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Développement des nouveaux outils de surveillance de l'émergence des bactéries à Gram négatif multirésistantes

Composition du Jury :

Pr BOUCHERIT. K	Université de Tlemcen, Algérie	Président du Jury
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« Une main habile sans tête qui la dirige est un instrument aveugle ».

Claude Bernard

SOMMAIRE

AVANT PROPOS

Le format de présentation de cette thèse correspond à une recommandation de la spécialité Maladies Infectieuses et Microbiologie, à l'intérieur du Master des Sciences de la Vie et de la Santé qui dépend de l'Ecole Doctorale des Sciences de la Vie de Marseille.

Le candidat est amené à respecter des règles qui lui sont imposées et qui comportent un format de thèse utilisé dans le Nord de l'Europe et qui permet un meilleur rangement que les thèses traditionnelles. Par ailleurs, la partie introduction et bibliographie est remplacée par une revue envoyée dans un journal afin de permettre une évaluation extérieure de la qualité de la revue et de permettre à l'étudiant de commencer le plus tôt possible une bibliographie exhaustive sur le domaine de cette thèse.

Par ailleurs, la thèse est présentée sur article publié, accepté, ou soumis associé d'un bref commentaire donnant le sens général du travail. Cette forme de présentation a paru plus en adéquation avec les exigences de la compétition internationale et permet de se concentrer sur des travaux qui bénéficieront d'une diffusion internationale.

Professeur Didier RAOULT

RESUME

L'augmentation et la dissémination de la résistance aux antibiotiques chez les bacilles à Gram-négatif, particulièrement les Entérobactéries, les bactéries du genre *Pseudomonas* et *Acinetobacter*, représentent un problème majeur de santé publique. Les infections nosocomiales causées par les bactéries multi-résistantes ont conduit non seulement à une augmentation de la mortalité, de la morbidité et du coût de traitement, mais aussi continuent à mettre en danger la vie des patients surtout immunodéprimés. L'utilisation abusive et non contrôlée des antibiotiques a grandement contribué à la large diffusion de la résistance aux antibiotiques. Cependant, des études récentes ont démontré que cette résistance pouvait émerger à partir de sources anciennes et/ou environnementales. Ainsi, face à cette préoccupation mondiale et suite à de nombreuses recommandations, plusieurs études épidémiologiques et moléculaires ont été rapportées afin de contrôler et surveiller la diffusion et la dissémination de la résistance aux antibiotiques. Il est cependant prioritaire de développer des nouveaux outils de surveillance de la résistance aux antibiotiques. C'est dans cette optique que ce projet de thèse s'articule avec comme objectifs :

- Le développement et la mise en place de nouveaux outils et logiciels de surveillance et de diagnostic des bactéries multi-résistantes,
- La réalisation des études d'épidémiologie moléculaire sur les isolats cliniques de bactéries multi-résistantes responsables d'épidémies.

Mots clés: bacilles Gram-négatif, isolats cliniques multi-résistants aux antibiotiques, outils de surveillance de la résistance aux antibiotiques, études moléculaires des mécanismes de résistance aux antibiotiques, études épidémiologiques.

SUMMARY

The increase and spread of multidrug-resistant (MDR) gram-negative bacteria especially Enterobacteriaceae, *Pseudomonas*, and *Acinetobacter* (E.P.A) species have become a major concern worldwide. The hospital-acquired infections caused by MDR bacteria have led not only to an increase in mortality, morbidity, and cost of treatment, but also continue to endanger the life of patients, especially those immunocompromised. Although, the frequent misuse of antibiotic drug has greatly contributed to worldwide dissemination of antibiotics resistance. Recent studies have shown that these resistance determinants could emerge from ancient or environmental sources. Front of this worldwide concern, and various recommendations, several epidemiological and molecular studies have been reported in order to control the spread and the dissemination of the antibiotic resistance. However, it is a priority to develop new tools for monitoring antibiotic resistance. Therefore, it is in this context that the project of this thesis was conducted with two essential objectives:

- The development and implementation of news tools and software for monitoring and diagnosis of potential MDR bacteria.
- The achievement of molecular epidemiology studies from clinical MDR bacteria responsible of outbreak.

Keywords: Gram-negative bacilli, multidrug-resistant clinical isolates, tools for monitoring antibiotic resistance, molecular studies of the mechanisms of antibiotic resistance, epidemiological studies.

INTRODUCTION

La découverte des antibiotiques, notamment la pénicilline en 1928 a sans doute été l'une des avancées thérapeutiques les plus importantes du vingtième siècle. L'utilisation de ces derniers depuis les années 1940 a considérablement réduit le taux de morbidité et de mortalité lié aux maladies infectieuses [1].

Cependant, leur utilisation à grande échelle a également conduit à l'émergence de la résistance aux antibiotiques. Les premières bactéries résistantes ont été identifiées dès les années 1940, avec notamment l'émergence de *Staphylococcus aureus* résistants à la pénicilline dès 1947, soit seulement quatre ans après l'utilisation à grande échelle de cet antibiotique [2]. A partir des années 1950, de nombreux antibiotiques ont été découverts ou synthétisés et pour chaque nouvelle classe développée, nous avons assisté par la suite à l'émergence de nouveaux mécanismes de résistance, entraînant la diffusion de bactéries pathogènes de plus en plus difficiles à traiter, comme ce fut le cas de la méticilline G, mise sur le marché en 1961 suivi de la découverte de *S. aureus* résistants en 1962 ; de l'ampicilline G en 1962 suivi de l'émergence d'Entérobactéries résistantes en 1964 ; puis des céphalosporines mise sur le marché en 1980 suivi de l'émergence d'entérobactéries résistantes en 1981 [1]. L'âge d'or de la recherche pharmaceutique sur les antibiotiques a duré jusqu'aux années 1980, expliquant le fait qu'à cette époque, la résistance aux antibiotiques, bien que connue et largement répandue, ne représentait pas encore une menace.

Dans les années 2000, selon les données de l'Organisation mondiale de la santé (OMS), les maladies infectieuses causées par les bactéries multi-résistantes (BMR) ont été à l'origine de 25% des décès dans le monde entier, dont 50% provenaient des pays en voie de développement. En Europe en 2007, 400000 infections ont été causées par les BMR dont 25000 décès ont été liés à ces bactéries qui n'ont pu être traitées faute d'antibiotiques efficaces. Le coût annuel de traitement de ces infections étant estimé à 1.5 milliard d'euros [3].

L'augmentation et la dissémination de la résistance aux antibiotiques chez les bacilles à Gram négatif, particulièrement les entérobactéries, les bactéries du genre *Pseudomonas* et *Acinetobacter* représentent un problème majeur de santé publique au niveau mondial. Les infections nosocomiales causées par ces BMR ont non seulement conduit à une augmentation de la mortalité, de la morbidité, et du coût de traitement, mais aussi continuent de mettre en danger la vie des patients surtout immunodéprimés en milieu hospitalier. Il est évident de noter que l'utilisation abusive et non contrôlée des antibiotiques a longtemps contribué à l'émergence et à la large diffusion des déterminants de la résistance « résistome », défini comme étant tous les gènes impliqués directement ou indirectement dans la résistance aux agents antimicrobiens [4]. Cependant, des études récentes ont pu démontrer que la résistance aux antibiotiques pouvait émerger à partir de sources anciennes , bien avant la découverte des antibiotiques, mais aussi de sources environnementales [5], telles les découvertes de l'équipe de recherche de l'Université de McMaster des gènes de résistance à différents antibiotiques dans les sédiments datant de plus

30000 ans du pergélisol dans les territoires du Yukon [6] et dans des échantillons provenant d'une grotte au Nouveau-Mexique, prélevés dans des zones isolées depuis plus de 4 millions d'années [7], ou la découverte d'une quantité importante de gènes de résistance aux antibiotiques dans les études métagénomiques à partir du sol et de l'eau [8, 9].

Face à cette préoccupation mondiale qui est l'émergence des BMR pathogènes résultant d'une part de l'utilisation abusive des antibiotiques et de la mobilisation des gènes de résistance à partir de réservoirs préexistants, et d'autre part de la capacité des bactéries à échanger du matériel génétique dans des conditions de pressions antibiotiques, il est primordial de conduire des études d'épidémiologie moléculaire afin de comprendre et de contrôler la diffusion et l'augmentation de la résistance aux antibiotiques.

