A ten-year surveillance study of carbapenemase-producing *Klebsiella pneumoniae* in a tertiary care Greek university hospital: predominance of KPC- over VIM- or NDM-producing isolates

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Resistance patterns and carbapenemase gene presence among *Klebsiella pneumoniae* isolates from the University General Hospital of Patras, Greece during a ten-year period were analysed under a surveillance programme for multi-drug-resistant bacteria. From 2005 to 2014, *K. pneumoniae* isolates from clinically significant specimens were identified by the Vitek 2 Advanced Expert System. Antibiotic susceptibility testing was performed by the agar disc diffusion method and Etest. The strains were tested for the presence of *bla*<sub>VIM</sub>, *bla*<sub>IMP</sub>, *bla*<sub>KPC</sub>, *bla*<sub>NDM</sub> and *bla*<sub>OXA-48</sub> genes by PCR. PFGE of chromosomal *XbaI* DNA digests was performed. A total of 3449 *K. pneumoniae* isolates were recovered during the last decade. Among them, 1668 (48 %) were carbapenemase-producing: 1333 (80 %) *K. pneumoniae* carbapenemase (KPC)-, 286 (17 %) Verona imipenemase (VIM), 45 (3 %) KPC- and VIM-, and four New Delhi metallo-beta-lactamase (NDM)-producing. Their resistance rates to gentamicin, colistin and tigecycline were 41 %, 23 % and 16 %, respectively. VIM-producing *K. pneumoniae* were isolated in 2005 and since 2008 have been endemic. KPC-producing *K. pneumoniae* (KPC-Kp) isolates were introduced in 2008 and until now represent the predominant carbapenemase-producing *K. pneumoniae* in our institution. PFGE of 97 KPC-Kp strains identified three types: A, 84 (87 %); B, 11 (11 %); and E, two (2 %). Eleven co-producing KPC and VIM *K. pneumoniae* isolates belonged to PFGE B. The four NDM-positives were classified to type F. The number of *K. pneumoniae* bacteraemias increased during the study period, which may be solely attributed to the increase of carbapenemase-producing isolates. The threat of carbapenemase-producing *K. pneumoniae* emphasizes the urgent need for implementation of infection control measures and budgetary allocations to infection control.

INTRODUCTION

Over recent years, a significant increase in infections caused by multidrug-resistant (MDR) Gram-negative bacteria, especially *Klebsiella pneumoniae*, has been observed worldwide (Tzouvelekis et al., 2012). Carbapenems are important therapeutic agents for treating infections caused by such pathogens. Their efficacy has declined over recent decades due to the emergence of carbapenemase-producing *K. pneumoniae* (CP-Kp) isolates (Queenan & Bush, 2007; Tzouvelekis et al., 2012).

Carbapenemases are beta-lactamases encoded by genes that can be found in mobile genetic elements, a fact that explains the way they spread so thoroughly (Queenan & Bush, 2007).
Concerning \textit{K. pneumoniae} isolates in Greece, carbapenemases are distinguished in two main groups, which can be differentiated by the hydrolytic mechanism in the active site. The first group’s enzymes (class A) have a serine-based hydrolytic mechanism and are represented mainly by \textit{K. pneumoniae} carbapenemase (KPC) (Queenan & Bush, 2007). The second group’s enzymes, containing a zinc atom (class B), are characterized as metallo-beta-lactamases (MBLs), with the primary representatives Verona imipenemase (VIM), imipenemase (IMP) and, more recently, New Delhi metallo-beta-lactamase (NDM) (Nordmann \textit{et al.}, 2011; Queenan & Bush, 2007).

VIM-producing \textit{K. pneumoniae} (VIM-Kp) isolates, initially isolated in Greece in 2002, were characterized by multiclonality and rapidly became endemic (Hasan \textit{et al.}, 2014; Vatopoulos, 2008). The introduction of KPC-producing \textit{K. pneumoniae} (KPC-Kp) in the Greek health system in 2007 changed abruptly the national epidemiology, since KPC-Kp rapidly substituted the previous VIM-Kp clones (Giakoupi \textit{et al.}, 2009; Maltezou \textit{et al.}, 2009). This phenomenon is probably due to the fact that most isolates belonged to a worldwide successful hyperendemic clone (ST258) (Giakoupi \textit{et al.}, 2009; Tzouvelekis \textit{et al.}, 2012). NDM-producing \textit{K. pneumoniae} (NDM-Kp) was isolated in early 2013 in Greece, provoking sporadic cases (Giakoupi \textit{et al.}, 2013; Spyropoulou \textit{et al.}, 2015).

