Research articles

KPC-2-PRODUCING *KLEBSIELLA PNEUMONIAE* INFECTIONS IN GREEK HOSPITALS ARE MAINLY DUE TO A HYPEREPIDEMIC CLONE

P Giakoupi¹, H Maltezou², M Polemis¹, O Pappa¹, G Saroglou³, A Vatopoulos (avatopou@nsph.gr)¹, the Greek System for the Surveillance of Antimicrobial Resistance⁴

1. Department of Microbiology, National School of Public Health Athens, Greece

2. Department for Interventions in Health-Care Facilities, Hellenic Center for Disease Control and Prevention, Athens, Greece

3. Department of Internal Medicine, Faculty of Nursing, University of Athens, Athens, Greece

4. The participating hospitals are listed at the end of the article

To verify the presence of *Klebsiella pneumoniae* carbapenemase-producing (KPC-producing) *Klebsiella pneumoniae* in Greece, we asked 40 Greek hospitals participating in the Greek System for the Surveillance of Antimicrobial Resistance (GSSAR) to apply a combination of the modified Hodge test plus EDTA synergy test on all *K. pneumoniae* clinical isolates obtained from February 2008 which displayed reduced susceptibility to carbapenems (MIC of imipenem ≥ 1 mg/L). The presence of the *blaKPC* gene was confirmed by PCR and sequencing. This procedure revealed the presence of KPC-2 in isolates from 173 patients in 18 hospitals during a period of 11 months. Of these, 166 isolates belonged to a single pulsotype a fact consistent with possible epidemic spread, whereas the remaining seven isolates were further classified into four different pulsotypes. *blaKPC-2* gene was found to be transferable by conjugation in the four pulsotypes other than the prevailing one. The emergence of a new carbapenemase gene in Greece, where high resistance rates to carbapenems in *K. pneumoniae* due to the spread of the VIM type metalloenzyme have been observed, emphasises the urgent need for the implementation of public health measures in the field of infection control and antibiotic consumption. It also underlines the need to supplement surveillance systems based on susceptibility data with the surveillance of resistance mechanisms.

Introduction

Resistance to carbapenems is one of the major threats for treatment of infections caused by Gram-negative bacteria, and the production of carbapenemases is the most important molecular mechanism both clinically and epidemiologically. Carbapenemases are beta-lactamases and are divided into two major molecular groups, differentiated by the hydrolytic mechanism in the active site. The first group contains at least one zinc atom at the active site, establishing them as metalloenzymes, represented mainly by Verona integron-encoded metallo-beta-lactamase (VIM) and IMP-type carbapenemases. The second group utilises serine at the active site and its main representatives are *Klebsiella pneumoniae* carbapenemase (KPC) type enzymes belonging to the Bush group 2f [1].

Two publications, one in late 2007 and the other in early 2008, reported infections due to KPC-producing *K. pneumoniae* in two patients, one in Sweden and the other in France. Both patients had originally been hospitalised in Crete, Greece [2,3].

Following these reports, in February 2008, the Department for interventions in healthcare facilities at the Hellenic Center for Disease Control and Prevention (HCDCP) in collaboration with the Greek System for the Surveillance of Antimicrobial Resistance (GSSAR) initiated a study aimed at confirming the presence of such clinical strains in Greece, and assessing the extent of their spread in the Greek hospitals. The objective was also to investigate the genetic relatedness of the respective bacterial strains and the transferability of the *blaKPC*-harbouring plasmids.

In this paper we report the preliminary results of this study. Part of these results have been presented at the 19th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) in Helsinki in May 2009.

Materials and methods

Study design

Written guidelines on how to detect isolates producing KPC were distributed to microbiology laboratories of the 40 hospitals that participate in the GSSAR*. These hospitals can be considered representative of all Greek hospitals, geographically, by type and by size. The laboratories were asked to screen all *K. pneumoniae* isolates displaying reduced susceptibility to carbapenems (minimum inhibitory concentration [MIC] of imipenem ≥ 1 mg/L), and, subsequently, send those identified as possible KPC-producing isolates to the microbiology laboratory at the National School of Public Health (NSPH) for confirmation and further analysis.