Par ailleurs, parmi les stratégies de surveillance, particulièrement dans le domaine de la microbiologie, nous assistons ces dernières années au développement de nouvelles techniques de surveillance de la résistance aux antibiotiques impliquant d'importantes ressources financières et intellectuelles à travers le monde. Cette surveillance demeure actuellement prioritaire pour les sociétés scientifiques et celles de santé publique, afin de détecter les épidémies dans leur stade précoce. Le développement de ces nouveaux outils de surveillance en temps réel, combiné au développement de nouveaux outils et logiciel bioinformatiques a révolutionné le monde de la recherche microbiologique et représente aujourd'hui le meilleur moyen de

prendre en charge le problème de la résistance aux antibiotiques particulièrement chez les bacilles à Gram négatif.

C'est dans cette optique que ce projet de thèse s'inscrit avec comme objectif principal le développement des nouveaux outils de surveillance de la résistance aux antibiotiques d'isolats cliniques multi-résistants aux antibiotiques, mais aussi l'étude d'épidémiologie moléculaire d'isolats cliniques multirésistants aux antibiotiques.

Ainsi ce manuscrit s'articule autour de trois chapitres présentés comme suit :

Chapitre I : Cette partie a été consacrée à une revue de littérature reprenant toutes les publications qui ont été rapportées à travers le monde décrivant les NDM-1 (Metallo-B-lactamase), qu'il s'agisse de cas autochtones, importés, ou décrits à partir de l'environnement. Pour rendre facile et rapide l'accès à toutes les publications, nous avons développé et mis en place une application interactive en ligne sur internet, tout à fait originale, permettant de surveiller en temps réel la diffusion de ce gène dans le monde en utilisant le logiciel Google Maps. Ce travail a été présenté sous forme d'une « e-Revue » **(Article 1)**.

Chapitre II : Dans cette partie, sont présentés les outils de surveillance de la résistance aux antibiotiques que nous avons développés tout au long de cette thèse. Nous avons mis au point une technique rapide utilisée en routine pour la détection phénotypique des souches bactériennes porteuses de carbapénèmases chez les bactéries à

Gram négatif par spectrométrie de masse (Maldi-Tof Ms). Le but étant d'identifier et de prévenir les épidémies et la propagation des gènes de résistance **(Article 2)**. En parallèle nous avons utilisé le MALDI-TOF Ms comme outil rapide et puissant pour déterminer la distribution épidémiologique d'une large série d'isolats cliniques de *K. pneumoniae* de différentes origines, à partir de patients atteints de divers syndromes infectieux. Au cours de ce travail nous avons corrélé entre les pathotypes, la distribution géographique et la clonalité des souches à l'aide des approches du MALDI-TOF MS et du data-mining **(Article 3)**. Nous avons également développé un outil bioinformatique « Clustering Hiérarchique » appliqué aux résultats des tests de sensibilité aux antibiotiques réalisés en routine dans les laboratoires de microbiologie clinique. Ce logiciel (MultiExperiment Viewer) peut surveiller en temps réel, qualitativement et quantitativement, la prévalence des phénotypes de résistance connus et inconnus **(Article 4)**.

Chapitre III : Dans ce chapitre, nous avons présenté l'ensemble des travaux réalisés sur l'épidémiologie moléculaire de la résistance aux carbapénèmes chez des souches cliniques appartenant au genre *Acinetobacter* (**Article 5 et 6**) et à l'espèce *Pseudomonas aeruginosa* **(Article 7)**.

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CHAPITRE I:

New Delhi métallo-β-lactamase dans le monde:
Une e-revue en utilisant Google Maps

AVANT PROPOS

La résistance aux antibiotiques est devenue une préoccupation mondiale [1,2]. Cette résistance est en grande partie liée à une diffusion et une propagation mondiale des gènes de résistance, particulièrement ceux codant pour la résistance aux carbapénèmes chez les bactéries Gram négatif [3,4]. Le résultat de cette diffusion a été l'émergence et la dissémination dans la majorité des pays du monde, de carbapénèmases de type VIM, IPM, KPC, OXA, et NDM-1 [5,6]. Cependant, une attention particulière est portée sur la nouvelle carbapénèmase, la New Delhi metallo-bêta-lactamase-1 (NDM-1), découverte en Décembre 2009 en Suède, à partir d'une souche clinique de *Klebsiella pneumoniae* responsable d'une infection urinaire chez un patient auparavant hospitalisé à New Delhi, Inde [7]. Cette souche porteuse de ce gène NDM-1 était résistante à tous les bêta-lactamines y compris les carbapénèmes.

Depuis cette découverte en Décembre 2009, plus de 300 publications ont été rapportées signalant la diffusion spectaculaire et inquiétante de ce gène dans plusieurs pays à travers le monde. Dans la plupart des cas, les patients avaient des liens avec la région indienne ou les pays des Balkans. Les cas des personnes infectés, originaires de ces régions, ayant séjourné dans ces régions et y avaient été hospitalisés, ou ont été potentiellement liés à d'autres patients hospitalisés dans ces régions.

Afin d'informer la communauté scientifique et médicale à travers le monde à propos de la dissémination des NDM-1, nous avons développé un outil interactif innovant de surveillance en temps réel de la diffusion de ce gène dans le monde en utilisant le logiciel Google Maps **(Article 1)**. A cet effet, nous avons répertorié tous les cas décrits de ce gène dans le monde à partir de la base de données PubMed, qu'il s'agisse de cas autochtones, importés, ou décrits à partir de l'environnement. Ce travail présenté sous forme d'une « e-Revue », nous a permis de suivre en temps réel l'évolution et la dissémination dans le monde de ce gène, mais aussi de découvrir d'autres foyers d'émergence de ce gène.

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Article 1:

New Delhi Metallo-β-lactamase around the world: An eReview using Google Maps

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New Delhi Metallo-β-lactamase around the world: An eReview using Google Maps

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Abstract

Gram-negative bacteria with carbapenem resistance conferred by New Delhi Metallo-β-lactamase-1 (NDM-1) are a major global health problem. To inform the scientific and medical community about worldwide NDM-1 isolates, we used the PubMed database to review publications from the first description of NDM-1 in 2009, and created a worldwide dissemination map using a web-based mapping application.

In the PubMed database, there were 33 reviews, and 136 case reports describing 950 NDM-1-producing bacteria from around the world. *Klebsiella pneumoniae* (n= 359) and *Escherichia coli* (n=268) were most commonly reported bacteria to carry the $bla_{\text{NDM-1}}$ gene. It has also been found in *Acinetobacter baumannii* (n=36), *Pseudomonas aeruginosa* (n=9) and in a wide variety of Gram-negative species. The first bacteria containing the NDM-1 enzyme were reported in India in 2009. Several infections due to imported isolates producing NDM-1 enzyme have also been reported in a number of countries, including the United Kingdom, Italy, and Oman. In most of the cases, patients had connections with the Indian Pacific region or the Balkan countries. Those infected were originally from those areas, had spent time there, had been hospitalized there, or they were potentially linked to other patients who had been hospitalized in those regions.

To prevent outbreaks of NDM-1 producing bacteria and to optimize antibiotic therapy, we strongly encourage epidemiologists to utilize these types of interactive tools for surveillance purposes and, more importantly, to communicate these data to other members of the research community and the general public in real time.

Introduction

New Delhi Metallo-β-lactamase-1 (NDM-1) is the most recently discovered transferable molecular class B β-lactamase. Unlike class A, C and D β-lactamases, NDM-1 has zinc ions at its active site, and it can hydrolyze all β-lactams except monobactam [1-3]. Most NDM-1-positive bacteria are resistant to a wide variety of drug classes and carry many other resistance mechanisms (to aminoglycosides, fluoroquinolones, macrolides and sulfonamides), leaving few or no therapeutic options [4-8]. The putative original source of the $bla_{\text{NDM-1}}$ gene could be from a chromosome of plant pathogens, such as *Pseudoxanthomonas* and related bacteria, which is widespread in the environment [9].

The first published reports of infections involved individuals who had received medical care in India, hence the name New Delhi Metallo-β-lactamase. The first NDM-1-positive bacteria came from a Swedish resident of Indian origin who contracted a urinary tract infection caused by carbapenem-resistant *Klebsiella pneumoniae* while he was in New Delhi in late 2007 [10]. The precise geographic origin and the time of the first appearance of this gene are unknown. At present, the isolates appearing worldwide have originated from infected people (with or without showing symptoms) on the Indian subcontinent who have then traveled elsewhere. However, it is presumed that there are other reservoirs of infected patients in the Balkan states. There is also an unknown burden in the Middle East

and Israel, where people often travel to and from the Indian subcontinent [11, 12].

Bacteria with NDM-1 have been recovered from many types of infections; it has been found in patients with urinary tract infections, pneumonia, septicemia, wound infections and device-associated infections [7, 13, 14]. Both hospital- and community-acquired infections have been reported [7, 13, 15]. The following principal factors have influenced the emergence of these infections: the increase in long-distance travel [16], the increase in international travel to access medical care [17] and widespread access to broad spectrum antibiotics. Antibiotics are often obtained without a prescription because of the strong economic incentives to sell and use them [18].