In the present study, resistance patterns and carbapenemase gene carriage among \textit{K. pneumoniae} isolates from a Greek university hospital during a ten-year period were investigated.

**METHODS**

This is a retrospective study that was carried out at the University General Hospital of Patras (UGHP), Greece, a 770-bed teaching hospital, from January 2005 to December 2014. The study was approved by the Ethical Committee of University Hospital of Patras (no. 18208/18-9-2013).

**Identification and antibiotic susceptibility testing.** \textit{K. pneumoniae} isolates (one per patient) from clinically significant specimens (blood, urine, catheter tips, sputum, bronchial secretions, pus, pleural and peritoneal fluids) from patients hospitalized in UGHP were identified by the Vitek 2 Advanced Expert System (bioMérieux). Antibiotic susceptibility testing was performed by the agar disc diffusion method against amoxicillin/clavulanic acid (AMC), ceftriaxone (CRO), cefazidime (CAZ), cefotaxin (FOX), aztreonam (ATM), imipenem (IPM), meropenem (MER), amikacin (AN), netilmicin (NET), sulphamethoxazol/trimethoprin (SXT), ciprofloxacin (CIP) and gentamicin (GM), while MICs of IPM, MER, colistin (COL) and tigecycline (TIG) were determined by Etest (bioMérieux). Colistin and tigecycline were tested in all isolates in Greece, carbapenemase-producing and non-producing, only in MDR isolates. Results were interpreted according to the EUCAST criteria (EUCAST, 2015).

**Phenotypic testing for carbapenemase production.** All isolates displaying reduced susceptibility to carbapenems (MIC IPM or MER $\geq$ 2 mg l$^{-1}$) were tested by applying the Hodge test for verification of carbapenemase production (EUCAST, 2015). Phenotypic identification of carbapenemases was performed by the EDTA synergy test (MER/MER-EDTA) and the boronic acid synergy test (MER/MER-boronic acid), which distinguishes the production of MBL and serine carbapenemase, as previously described (Tsakris \textit{et al.}, 2010).

**Genotypes of isolates.** Detection of \textit{bla} genes, encoding important carbapenemase types for our area, was performed by PCR using specific primers for \textit{bla}$_{TEM}$, \textit{bla}$_{BDM}$, \textit{bla}$_{KPC}$, \textit{bla}$_{OXA-48}$ and \textit{bla}$_{NDM}$ according to published protocols (Nordmann \textit{et al.}, 2011; Queenan & Bush, 2007). PFGE of chromosomal \textit{XbaI} DNA digests was performed among 97 representative KPC-Kp isolates. One isolate was recovered in 2008, and 96 from 2009–2014 (including the first 16 isolates per year from different hospital departments). Moreover, 12 VIM-producing isolates from 2008, 11 KPC and VIM co-producing isolates from 2010–2014, as well as the four NDM-positives from 2013 and 2014, were analysed. Clones were interpreted according to established criteria and identified by capital letters (Tenover \textit{et al.}, 1995).

**Statistical analysis.** SPSS version 19.0 software was used for data analysis. Incidence of carbapenemase-producing and non-producing \textit{K. pneumoniae} bloodstream infection was calculated per 10 000 patient-days. Categorical variables were analysed by using either the Fisher exact test or the chi$^2$ test, as appropriate. Trends of carbapenemase-producing and non-producing rates over the 10 years were assessed using Spearman’s correlation analysis. A \textit{P} value of $<$0.05 was considered significant.

