plates containing 100 mg/l of efflux pump inhibitor phenylalanine–arginine β-naphthylamide hydrochloride (PAβN; Sigma-Aldrich, USA) were prepared. Selected isolates were swabbed on plates with and without PAβN, and Etest strips of ertapenem, cefixime, and levofloxacin (Liofilchem, Via Scozia, Italy) were placed on the plates. After overnight incubation at 37 °C, MICs in the presence and the absence of the inhibitor were compared, and a two-fold decrease in MIC in the presence of PAβN was considered as indicative of efflux activity.[15,16] *E. coli* ATCC 25922 was used as internal control.

2.3. Genotypic analysis

The presence of various types of beta-lactamase genes was screened for by PCR using primers designed by the Institut Pasteur, Unité des Agents Antibactériens, Paris, France.[17] When ESBLs were detected by phenotypic tests, PCR experiments to detect $bla_{\text{CTX-M group 1}}$ and bla_{SHV} genes were performed. When hyperproduction of AmpC was suspected by the cloxacillin test, multiplex PCR for plasmid-encoded AmpC genes was performed, including bla_{ACC}, bla_{FOX}, bla_{MOX}, bla_{DHA}, bla_{CIT}, and bla_{EBC}. To detect carbapenemases, strains were tested for bla_{KPC}, bla_{GES}, $bla_{\text{OXA-48}}$, bla_{VIM}, $bla_{\text{IMP-1}}$, $bla_{\text{IMP-2}}$, and bla_{NDM} genes. For OXA-48 producers, the presence of insertion sequence IS*1999* of transposon Tn1999 known to carry the $bla_{\text{OXA-48}}$ gene was investigated, as described by Aubert et al.[18]

2.3.1. Conjugation experiments with Klebsiella pneumoniae

Conjugation experiments were performed with *K. pneumoniae* Kpd2, using the recipient nalidixic acid-resistant *E. coli* K12, as described previously,[19] but changing the mating broth medium to brain–heart infusion.