Given the volume of international travel, the quality of care in developing countries, and the number of humans carrying NDM-1-positive bacteria, it is likely that these bacteria will continue to spread worldwide [19]. There has been an increase in the number of articles referencing New Delhi Metallo-β-lactamase added to the PubMed database (Figure 1), but the current spread of NDM-1-positive bacteria is likely broader than the published reports suggest. We used in this article Google Maps to conduct an eReview of all published isolates worldwide in order to simplify and accelerate access to documentation, organize information about NDM-1-producing bacteria and provide real-time information to the scientific and medical community about NDM-1 isolates around the world. Few studies have used this type of automated system to investigate, in real

time, web-based electronic reports for the purpose of monitoring the spread and the expansion of infectious diseases, such as H1N1 Influenza and Dengue virus [20, 21]. Google Maps is a widely available, free, and extremely powerful tool for visualization with a simple, intuitive interface that requires little training or experience to operate. It can be run on any common desktop computer or laptop, and there is also a Google Maps application available for mobile phones [22].

Because a visual representation of scientific data is more informative than a written description, this article describes the development of an internet-based mapping and geo-referencing application for tracking the worldwide dissemination of NDM-1 producing bacteria. We analyzed in this article the medical literature from the first case report in 2009 until December 31, 2012.

Methods

Literature search in the PubMed database

We started by retrieving all of the published articles from the PubMed database that were related to "NDM-1" or "New Delhi Metallo-β-lactamase-1" producing bacteria, from the first description in 2009. In an Excel file, we summarized these articles in a table containing all useful information about the isolates of NDM-1-producing bacteria. Specifically, we examined the year of detection, the city and country, the bacterial species that produced the NDM-1 enzyme, the number of isolates, the type of isolates, the link to the isolates description in PubMed database, the title and the full

reference for the published article. An isolate of NDM-1-producing bacteria was defined as a patient from whom one or more Gram-negative bacteria had been isolated that expressed the NDM-1 enzyme, with the test result confirmed by an expert laboratory. We categorized isolates into five different types: imported human infection isolates (isolates involving patients with a history of recent travel or contact with healthcare facilities abroad before the detection of $bla_{\text{NDM-1}}$ gene), autochthonous human infection isolates (local reports of patients with an infection caused by NDM-1-positive bacteria who did not have contact with a travel-associated isolates), autochthonous human carriage isolates (local carriage of NDM-1-producing bacteria in patients who did not have contact with a travel-associated isolates), autochthonous environmental isolates (a description of NDM-1-producing bacteria in the hospital or the external environment) and autochthonous human carriage and environmental isolates (a description of the two types of isolates in the same article).

Construction of Google Maps

We used Google Maps to create an electronic map depicting the geographic locations of the NDM-1-producing bacteria listed in our database. Google provides full documentation for Google Maps, tutorials and other materials to help users take full advantage of the application (https://maps.google.com). The locations on the map were tagged using different symbols for each type of isolates, as described above (Figure 2). Clicking the tags will provide a display of the

important information about the selected article (the same information stored in the columns in the database). If there are several tags within close proximity to one another, the tags expand outward to facilitate selection of a single tag. Google Maps navigation controls in the upper left portion of the screen can be used to zoom in on an area of interest. Alternatively, one can double-click on one of the interesting locations in the table of contents on the left-hand side of the screen to access information about the selected article.

Data storage and analysis

The data from all articles were recorded in an Excel file (Microsoft, Redmond, WA, USA) and analyzed using the same software. We discussed in this paper about the medical literature from the first case report in 2009 until December 31, 2012.

Results

Google Maps eReview

To visualize the isolates of NDM-1-producing bacteria that have appeared around the world since the first description, we have developed a Google Maps application as described in the methods section that is regularly updated and freely available at the following website: http://www.ifr48.com/spip.php?article21. As soon as an article with the keyword "NDM-1" or "New Delhi Metallo-β-lactamase-1" is added to the PubMed database, we automatically receive an alert by email. In less than 10 minutes, we are able to analyze the article, extract the relevant information about the isolates,

add it to our own database and update the map so that the information is freely accessible. Other NDM enzymes are not included in the manuscript but have been added in the Google map website.

Distribution of bacteria carrying NDM-1

From its first description in 2009 through December 31, 2012, there have been 33 reviews describing the *bla*$_{\text{NDM-1}}$ gene [3, 13, 18, 23-52], and 136 case reports in the PubMed database, reporting 950 NDM-1- producing bacteria from around the world. There have been 13 articles describing autochthonous environmental and human carriage isolates, reporting 54 (5,68%) and 172 (18,10%) NDM-1-producing bacteria, respectively; 57 articles describing autochthonous human infection isolates with 571 (60,11%) NDM-1-producing bacteria and 66 articles describing imported human infection isolates with 153 (16,11%) NDM-1-producing bacteria.

Klebsiella pneumoniae (n= 359) and *Escherichia coli* (n=268) were the most commonly described bacteria carrying the *bla*$_{\text{NDM-1}}$ gene (Figure 3). This gene has also been recorded in clinical Enterobacteriaceae other than *K. pneumoniae* and *E. coli* (Table 1). NDM-1 has been found in clinical *Acinetobacter baumannii* (n=36), *Pseudomonas aeruginosa* (n=9) and in a wide variety of non-fermenting Gram-negative species (Table 1).

The distribution of autochthonous isolates of NDM-1-producing bacteria by country

In India, bacteria containing *bla*$_{\text{NDM-1}}$ gene have been identified in many different cities, including Chennai, Guwahati, Varanasi, Mumbai, Haryana, Kolkata, New Delhi, Pune, Bangalore, and Assam. There have been 374 bacteria responsible for autochthonous human infection isolates; 21 bacteria were responsible for autochthonous human carriage isolates, and 22 isolates were identified in the environment. In Pakistan, 101 bacteria were responsible for autochthonous human carriage isolates, and 32 bacteria were responsible for autochthonous human infection isolates described in nine cities. In China, 16 bacteria were responsible for autochthonous human infection isolates described in eight cities, 49 bacteria were responsible for autochthonous human carriage isolates, and 30 isolates were identified in the environment. Overall, 149 bacteria responsible for autochthonous human infection isolates have been identified in the United Kingdom (n=23), Canada (n=18), Bangladesh (n=17), Singapore (n=15), Israel (n=10), Serbia (n=8), Kenya (n=7), Kosovo (n=7), Thailand (n=6), France (n=4), Japan (n=4), Morocco (n=4), South Korea (n=4), Sweden (n=4), Switzerland (n=3), Afghanistan (n=2), Guatemala (n=2), South Africa (n=2), Vietnam (n=2), United Arab Emirates (n=2), Iran (n=1), Mauritius (n=1), Netherlands (n=1), Spain (n=1), and Taiwan (n=1). Details are included in Figure 4A. Table 2 summarizes the distribution of bacteria carrying the *bla*$_{\text{NDM-1}}$ gene, grouped according to the type of autochthonous isolates reported

in all the 29 countries. The year of the first description is indicated for each country. The first bacteria producing the NDM-1 enzyme responsible for autochthonous human infection isolates were isolated from India in 2006 [6], followed by Kenya in 2007 [53] and the Netherlands in 2008 (a putative secondary transmission) [54].

The distribution of imported isolates of NDM-1-producing bacteria by country

Several imported isolates of NDM-1-producing bacteria have been reported in a number of countries in different geographical locations, but most of them have been reported in the United Kingdom (n=44) (Table 3). The first imported isolates of NDM-1-producing bacteria was reported in 2007 in Germany [55], followed by two isolates in 2008 in the United Kingdom [7] and the Netherlands [54]. In most of the cases, patients had connections to other countries or regions such as the Indian subcontinent (n=121), the Balkan countries (n=11), Africa (n=9), the Middle East (n=7), and East Asia (n=5). The patients were originally from these areas, they had spent time or been hospitalized there, or they may have been secondary isolates linked to other hospitalized patients who had recently returned from these areas. Figure 4B shows the putative countries of origin for the imported isolates of NDM-1-producing bacteria. The majority of these patients were admitted to foreign hospitals because of an accident or an illness that occurred during their travel, although a minority of patients were traveling for medical reasons.