**RESULTS**

**Antibiotic susceptibility**

\textit{K. pneumoniae} isolates \textit{(n=3449)} were recovered from 1619 (47 \%) urine specimens, 631 (18 \%) blood cultures, 279 (8 \%) pulmonary specimens (sputum and bronchial secretions), 231 (7 \%) central venous catheter tips, and 689 (20 \%) from other specimens (pus, wounds, pleural, peritoneal fluids, etc.). A total of 1668 (48 \%) isolates produced one or more carbapenemase; 1333 (80 \%) were KPC-, 286 (17 \%) VIM-, 45 (3 \%) KPC- and VIM- and four (0.2 \%) NDM-producing, respectively. Resistance rates to antibiotics tested in relation to the presence of carbapenemase genes are presented in Fig. 1. High resistance rates ($>$85 \%) to all beta-lactams, AN, NET, CIP and SXT among CP-Kp isolates (KPC- or MBL-producing) were recognized. Resistance was 41 \% to gentamicin (27 \% in KPC-, 56 \% in MBL-producing), 23 \% to colistin (29 \% vs 16 \%) and 16 \% to tigecycline (23 \% vs 5 \%), respectively. \textit{K. pneumoniae} strains were more commonly isolated from internal medicine departments (1473; 43 \%), followed by surgical departments (696; 20 \%), intensive care units (ICU; 611; 18 \%), emergency department (405; 12 \%) and paediatric departments (264; 8 \%). Resistance rates to the antibiotics tested and presence of carbapenemase genes according to department of isolation are shown in Fig. 2. Resistance to most antibiotics and the presence of CP-Kp were higher in the ICUs, followed by internal medicine and surgical departments, while, less than 10 \% of isolates from emergency and paediatric departments were CP-Kp.
**Fig. 1.** Resistance rates of *K. pneumoniae* strains to antibiotics tested. Imipenem, meropenem, colistin and tigecycline resistance was evaluated with Etest. Colistin and tigecycline were tested in all carbapenemase-producing isolates and only in MDR-strains among the non-carbapenemase-producing isolates. \(^a\) includes KPC-positive or KPC- and VIM-positive isolates; \(^b\) includes VIM-positive or NDM-positive isolates.

**Fig. 2.** Resistance rates and presence of carbapenemase genes among *K. pneumoniae* strains according to department of isolation. Imipenem, meropenem, colistin and tigecycline resistance was evaluated with Etest. Colistin and tigecycline resistance rates are those in carbapenemase-producing isolates only.
Evolution of carbapenemase-producing isolates

CP-Kp isolates were introduced into our hospital in 2005, when 20 VIM-Kp strains were isolated (Fig. 3a). Such isolates disseminated, especially in the ICU, leading to an outbreak of bloodstream infections in 2007 with 45 VIM-Kp bacteraemias (Fig. 3b). The annual distribution of VIM-Kp isolates is depicted in Fig. 3. In September of 2008, the first KPC-Kp strain was isolated in our hospital (Fig. 3a). KPC-Kp disseminated rapidly during the following years and predominated from 2009 to 2014, leading to the disappearance of VIM-Kp. In 2010, three isolates carrying both \( \text{bla}_{KPC} \) and \( \text{bla}_{	ext{VIM}} \) were identified and until the end of the study period a total of 45 such isolates were identified. NDM-Kp was isolated for the first time in 2013, followed by three more isolations in 2014. (Spyropoulou et al., 2015)

Genotypes of isolates

PFGE of 97 KPC-Kp strains identified three types: most isolates (84; 87%) belonged to PFGE type A, 11 (11%) to type B and two (2%) to type E. Moreover, 11 isolates carrying both \( \text{bla}_{KPC} \) and \( \text{bla}_{	ext{VIM}} \) belonged to type B. VIM-Kp isolates were assigned to different PFGE types, whereas NDM strains belonged to a common PFGE type F.