Susceptibility testing

Susceptibility tests were performed by the agar dilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [4]. MIC of imipenem was determined by Etest according to the instructions of the manufacturer (AB Biodisk, Solna, Sweden).

Phenotypic detection of the KPC enzyme

Preliminary phenotypic detection of the KPC enzyme performed by the hospital laboratories was based on the combination of a bioassay test - the Hodge (cloverleaf) test - and the EDTA synergy test [5].

A possible case of KPC-producing *K. pneumoniae* was defined as an isolate which displayed reduced susceptibility to carbapenems (MIC of imipenem ≥ 1 mg/L) and tested positive in modified Hodge test for the presence of carbapenemase activity, and negative in EDTA synergy test for the presence of metalloenzymes.

A possible case of VIM-producing *K. pneumoniae* was defined as an isolate which displayed reduced susceptibility to carbapenems (MIC of imipenem ≥ 1 mg/L) and tested positive in Hodge (cloverleaf) test for the presence of carbapenemase activity and positive in EDTA synergy test for the presence of metalloenzymes.

Both tests were negative in strains not producing carbapenemase.

Hodge (cloverleaf) test (bioassay)

The indicator organism, *Escherichia coli* ATCC 25922, at a turbidity of 0.5 McFarland standard, was used to inoculate the surface of a Mueller Hinton agar plate, and the test strain was heavily streaked from the centre to the plate periphery. After the plate was allowed to stand for 15 minutes at room temperature, a 10 µg IPM disk was placed at the centre of the streak, and the plate was incubated overnight. The presence of an even slightly distorted inhibition zone was interpreted as a positive result for carbapenem hydrolysis.

EDTA synergy test

The test strain, at a turbidity of 0.5 McFarland standard, was used to inoculate the surface of a Mueller Hinton agar plate. Disks of imipenem (10 µg), meropenem (10 µg), ceftazidime (30 µg) and piperacillin (100 µg) were placed at a 20 mm centre-to-centre distance from a disk containing 930 µg EDTA. The plate was incubated overnight. The presence of distorted inhibition zones to either antibiotic disk was interpreted as a positive result for metallo-beta-lactamase production.

Confirmation of the presence of the blaKPC gene

The presence of the *blaKPC* gene was confirmed by PCR using forward and reverse primers proposed by Queenan and Bush [1], and subsequent sequencing on both strands of the PCR products.

Molecular typing was performed by pulsed field gel electrophoresis (PFGE) of *Xba*I-restricted genomic DNA as described previously [6]. Restriction fragments were separated through a 1% agarose using a contour-clamber homogeneous electric field DRII apparatus (BioRad, Milano, Italy). Gel Compar II was used for classification of the isolates into PFGE types.

Strains

*bla*VIM and extended-spectrum beta-lactamase (ESBL)-producing *K. pneumoniae* isolates used in this study for the quality assessment of the phenotypic tests came from the collection of the microbiology laboratory at NSPH.

Conjugation

Conjugal transfer of antibiotic resistance was performed in mixed broth cultures as described previously [7] using the

Escherichia coli strain 1R716 (StrR, lac-) as a recipient. Transconjugant clones were selected in McConkey agar containing streptomycin 1000 µg/ml plus ampicillin 100 µg/ml.

Results

From February 2008 until December 2008, 21 hospitals sent us a total of 225 *K. pneumoniae* isolates, from an equal number of patients, phenotypically considered as possible KPC-producers. Hospitals sent different numbers of isolates (ranging from one to 37). Further analysis by PCR and sequencing at the NSPH laboratory confirmed 173 (77%) isolates from 18 hospitals to harbour *blaKPC-2* gene. The remaining 52 isolates were found to be VIM-producers.

Interestingly, when the two phenotypic tests were repeated at the NSPH, the results indicated possible KPC production in 171 of the 173 PCR-confirmed KPC-2-producing isolates and in none of the 52 VIM-producing isolates. Two isolates that showed a positive bioassay test and a positive EDTA synergy test due to VIM-1 production, exhibited resistance to aztreonam and were found to concurrently produce KPC-2 enzyme.