Discussion

The current data indicate an increase in the spread of NDM-1 and other carbapenemases producing bacteria all over the world [2, 7, 56]. In this study, we described 950 NDM-1-producing bacteria from different types of isolates reported in 55 countries between 2006 and December 31, 2012, with the majority of isolates occurring in India, Pakistan and China. It is probable that the number of isolates reported underestimates the true number of isolates because most countries do not report infections with highly resistant bacteria and many isolates are not confirmed. In some cases, the patient is asymptomatic, so the infection is not detected. In addition, microbiological guidance on the detection and the identification of carbapenemase producing bacteria is only available in a minority of countries [43, 57]. The highest concentration of NDM-1-producing bacteria per million square kilometers of land was found between 30 and 60 degrees north latitude, with the main hotspots on the Indian subcontinent and in the Balkan states. Moreover, the majority of the imported isolates described in our survey involved patients with a history of recent travel or hospital admission on the Indian subcontinent or in Balkan countries [4, 7, 58]. In 2008, India and Pakistan received an estimated 5 million visitors, and an estimated 10 million residents left the country, which amounts to a dispersion of 20 million people [59]. In some cases, it should be noted that travel alone was sufficient to acquire antibiotic-resistant bacteria carrying the NDM-1encoded gene [60].

In view of this situation, we believe that an immediate response to the emergence of NDM-1-producing bacteria and other carbapenemases should be an urgent priority worldwide. At a local level, patients with a history of travel to or native from high-risk areas should be screened for NDM-1-producing bacteria [61-64]. This screening should prevent the development of outbreaks and help to optimize antibiotic therapy in the carriers who subsequently develop infections. At the international level, the response to growing multi-drug resistance of Gram-negative bacteria should be the implementation of a worldwide surveillance network to discover and report emerging resistance traits [31]. To the best of our knowledge, this study is the first time that Google Maps has been used as an interactive and free tool to document all reported isolates of NDM-1 worldwide. It offers a new way to monitor genes responsible for antibiotic resistance, unlike other works that report on the bacteria responsible for infection disease. Such a development is important because we are now witnessing outbreaks of resistance genes, not bacteria.

Google Maps can be advantageous to the scientific and medical community for a number of reasons. It facilitates the following tasks: counting the isolates producing antibiotic resistance genes, estimating the prevalence of each species, differentiating between the different types of reported isolates, visualizing the relationship between the circulation of antibiotic resistance genes and the worldwide human traffic patterns, identifying the origin and the reservoir of the

antibiotic resistance gene, and communicating information about the local and worldwide dissemination of antibiotic resistance genes in real time. The advantages of Google Maps also include the immediate access to the PubMed publications from the link in the isolates description and the real-time update of the map as soon as an article is added on PubMed database. Google Maps represents a new generation of interactive review capability; it is easy to use, it does not require PDF, and it is accessible everywhere by everyone, facilitating the diffusion and the circulation of knowledge.

Simple mapping in public health is not new. The cholera map by John Snow marked a critical turn in the use of maps to understand geographic patterns of disease [65]. Moreover, the geographic distribution of scientific data is a growing area of interest in many fields, including infectious diseases [66, 67], paleontology [68], natural products research [22], microbial marine biology [69], ecology [70], and archaeology [71]. It allows the presentation of data (even old data) in new ways. For example, a paper examined the geographic origins of emerging infectious diseases from 1940 to 2004, showing non-random global patterns [72]. Another online, real-time disease outbreak monitoring system, "HealthMap", developed by the Brownstein team in 2008, has demonstrated the effectiveness of collecting new media sources for improved situational awareness of infectious disease worldwide [73].

Given the popularity of Google Maps, it is almost certain that Google will continue to add new features, such as higher resolution,

more options for the maps, three-dimensional views, and a Smartphone application. Smartphone applications are a growing field that offers novel approaches, with software that allows data entry and retrieval of data from the maps using a mobile phone [74, 75]. The possibilities are vast, and to better convey information, keeping an open mind and testing many different visual representations are most likely the best pieces of advice we can propose. We strongly encourage epidemiologists to embrace this type of data collection by using these types of interactive tools for surveillance purposes and perhaps more importantly to communicate these data to other members of the research community and the general public in real time. Using detailed maps to convey such data visually helps to break down communication barriers, bringing diverse research ideas together [22].

Figure legends

Figure 1. The number of articles with keyword "NDM-1" or “New Delhi Metallo-β-lactamase-1” added to the PubMed database (per year).

Figure 2. A screenshot of reported isolates carrying the *bla*$_{\text{NDM-1}}$ gene shown in Google Maps (http://www.ifr48.com/spip.php?article21)

Figure 3. The distribution of isolates carrying the *bla*$_{\text{NDM-1}}$ gene
1: Acinetobacter pittii (n=27), *Acinetobacter lwoffii* (n=20), *Acinetobacter* sp. (n=13), *Pseudomonas aeruginosa* (n=9), *Moraxella* ssp.(n=8), *Acinetobacter* spp. (n=7), *Comamonas testosteroni* (n=7), *Pseudomonas* sp. (n=7), *Stenotrophomonas maltophilia* (n=5), *Vibrio cholerae* (n=3), *Achromobacter* spp. (n=2), *Acinetobacter johnsonii* (n=2), *Alcaligenes faecalis* (n=2), *Pseudomonas pseudoalcaligenes (*n=2), *Pseudomonas putida* (n=2), *Acinetobacter junii* (n=1), *Acinetobacter ursingii* (n=1), *Aeromonas caviae* (n=1), *Kingella denitrificans* (n=1), *Methylobacterium* spp. (n=1), *Pseudomonas oryzihabitans* (n=1), *Suttonella indologenes* (n=1)
2: Citrobacter freundii (n=16), *Citrobacter* sp. (n=15), not determined enterobacteriaceae (n=15), *Citrobacter* spp. (n=13), *Klebsiella* spp. (n=10), *Morganella morganii* (n=8), *Enterobacter* spp. (n=7), *Providencia rettgeri* (n=6), *Klebsiella oxytoca* (n=5), *Proteus mirabilis* (n=4), *Providencia stuartii* (n=3), *Enterobacter aerogenes* (n=2), *Proteus* spp. (n=2), *Citrobacter braakii* (n=1), *Proteus vulgaris* (n=1), *Providencia* spp. (n=1), *Salmonella enterica* (n=1), *Salmonella* spp. (n=1), *Shigella boydii* (n=1)

Figure 4. The worldwide distribution of published isolates producing NDM-1 enzyme

Tables

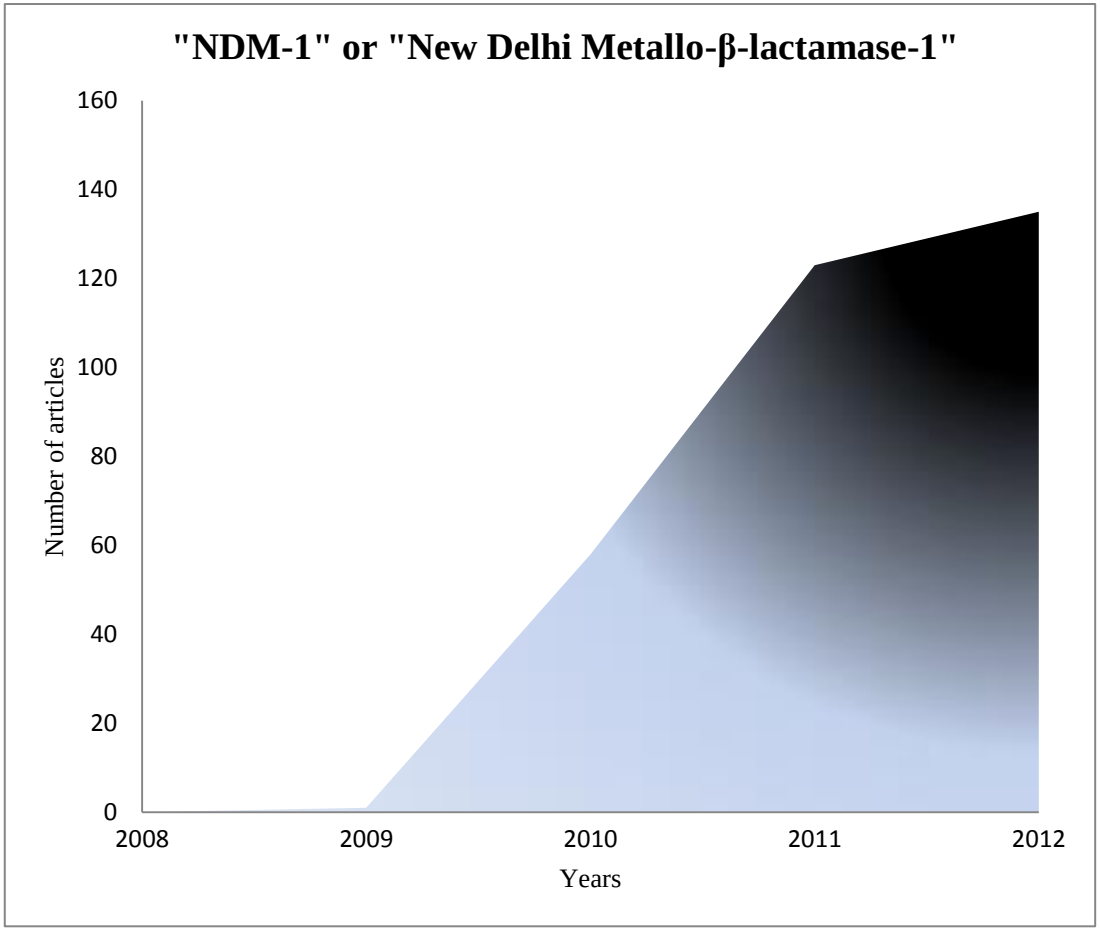


Figure 1. The number of articles with keyword "NDM-1" or "New Delhi Metallo-β-lactamase-1" added to the PubMed database (per year)