Annual trends

An increasing isolation rate of \( K. \text{pneumoniae} \) from all specimens \((r=0.998, P<0.001)\) was observed during the study period (Fig. 3a). This is due to an increase of both CP-Kp \((r=0.998, P<0.001)\) and non-carbapenemase-producing isolates \((r=0.661, P=0.038)\). Colistin resistance rates increased from 0% in 2005 to 39% in 2014 \((r=0.881, P=0.001)\), tigecycline resistance rates from 0% to 33% \((r=0.917, P<0.001)\), while gentamicin resistance rates remained quite stable \((from 30\% to 36\%; r=−0.091, P=0.803)\). Since all isolates from different specimens were included, a proportion of these may not reflect true infection. For this reason, the annual distribution of \( K. \text{pneumoniae} \) bloodstream infections is shown in Fig. 3(b). The increasing trend of \( K. \text{pneumoniae} \) bacteraemia incidence \((r=0.915, P<0.001)\) was due to an increase of CP-Kp \((r=0.912, P<0.001)\), while the rate of non-CP-Kp \((r=0.237, P=0.510)\) stayed stable during the decade. During the ten-year period, overall incidence of CP-Kp bacteraemia was 2.4 per 10,000 patient-days, increasing from 0.5 in 2005 to 4.2 in 2014, while overall incidence of non-CP-Kp bacteraemia was 1.1 per 10,000 patient-days and remained stable during the study period.

DISCUSSION

The global dissemination of CP-Kp poses a major threat to health care systems worldwide (Tzouvelekis et al., 2012). The present report shows that during the last decade almost half (48%) of the \( K. \text{pneumoniae} \) isolates in our institution produced at least one carbapenemase. Indeed, the mosaic consists of four different types of CP-Kp (VIM-Kp, KPC-Kp, VIM- and KPC-Kp, and NDM-Kp).

VIM-Kp was isolated in 2005 leading to a wider dissemination, especially in the ICU during the following years, also reflecting the polyclonal outbreaks as described in hospitals throughout Greece during the same period (Hasan et al., 2014; Vatopoulos, 2008). KPC-Kp was first isolated in 2008 and outnumbered VIM-Kp the following year (66 KPC-Kp vs 41 VIM-Kp isolates in 2009). This is peculiar, since VIM-Kp isolates in 2009 as compared with KPC-Kp isolates from the same year were more resistant to meropenem (98% vs 83%; \( P=0.027 \)), gentamicin (37% vs 5%; \( P<0.001 \)) and colistin (49% vs 11%; \( P<0.001 \)), while no differences in resistance to the remaining antibiotics, including tigecycline, were observed. Even though KPC-Kp was susceptible to the antibiotics most commonly used (carbapenems, colistin, gentamicin and tigecycline) to treat CP-Kp infections, it successfully disseminated by supplanting VIM-Kp. This may be attributed to the wider dissemination of KPC-Kp in non-ICU wards as compared with VIM-Kp in 2009 (47 KPC-Kp, 71% vs 20 VIM-Kp,
49 %; $P=0.025$) (Papadimitriou-Olivgeris et al., 2015). The fact that VIM-Kp spread is mainly based on its establishment in ICUs is also strengthened by such an outbreak in an Italian hospital, involving three different wards, where the ICU offered the necessary epidemiological link for their dissemination in non-ICU wards (Cagnacci et al., 2008).

The expansion of KPC-Kp in our setting after 2009 may be due to the spread of the main PFGE type A (87 %) that is part of the hyperendemic clone ST258. (Giakoupi et al., 2009; Giani et al., 2013). Genetic homogeneity is probably consistent with the clonal spread of KPC-Kp isolates, explaining the fact that after 2009 such isolates accounted for more than 90 % of CP-Kp in different countries (Giakoupi et al., 2009; Giani et al., 2015; Pollett et al., 2014). On the contrary, VIM-Kp strains in our setting were characterized by multi-clonality, as previously reported (Hasan et al., 2014; Vatopoulos, 2008). NDM-Kp isolates in Greece, including those of the present study, are classified in the same PFGE type, although they originated from different parts of the country as sporadic cases. Some were imported from countries neighbouring northern Greece, while others lacking clear epidemiological links are reported as indigenous strains (Giakoupi et al., 2013; Papagiannitsis et al., 2012).

A similar evolution was reported from the Hippokration General Hospital of Thessaloniki, Greece, where KPC-Kp was first isolated in 2007 and outnumbered VIM-Kp in 2009, which had predominated since 2004 (Zagorianou et al., 2012). In Italy, where the first VIM-Kp outbreak took place in 2008, a multi-centre survey in 2011, including 25 Italian hospitals, showed a predominance of KPC-Kp over VIM-Kp (91 % vs 7 %), underlying its predominance, even though only 12 % among K. pneumoniae isolates were carbapenemase-producers (Giani et al., 2013). KPC-Kp is endemic in many countries worldwide (e.g. Greece, Italy, Poland, USA, Brazil, Colombia, Argentina, China) and has been isolated from other countries, reflecting its predominance over other carbapenemases (Munoz-Price et al., 2013).