The validity of the proposed combination of the phenotypic tests for the detection of the various carbapenemases was further evaluated using 34 VIM-producing and 41 ESBL-producing *K. pneumoniae* available in the microbiology laboratory at NSPH. The tests were able to identify all but three VIM-producing isolates which displayed a falsely negative bioassay and a positive EDTA test. The results were negative for all ESBL-producing isolates.

To estimate the probable period of emergence of the KPC-2-producing *K. pneumoniae* in Greece, all carbapenem-non-susceptible isolates in the collection of the microbiology laboratory at NSPH (which serves as the reference centre for carbapenem-resistant Enterobacteriaceae) were screened and found negative for the presence of *blaKPC-2* gene. This collection of samples covered a period of seven years (January 2001 – December 2007).

Geographical distribution

The 171 confirmed KPC-2-producing *K. pneumoniae* isolates were obtained from three hospitals in Crete, 14 hospitals in the Athens – Piraeus area, and one hospital in Thessaloniki.

PFGE patterns of the *Xba*I restriction fragments of KPC-2-producing *K. pneumoniae* isolates are shown in the Figure. Isolates producing KPC-2 were classified into five pulsotypes displaying 90% similarity within each type. Pulsotype A included 166 isolates, pulsotype B consisted of one isolate, and pulsotypes C, D and E included two isolates each. The two isolates producing both VIM-1 and KPC-2 belonged to pulsotype C (Table).

Pulsotype A was found in all but one hospital a fact consistent with possible epidemic spread, whereas pulsotypes B and C were found exclusively in Crete, each in different hospitals, together with pulsotype A. Pulsotype E was found in one hospital in Crete and in one hospital in Athens, whereas pulsotype D was found only in one hospital in Athens.

Pulsotype A was also found to be indistinguishable from the clinical strains isolated from patients in Sweden and France initially hospitalised in Greece [2,3], as well as from a patient transferred

to France from Israel and already known in Israel to be infected by this strain [V. Jarlier, personal communication].

Sensitivity testing

Sensitivity testing revealed that all KPC-2-producing isolates were resistant to the combinations of penicillin with beta-lactamase inhibitors, as well as to ceftazidime and aztreonam and non-susceptible to cefoxitin, cefotaxime and cefepime. Colonies observed within the ellipses of inhibition made determination of the imipenem MIC difficult (see also reference 8). Concerning other drug classes, isolates of A, C and D types were resistant to aminoglycosides (except gentamycin), cotrimoxazole and quinolones and only tetracycline and tigecycline were shown efficacious in all types (Table). Type B strain was sensitive to all other drug classes tested.

Conjugation

Conjugal transfer was attempted with two isolates per hospital for pulsotype A and with all isolates belonging to the other pulsotypes. Transconjugants at a rate of 10^{-6} were recovered from isolates of pulsotype B, C, D and E but not from the prevailing pulsotype A. Presence of *bla*KPC-2 gene in all transconjugants was confirmed by PCR (only the *bla*KPC-2 gene and not the *bla*VIM gene from the pulsotype C isolate was transferred). Transconjugants were resistant to combinations of penicillin with beta-lactamase inhibitors and aztreonam, but were susceptible to all other oxyimino-beta-lactams. MIC values of imipenem were two to five doubling dilutions higher than that of the recipient (recipient's MIC of imipenem was 0.25 mg/L), but remained within the fully susceptible area as determined by the CLSI criteria [6]. Transconjugants from pulsotypes B, C and E were susceptible to all other drug classes, whereas in transconjugant from pulsotype D resistance to all other drug classes except quinolones was transferred. Work is in progress for the further characterisation of the genetic environment of the *bla*KPC-2 gene.

Discussion

The occurrence of KPC-producing *K. pneumoniae* seems to be an emerging public health problem in various parts of the world [9,10], although, to date, widespread hospital outbreaks have been reported mainly in the United States [11,12] and Israel [13,14]. Greece seems to be the third country facing a similar widespread problem. International cooperation through the early publication of reports [2,3] proved very helpful for the timely mobilisation of the Public Health System in Greece and the early detection of this epidemic.