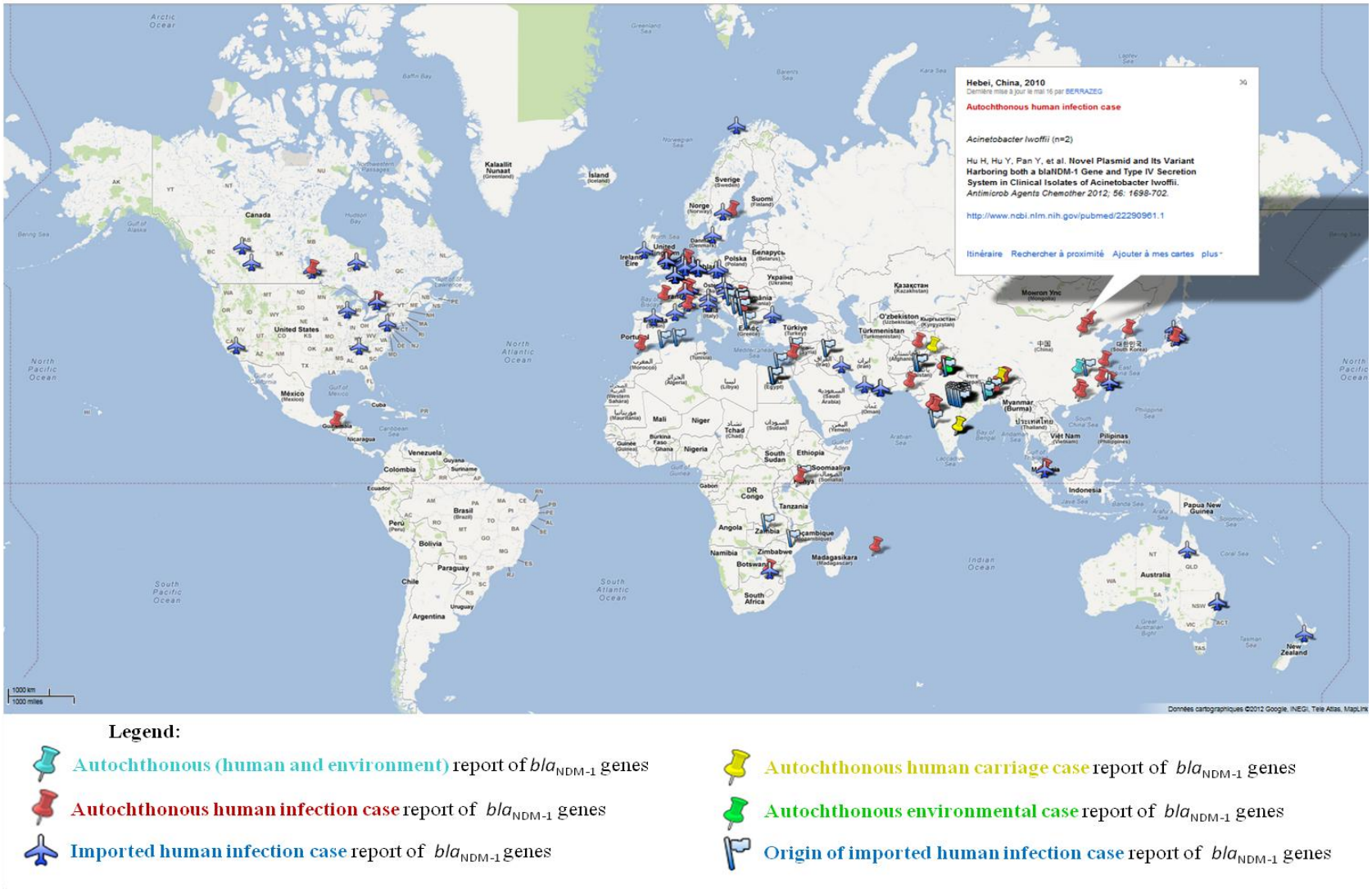
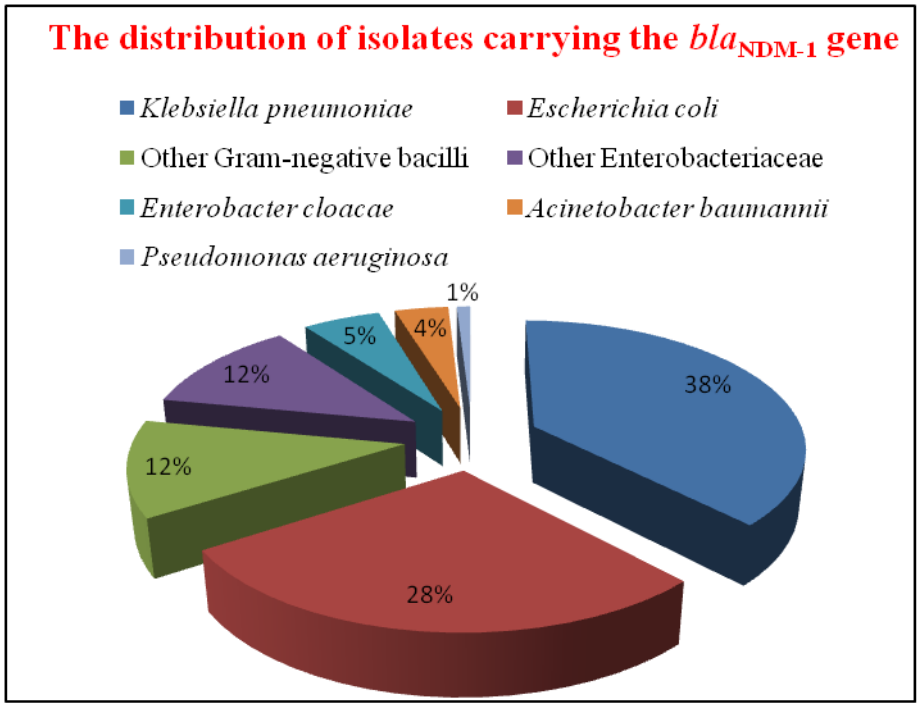


Figure 2. A screenshot of reported isolates carrying the *bla*$_{\text{NDM-1}}$ gene shown in Google Maps (http://www.ifr48.com/spip.php?article21)

(A)



(B)

Species	Number of isolates	%
Klebsiella pneumoniae	359	37.79
Escherichia coli	268	28.21
Other Gram-negative bacilli[1]	114	12.00
Other Enterobacteriaceae [2]	112	11.79
Enterobacter cloacae	52	5.47
Acinetobacter baumannii	36	3.79
Pseudomonas aeruginosa	9	0.95
Total	950	100.00