During the study period, K. pneumoniae bloodstream infections increased from 17 episodes in 2005 to 96 in 2014. Despite the fact that bacteraemic episodes due to non-carbapenemase-producing isolates remained stable, a steady increase in the number due to CP-Kp was observed. During 2005–2008 these were due to VIM-Kp in the adult ICUs, while from 2009 onwards the increase was due to KPC-Kp, which became endemic in most departments. This rendered K. pneumoniae the most commonly isolated pathogen from bloodstream infections in our hospital. Even though a similar steep increase in the CP-Kp incidence was observed in a tertiary hospital in Genoa, Italy, during an eight-year period, incidence of CP-Kp bacteraemias per 10 000 patient-days remained significantly lower as compared with ours (0.9 vs 2.4 per 10 000 patient-days) (Alicino et al., 2015). In addition to the narrow armamentarium against them, surveillance of these pathogens is absolutely necessary (Papadimitriou-Olivgeris et al., 2014b; Tzouvelekis et al., 2012). Even though standard infection control measures, including hand hygiene, contact precautions, surveillance for colonization in high-risk patients, isolation and environment disinfection, were implemented, dissemination of CP-Kp was not contained (Papadimitriou-Olivgeris et al., 2015). There are several reasons for the failure of infection control practices: compliance with hand hygiene and contact precautions was low (Fafliora et al., 2014), and carbapenems that more often than other antibiotics have been implicated as risk factors for CP-Kp acquisition were among the most commonly used antibiotics (Papadimitriou-Olivgeris et al., 2015). However, the most important factor is considered to be the economic crisis that led to hospital budgetary allocation from infection control. Moreover, the low nurse-to-patient ratio in Greek hospitals is the determining element that influenced CP-Kp dissemination, as was proven in the ICU environment (Papadimitriou-Olivgeris et al., 2015).

K. pneumoniae is the second most common cause of urinary tract infection (Flores-Mireles et al., 2015). It is noteworthy that 9 % of K. pneumoniae samples isolated from urine specimens from patients arriving at the emergency department were CP-Kp. This percentage is substantially higher than that reported from an Italian study (2 %) (Giani et al., 2013). It is important that in countries with high rates of CP-Kp, clinicians should thoroughly ask patients at emergency departments about previous hospitalization, which may warrant treatment with more broad-spectrum antibiotics.

Colistin, tigecycline and gentamicin remain among the last therapeutic options against CP-Kp infections. Resistance rates of such isolates increased steadily during the study period, reaching 30 % in 2014. As was previously shown, the crucial factors for development of colonization by colistin- or tigecycline-resistant CP-Kp were the proximity to colonized patients and previous administration of these antibiotics (Giani et al., 2015; Papadimitriou-Olivgeris et al., 2014a). Similar increase in resistance to the aforementioned antibiotics among CP-Kp is reported worldwide, posing a serious threat since these remain the last treatment options in our armamentarium (Giani et al., 2015; Meletis et al., 2015; Munoz-Price et al., 2013; Tzouvelekis et al., 2012; Zagorianou et al., 2012). The increase of colistin-resistant CP-Kp was even higher in the report of Giani et al. (2015), reaching 57 % in 2013.

In conclusion, an increase in K. pneumoniae bloodstream infections was observed during the last decade, and is solely attributed to the increase in CP-Kp isolates. An initial outbreak (2005–2008) of VIM-Kp was replaced in 2009 and onwards by KPC-Kp, which became predominant. Resistance rates to colistin, tigecycline and gentamicin increased and reached 30 % in 2014, a fact that possibly contributes to the high morbidity and mortality rates associated with infections by such isolates. The main concern is whether history will repeat itself with
NDM-Kp, which was initially isolated in 2013 in our settings. Reinforcing infection control practices and, most importantly, a generous increase in the nosocomial budget are imperative in order to terminate the steep increase in CP-Kp bacteraemias and contain the epidemic.

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