A combination of the modified Hodge (cloverleaf) test with the EDTA synergy test was used by the hospital laboratories in Greece for the preliminary detection of the KPC-producing *K. pneumoniae*. Similar approaches have been described for the phenotypic detection of the carbapenemase-producing *Enterobacteriaceae* [15,16,17,18]. However various authors suggest the use of boronic acid disk potentiation tests for the detection of the KPC enzymes [19,20,21]. Although in the reference laboratory this approach showed high sensitivity and specificity for both detection of carbapenemases and discrimination of the KPC and VIM enzymes, the high number of errors in identifying the type of carbapenemase among the test results obtained initially by the hospital laboratories,

FIGURE

PFGE analysis of XbaI-digested genomic DNA from representative KPC-2-producing *Klebsiella pneumoniae* described in this study

Legend:
lanes 1 and 14: Lambda Ladder PFG Marker (New England Biolabs, Ipswich, MA);
lanes 2-7: pulsotype A;
lane 8: pulsotype B;
lane 9: pulsotype C;
lane 10: pulsotype D;
lane 11: pulsotype E;
lanes 12 and 13: VIM-producing *K. pneumoniae*

TABLE

Summary of epidemiological data and information on antibiotic susceptibility and transferability of the KPC-2-producing *Klebsiella pneumoniae* isolates described in this study

Pulsotype	Number of isolates	Number of hospitals	Resistance to other drug classes*	<i>bla</i> KPC gene transferred via conjugation	Other drug classes transferred
A	166	18	an, net, tb, spt, sxt, c, cip	No	
B	1	1	—	Yes	
C	2	1	an, net, tb, spt, sxt, cip	Yes	—
D	2	1	gm, an, net, tb, spt, sxt, c, cip	Yes	gm an net tb spt sxt c
E	2	2	net, tb, spt, sxt, c, cip	Yes	—

*Abbreviations:
an - amikacin, net - metilmicin, tb - tobramycin, spt - spectinomycine, sxt - cotrimoxazole, c - chloramphenicol, cip - ciprofloxacin, gm - gentamicin.
—: negative result

underline the importance of experience needed in performing and interpreting such tests.

Furthermore it is well recognised that these enzymes confer low level resistance, lower than the established breakpoints [8]. Since this study was confined to strains exhibiting a MIC value of imipenem not less than 1 mg/L, the possible presence of undetected strains exhibiting lower MICs can not be excluded and thus it is possible that the overall prevalence has been underestimated.

These represented the major limitations of our study which made it difficult to assess the exact prevalence of the KPC-producing isolates in Greece as well as the exact date of the first isolation in each hospital.

With these limitations in mind, however, it can be deduced, mainly from the two published reports on Swedish and French patients hospitalised in Crete [2,3], from the results of an epidemiological study subsequently performed in Crete [22] and from the fact that no KPC-producing *K. pneumoniae* were found in the collection of the reference laboratory, that the first KPC-2-producing isolates seem to have emerged in Crete in spring 2007 [2,22]. The rapid mainly monoclonal epidemic spread in the rest of the country could at least partly be explained by the movement of patients among hospitals, a well known practice in Greece.

Currently, the KPC-2 enzyme seems to spread in Greece in *K. pneumoniae* and other *Enterobacteriaceae* in parallel with the well established VIM-type [23]. Data from the Greek System for the Surveillance of Antimicrobial Resistance (www.mednet.gr/whonet) show that there has been an increase in the resistance rates to imipenem in *K. pneumoniae* during the last three years [23].

Consumption of antibiotics in hospitals in Greece, overall and of the newer beta-lactam antibiotics (third generation cephalosporins and carbapenems), is reported to be the highest in Europe [24]. Carbapenems and third generation cephalosporins can act as selective factors for both *blaVIM* and *blaKPC* genes. Interestingly, VIM- producing *K. pneumoniae* were shown to cause a polyclonal epidemic in Greece [5,23], while KPC-2-producing *K. pneumoniae* isolates were found to belong to a single PFGE genotype in the vast majority of cases. Genetic homogeneity is probably consistent with the recent introduction and clonal spread of KPC-2-producing isolates. It also implies that infection control is an important public health strategy for the containment of the KPC-producing mechanism.