Figure 3. Distribution of isolates carrying the bla_{NDM-1} gene

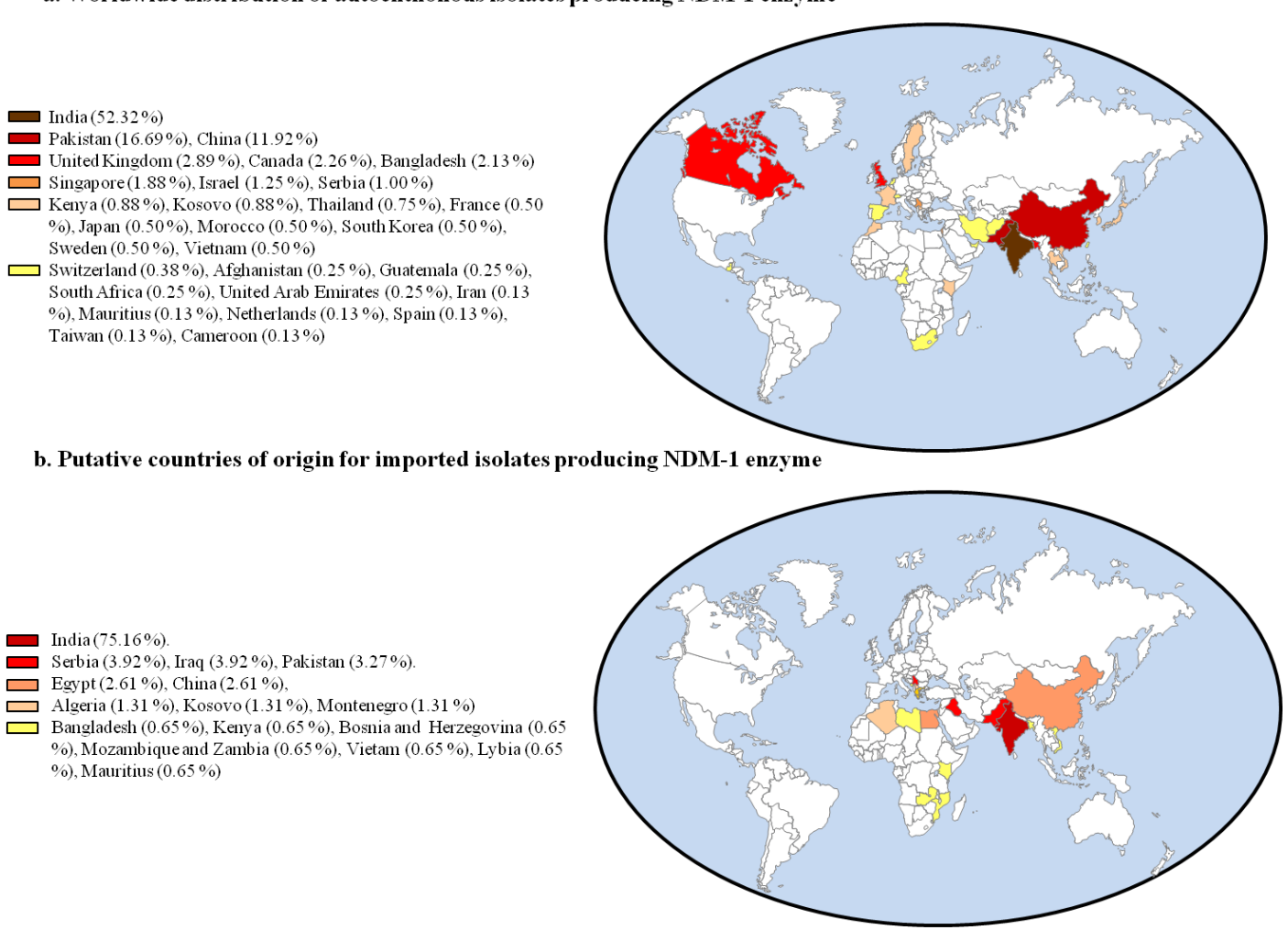


Figure 4. Worldwide distribution of published isolates producing NDM-1 enzyme

Table 1. Distribution of reported NDM-1-producing isolates among bacterial species.

Species	Number of isolates	Percentage of total
Klebsiella pneumoniae	359	37,79
Escherichia coli	268	28,21
Enterobacter cloacae	52	5,47
Acinetobacter baumannii	36	3,79
Acinetobacter pittii	27	2,84
Acinetobacter lwoffii	20	2,11
Citrobacter freundii	16	1,68
***Citrobacter* sp.**	15	1,58
Enterobacteriaceae	15	1,58
***Acinetobacter* sp.**	13	1,37
***Citrobacter* spp.**	13	1,37
***Klebsiella* spp.**	10	1,05
Pseudomonas aeruginosa	9	0,95
***Moraxella* ssp.**	8	0,84
Morganella morganii	8	0,84
***Acinetobacter* spp.**	7	0,74
Comamonas testosteroni	7	0,74
***Enterobacter* spp.**	7	0,74
***Pseudomonas* sp.**	7	0,74
Providencia rettgeri	6	0,63
Klebsiella oxytoca	5	0,53
Stenotrophomonas maltophilia	5	0,53
Proteus mirabilis	4	0,42
Providencia stuartii	3	0,32
Vibrio cholerae	3	0,32
***Achromobacter* spp.**	2	0,21
Acinetobacter johnsonii	2	0,21
Alcaligenes faecalis	2	0,21
Enterobacter aerogenes	2	0,21
***Proteus* spp.**	2	0,21
Pseudomonas pseudoalcaligenes	2	0,21

Species	Number of isolates	Percentage of total
Pseudomonas putida	2	0,21
Acinetobacter junii	1	0,11
Acinetobacter ursingii	1	0,11
Aeromonas caviae	1	0,11
Citrobacter braakii	1	0,11
Kingella denitrificans	1	0,11
***Methylobacterium* spp.**	1	0,11
Proteus vulgaris	1	0,11
***Providencia* spp.**	1	0,11
Pseudomonas oryzihabitans	1	0,11
Salmonella enterica	1	0,11
***Salmonella* spp.**	1	0,11
Shigella boydii	1	0,11
Suttonella indologenes	1	0,11
Total	950	100,00

Table 2. Countries reporting autochthonous isolates of NDM-1-producing bacteria

Type of isolates	**Country**	**Cities**	**Number of isolates**	**First description**
Human infection	Afghanistan	Kaboul	2	2011
	Bangladesh	Dhaka	17	2008
	Canada	Toronto, Winnipeg, Brampton	18	2009-2010
	China	Hunan, Fujian, Hangzhou, Hebei, Hong Kong, Guangzhou, Beijing, Chongqing	16	2009-2012
	France	Bordeaux, Lyon, Toulon	4	2011
	Guatemala	Not available	2	2011
	India	Chennai, Guwahati, Varanasi, Mumbai, Haryana, Kolkata, New Delhi, Pune, Assam, Bangalore	374	2006-2007
	Iran	Tehran	1	2011
	Israel	Tel Aviv, Jerusalem	10	2010
	Japan	Saitama, Tokyo	4	2010
	Kenya	Nairobi	7	2007-2009
	Kosovo	Pristina	7	2010
	Mauritius	Quatre Bornes	1	2009
	Morocco	Rabat, Taza	4	2011
	Netherlands	Enschede	1	2008
	Pakistan	Charsadda, Faisalabad, Gujrat, Hafizabad, Karachi, Lahore, Rahim Yar Khan, Sheikhupura	32	2009

	Serbia	Belgrade	8	2010
	Singapore	Singapore	15	2011
	South Africa	Johannesburg	2	2011
	South Korea	Seoul	4	2010
	Spain	Madrid	1	2012
	Sweden	Stockholm	4	2011
	Switzerland	Geneva	3	2009-2010
	Taiwan	Taipei	1	2011
	Thailand	Khon Kaen	6	2010
	Vietnam	Hanoi	2	2010
	United Arab Emirates	Abu Dhabi	2	2011
	United Kingdom	10 cities (not available)	23	2011
Human carriage	Cameroon	Douala	1	2012
	China	Hunan, Beijing	49	2011
	India	Kolkata, Chennai, Guwahati	21	2009
	Pakistan	Rawalpindi	101	2010
Environmental	China	Beijing, Chengdu	30	2012
	India	Kolkata, New Delhi	22	2010
	Vietnam	Hanoi	2	2011

Table 3. Countries reporting imported isolates of NDM-1-producing bacteria

Type of isolates	Country	Cities	Imported from	Number of isolates	First description
Human infection	Australia	Sydney	India, Bangladesh	3	2010
	Austria	Graz	India, Kosovo	2	2009-2011
	Belgium	Yvoir, Brussels, Antwerp, Namur	Algeria, Pakistan, Montenegro, Serbia, Kosovo	6	2010
	Canada	Brampton, Calgary, Toronto, Winnipeg	India	7	2010
	China	Hong Kong	India	1	2010
	Croatia	Zagreb	Bosnia and Herzegovina	1	2009
	Czech Republic	Prague, Plzen	Egypt	3	2011
	Denmark	Hvidovre, Copenhagen	Pakistan, Lybia	2	2011
	France	Marseille, Lyon, Paris, Saint Pierre	India, Algeria, Serbia, Iraq, Mauritius	11	2010
	Germany	Frankfurt, Bonn	Egypt, Serbia, India	3	2007
	Ireland	Dublin	India	1	2011

Italy	Siena, Bologna	India	14	2009-2010
Japan	Soka, Tochigi, Niigata, Tokyo	India	4	2009
Kuwait	Jabriya	India	2	2010-2011
Lebanon	Beirut	Iraq	4	2008-2011
Netherlands	Enschede, Utrecht	Serbia, India	3	2008
New Zealand	Porirua	India	4	2009-2010
Norway	Tromsø	India	2	2010
Oman	Muscat	India	14	2010
Singapore	Singapore	India	1	2010
South Africa	Johannesburg	Mozambique, Zambia	1	2010
Spain	Madrid, Barcelona	India	2	2011
Sweden	Örebro	India	1	2009
Switzerland	Geneva	Serbia, India	2	2009-2010
Taiwan	Taipei	China, India	5	2010
Turkey	Istanbul	Iraq	1	2011
United Kingdom	London, Brustol	Kenya, India	44	2008
United States	Los Angeles, Chicago, Atlanta, Providence	Pakistan, India, Vietnam	9	2010

1

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Table. Summary of articles describing NDM-1-positive bacteria, from the first report through December 31, 2012

Year	City	Country	Number of bacteria	Species	Imported Vs autochthonous isolates	Type of isolates	PubMed Link	Title	Reference
2012	Hvidovre	Denmark	1	*Escherichia coli* (n=1)	Imported isolates from Pakistan	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22532468	An NDM-1-producing *Escherichia coli* obtained in Denmark has a genetic profile similar to an NDM-1-producing *E. coli* isolate from the UK	[1]
2009-2010	Porirua	New Zealand	4	*Escherichia coli* (n=2) *Klebsiella pneumoniae* (n=1) *Proteus mirabilis* (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22526013	Identification and molecular characterization of New Delhi metallo-β-lactamase-1 (NDM-1)- and NDM-6-producing Enterobacteriaceae from New Zealand hospitals.	[2]
2011	Not available	Guatemala	2	*Klebsiella pneumoniae* (n=2)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22461309	Emergence of NDM-1-producing *Klebsiella pneumoniae* in Guatemala	[3]
2011	Bordeaux	France	1	*Klebsiella pneumoniae* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22450982	Autochthonous isolates of NDM-1-producing *Klebsiella pneumoniae* resistant to colistin in a French community patient	[4]
2011	Praha	Czech Republic	1	*Acinetobacter baumannii* (n=1)	Imported isolates from Egypt	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22449869	Carbapenem-resistant *Acinetobacter baumannii* carrying the NDM-1 gene, Czech Republic, 2011	[5]
2011	Hunan	China	4	*Escherichia coli* (n=2) *Klebsiella pneumoniae* (n=1) *Enterobacter aerogenes* (n=1)	Autochthonous isolates	Human isolates and human carrying isolates	http://www.ncbi.nlm.nih.gov/pubmed/22438435	Emergence of NDM-1-producing Enterobacteriaceae in China.	[6]
2010	Dhaka	Bangladesh	14	*Klebsiella pneumoniae* (n=7) *Acinetobacter baumannii* (n=3) *Escherichia coli* (n=2) *Proteus rettgeri* (n=1) *Citrobacter freundii* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22422273.1	Emergence of multidrug-resistant NDM-1-producing Gram-negative bacteria in Bangladesh	[7]
2012	Soka Saitama	Japan	2	*Escherichia coli* (n=1) *Klebsiella pneumoniae* (n=1)	Imported isolates from India Autochthonous isolates	Human isolates Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22413529.1	New Delhi metallo -beta-lactamase-1 (NDM-1) producing bacteria	[8]

2010	Tokyo	Japan	2	Enterobacteriaceae (n=2)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22413516.1	Three month survey of multidrug-resistant Enterobacteriaceae in Japan	[9]
2012	Lyon	France	3	*Escherichia coli* (n=2) *Citrobacter* spp. (n=1)	Autochthonous isolates Imported isolates from India	Human isolates Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22404570.1	Nosocomial transmission of NDM-1-producing *Escherichia coli* within a non-endemic area in France	[10]
2010-2011	Muscat	Oman	12	*Escherichia coli* (n=1) *Klebsiella pneumoniae* (n=11)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22404169.1	NDM-1, OXA-48 and OXA-181 carbapenemase-producing Enterobacteriaceae in Sultanate of Oman	[11]
2009-2010	Winnipeg	Canada	2	*Escherichia coli* (n=1) *Klebsiella pneumoniae* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22398651.1	Carbapenem-resistant Gram-negative bacilli in Canada 2009-10: results from the Canadian Nosocomial Infection Surveillance Program (CNISP)	[12]
2011	Istanbul	Turkey	1	*Klebsiella pneumoniae* (n=1)	Imported isolates from Baghdad, Iraq	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22391536.1	NDM-1-Producing *Klebsiella pneumoniae* Now in Turkey	[13]
2011	Madrid	Spain	1	*Klebsiella pneumoniae* (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22383442.1	Abdominal abscess due to NDM-1-producing *Klebsiella pneumoniae* in Spain	[14]
2009	Zagreb	Croatia	1	*Klebsiella pneumoniae* (n=1)	Imported isolates from Bosnia and Herzegovina	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22377049.1	NDM-1-producing *Klebsiella pneumoniae*, Croatia	[15]
2011	Plzen	Czech Republic	2	*Acinetobacter baumannii* (n=2)	Imported isolates from Egypt	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22370014.1	NDM-1 producing *Acinetobacter baumannii* isolated from a patient repatriated to the Czech Republic from Egypt, July 2011	[16]
2011	Dublin	Ireland	1	*Klebsiella pneumoniae* (n=1)	Imported isolates from India (Kolkata)	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22370013.1	Isolation of NDM-1-producing *Klebsiella pneumoniae* in Ireland, July 2011	[17]
2012	Fujian	China	1	*Acinetobacter baumannii* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22357496.1	Sensitive and rapid detection of the new delhi metallo-Beta-lactamase gene by loop-mediated isothermal amplification.	[18]
2011	Chennai Haryana Various cities Stockholm	India India United Kingdom Sweden	39	*Klebsiella pneumoniae* (n=16) *Klebsiella pneumoniae* (n=6) *Klebsiella pneumoniae* (n=13) *Klebsiella pneumoniae* (n=4)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22354295.1	Diverse Sequence Types of *Klebsiella pneumoniae* Contribute to the Dissemination of blaNDM-1 in India, Sweden, and the United Kingdom	[19]
2011	Yvoir	Belgium	1	*Acinetobacter baumannii* (n=1)	Imported isolates from Algeria	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22345387.1	Emergence of NDM-1-producing *Acinetobacter baumannii* in Belgium	[20]

2012	Chennai	India	2	*Acinetobacter baumannii* (n=2)	Autochthonous isolates	human carrying is olates	http://www.ncbi.nlm.nih.gov/pubmed/22335806.1	A study on the isolation rate and prevalence of drug resistance among microorganisms isolated from multiorgan donor and donor corneal rim along with a report on the existence of blaNDM-1 among Indian population.	[21]
2008	Enschede	Netherlands	2	*Klebsiella pneumoniae* (n=1) *Klebsiella pneumoniae* (n=1)	Imported isolates Belgrade, Serbia Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22330915.1	A Isolates of New Delhi Metallo-β-Lactamase 1 (NDM-1)-Producing *Klebsiella pneumoniae* with Putative Secondary Transmission from the Balkan Region in the Netherlands	[22]
2011	Paris	France	1	*Acinetobacter baumannii* (n=1)	Imported from Oran, Algeria	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22290985.1	NDM-1-producing *Acinetobacter baumannii* from Algeria	[23]
2011	Kabul	Afghanistan	2	*Providencia stuartii* (n=2)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22290972.1	Complete sequence of a novel 178-kilobase plasmid carrying bla(NDM-1) in a *Providencia stuartii* strain isolated in Afghanistan	[24]
2010	Hebei	China	2	*Acinetobacter lwoffii* (n=2)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22290961.1	Novel plasmid and its variant harboring both a bla(NDM-1) gene and type IV secretion system in clinical isolates of *Acinetobacter lwoffii*	[25]
2010	Johannesburg	South Africa	1	*Enterobacter cloacae* (n=1)	Imported isolates from Mozambique and Zambia	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22273027.1	NDM-1 has arrived: first report of a carbapenem resistance mechanism in South Africa	[26]
2011	Kolkata	India	5	*Escherichia coli* (n=1) *Klebsiella pneumoniae* (n=1) *Acinetobacter baumannii* (n=1) *Stenotrophomonas maltophilia* (n=1) *Enterobacter aerogenes* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22269475.1	Polyethylene glycol-stabilized sulphur nanoparticles: an effective antimicrobial agent against multidrug-resistant bacteria	[27]
2010	Seoul	South Korea	4	*Klebsiella pneumoniae* (n=4)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22259206.1	Nosocomial clustering of NDM-1-producing *Klebsiella pneumoniae* sequence type 340 strains in four patients at a South Korean tertiary care hospital	[28]
2010	Diverscities	India	1	*Escherichia coli* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22252797.1	NDM-4 metallo-β-lactamase with increased carbapenemase activity from *Escherichia coli*	[29]