The spread of KPC-2 via indistinguishable pulsotypes, as described in this study, was also shown in outbreaks in New York [25]. However, the location of *blaKPC*-2 gene on transferable plasmids as in the case of pulsotypes B to E observed in this study, could contribute to its further spread among clones and bacterial species. Transferable plasmids indistinguishable by restriction profile analysis were implicated in the dissemination of KPC-2 in various instances [26]. Moreover, the ability of *blaKPC*-2 gene to coexist with *blaVIM* gene observed in this study, as well as with other newer beta-lactam-resistant determinants recently described [9] can lead to difficult to treat bacterial infections.

The observed similarity between the Greek major clone and the isolate from Israel could be regarded as consistent with the possible spread of the Israeli clone in Greece, a hypothesis that must be further evaluated. It is important to note however that in

Israel, the *blaKPC*-2 gene was found on six different pulsotypes of *K. pneumoniae* [13] whereas the *blaKPC*-3 gene was found to spread monoclonally [13]. Recently, the possible spread of strains carrying the *blaKPC*-3 gene from Israel to the United Kingdom has been reported [27].

In conclusion, resistance to carbapenems in *K. pneumoniae* in Greece seems to be due to the contemporary spread of two resistance mechanisms: the already established VIM type metalloenzyme characterised mainly by polyclonal spread and transferable plasmids [23] and the KPC-2 shown in our study to spread mainly monoclonally in an epidemic mode. Currently, there are no confirmed clinical data to assess the possible implication of the presence of carbapenemase-producing organisms in infections treated with carbapenems [23,28]. However, our data emphasise the urgent need for implementation of public health measures in the field of infection control and antibiotic consumption. They also underline the inadequacy of the surveillance systems that are exclusively based on antibiotic susceptibility data in elucidating the resistance phenomenon, and thus emphasise the need to supplement these systems with the surveillance of the resistance mechanisms at the molecular level. Understanding these complex processes at the hospital, country, national and even international level is an important prerequisite for instituting properly designed public health measures.

* The following hospitals of the Greek System for the Surveillance of Antimicrobial Resistance (<http://www.mednet.gr/whonet/>) participated in the present study:

"Venizelio" General Hospital, Herakleion, Crete (M Ventouri, V Liakou); "Onassio" Cardiac Surgery Centre, Athens (A Tasouli, S Geroulanos); General Hospital of Chania, Crete (G Alevraki, K Tsaferaki); "Evangelismos" General Hospital, Athens (E Platsouka, O Panirara); "Agios Panteleimon" General Hospital, Nikaia, Piraeus (P Karle, D Mylona-Petropoulou); "Agios Pavlos" General Hospital, Thessaloniki (H Kakasi, B Galanopoulou-Gkiousera); Naval Hospital, Athens (E Mournianakis, G Tottos); "Agia Olga" General Hospital, Athens (I Mellas, Z Roussou); 251 Air Force Hospital, Athens (H Douma-Zaharopoulou, G Katsanis); "Amalia Flemming" General Hospital, Melissia, Athens (A Karaitianou, G Kouppari); "Izannio" General Hospital, Piraeus (O Zarkotou, K Themeli-Digalaki); Emergency Hospital "KAT", Athens (S Tsiplakou, V Papaioannou); Hippocraton General Hospital, Athens (A Xanthaki, M Toutouza); "G. Gennimatas" General Hospital, Athens (H Vagiakou, H Malamou-Ladas); "Asklipio" General Hospital, Voula, Piraeus (D Kairis, X Koutsia); "Sotiria" General Hospital, Athens (H Moraitou, S Kanavaki); "Sismanoglio" General Hospital, Athens (M Kanellopoulou, E Papafrangas); "Demokritos" University General Hospital, Alexandroupoli (M Panopoulou, S Ktenidou-Kartali); General Hospital of Ierapetra, Ierapetra, Crete (M Kotsidoniotali); "Attiko" University General Hospital, Athens (S Vourli, L Zerva); "Laiko" General Hospital, Athens (A Pantazatou, A Avlami).

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