2011	Abu Dhabi	United Arab Emirates	2	*Acinetobacter baumannii* (n=2)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22192275	NDM-2 carbapenemase-producing *Acinetobacter baumannii* in the United Arab Emirates	[30]
2011	Guangzhou	China	1	*Acinetobacter junii* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22181044	Identification of New Delhi metallo-β-lactamase gene (NDM-1) from a clinical isolate of *Acinetobacter junii* in China	[31]
2011	Bologna	Italy	6	*Klebsiella pneumoniae* (n=5) *Escherichia coli* (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22152705	Outbreak of NDM-1-producing Enterobacteriaceae in northern Italy, July to August 2011.	[32]
2009-2010	Geneva	Switzerland	1	*Acinetobacter baumannii* (n=1)	Imported isolates from Serbia	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22143526	Tn125-related acquisition of blaNDM-like genes in *Acinetobacter baumannii*	[33]
2010	Pune	India	20	*Acinetobacter* sp. (n=13) *Pseudomonas* sp. (n=7)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22137509	Prevalence of New Delhi metallo-β-lactamase (NDM-1)-positive bacteria in a tertiary care center in Pune, India	[34]
2011	Singapore	Singapore	1	*Klebsiella pneumoniae* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22126430	Successful treatment of NDM-1 *Klebsiella pneumoniae* bacteriemia in a neutropenic patient	[35]
2011	Johannesburg	South Africa	2	*Klebsiella pneumoniae* (n=2)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22116157	Emergence of New Delhi metallo-beta-lactamase (NDM-1) and *Klebsiella pneumoniae* carbapenemase (KPC-2) in South Africa	[36]
2010-2011	Jabriya	Kuwait	2	*Klebsiella pneumoniae* (n=2)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22113192	Emergence of nosocomial New Delhi metallo-β-lactamase-1 (NDM-1)-producing *Klebsiella pneumoniae* in patients admitted to a tertiary care hospital in Kuwait	[37]
2010	Taipei	Taiwan	4	*Klebsiella oxytoca* (n=4)	Imported isolates from Nanchang, China	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22083082	Pelvic abscess caused by New Delhi metallo-β-lactamase-1-producing *Klebsiella oxytoca* in Taiwan in a patient who underwent renal transplantation in China	[38]
2012	Not available	India	2	*Enterobacter cloacae* (n=1) *Klebsiella pneumoniae* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22034164	NDM-1-producing *Enterobacter cloacae* and *Klebsiella pneumoniae* from diabetic foot ulcers in India	[39]
2009	Quatre Bornes	Mauritius	1	*Klebsiella pneumoniae* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22006002	NDM-1-producing *Klebsiella pneumoniae* in Mauritius	[40]
2010	Chennai	India	1	*Klebsiella pneumoniae* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21990048	Emergence of a *Klebsiella pneumoniae* isolate co-producing NDM-1 with KPC-2	[41]

								from India.	
2011	Not available	USA	1	*Salmonella* spp. (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21968356	First NDM-positive *Salmonella* sp. strain identified in the United States.	[42]
2011	Toulon	France	1	*Klebsiella pneumoniae* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21960718	Emergence of an autochthonous and community-acquired NDM-1-producing *Klebsiella pneumoniae* in Europe	[43]
2011	London	United Kingdom	1	*Escherichia coli* (n=1)	Imported isolates from Goa, India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21930874	A novel variant, NDM-5, of the New Delhi metallo-β-lactamase in a multidrug-resistant *Escherichia coli* ST648 isolate recovered from a patient in the United Kingdom	[44]
2010	Kolkata	India	22	*Escherichia coli* (n=18) *Klebsiella pneumoniae* (n=2) *Stenotrophomonas maltophilia* (n=1) *Acinetobacter baumannii* (n=1)	Autochthonous isolates	Human isolates and human carrying isolates Environment Human carrying isolates Human carrying isolates	http://www.ncbi.nlm.nih.gov/pubmed/21930573	Transmission of imipenem resistance determinants during the course of an outbreak of NDM-1 *Escherichia coli* in a sick newborn care unit.	[45]
2011	Rabat	Morocco	3	*Klebsiella pneumoniae* (n=3)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21930570.1	Emergence of NDM-1-producing *Klebsiella pneumoniae* in Morocco	[46]
2011	Calgary	Canada	2	*Klebsiella pneumoniae* (n=2)	Imported isolates from New Delhi, India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21924993	The characteristics of NDM-producing *Klebsiella pneumoniae* from Canada.	[47]
2011	Paris Various cities	France India	1 3	*Escherichia coli* (n=1) *Escherichia coli* (n=1) *Klebsiella pneumoniae* (n=1) *Providencia stuartii* (n=1)	Imported isolates from India Autochthonous isolates	Human isolates Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21859933	Genetic features of blaNDM-1-positive Enterobacteriaceae.	[48]
2011	London	United Kingdom	1	*Escherichia coli* (n=1)	Imported isolates from India and Kenya	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21846669	Breakthrough bacteriemia due to tigecycline-resistant *Escherichia coli* with New Delhi metallo-β-lactamase (NDM)-1 successfully treated with colistin in a patient with calciphylaxis.	[49]

2011	Tel Aviv	Israel	5	*Acinetobacter baumannii* (n=5)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21825296.1	Dissemination of an NDM-2-producing *Acinetobacter baumannii* clone in an Israeli rehabilitation center.	[50]
2010	Pristina	Kosovo	7	*Acinetobacter baumannii* (n=1) *Klebsiella pneumoniae* (n=2) *Escherichia coli* (n=2) *Citrobacter freundii* (n=1) *Proteus vulgaris* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21798461.1	Balkan NDM-1: escape or transplant?	[51]
2010	Rawalpindi	Pakistan	64	*Escherichia coli* (n=30) *Enterobacter cloacae* (n=21) *Citrobacter* sp. (n=3) *Citrobacter freundii* (n=4) *Citrobacter braakii* (n=1) *Klebsiella pneumoniae* (n=3) *Providencia rettgeri* (n=2)	Autochthonous isolates	Human carrying isolates	http://www.ncbi.nlm.nih.gov/pubmed/21788293.1	Prevalence of fecal carriage of Enterobacteriaceae with NDM-1 carbapenemase at military hospitals in Pakistan, and evaluation of two chromogenic media.	[52]
2010	Sydney	Australia	1	*Escherichia coli* (n=1)	Imported isolates from Bangladesh	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21746951.1	Analysis of the resistome of a multidrug-resistant NDM-1-producing *Escherichia coli* strain by high-throughput genome sequencing	[53]
2011	Beijing	China	1	*Acinetobacter lwoffii* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21742884.1	Whole-genome sequence of a multidrug-resistant clinical isolate of *Acinetobacter l woffii*.	[54]
2011	Barcelona	Spain	1	*Escherichia coli* (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21730115.1	First description of an *Escherichia coli* strain producing NDM-1 carbapenemase in Spain.	[55]
2007	Frankfurt	Germany	1	*Acinetobacter baumannii* (n=1)	Imported isolates from Serbia	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21693460.1	Molecular characterization of blaNDM-1 in an *Acinetobacter baumannii* strain isolated in Germanyin 2007.	[56]

2009	8 cities	India	33	*Klebsiella pneumoniae* (n=18) *Escherichia coli* (n=8) *Enterobacter cloacae* (n=5) *Providencia rettgeri* (n=1) *Morganella morganii* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21676902.1	Increasing prevalence and dissemination of NDM-1 metallo-β-lactamase in India: data from the SMART study (2009).	[57]
2011	10 cities Karachi Assam	United Kingdom Pakistan India	10 7 1	*Escherichia coli* (n=10) *Escherichia coli* (n=7) *Escherichia coli* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21669947.1	Phylogenetic diversity of *Escherichia coli* strains producing NDM-type carbapenemases.	[58]
2011	Paris	France	1	*Escherichia coli* (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21653781.1	Early detection of colonization by VIM-1-producing *Klebsiella pneumoniae* and NDM-1-producing *Escherichia coli* in two children returning to France.	[59]
2011	Paris	France	1	*Escherichia coli* (n=1)	Imported isolates from Serbia	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21653599.1	Long-term carriage of NDM-1-producing *Escherichia coli*.	[60]
2010	Belgrade	Serbia	7	*Pseudomonas aeruginosa* (n=7)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21646490.1	Emergence of NDM-1 metallo-β-lactamase in *Pseudomonas aeruginosa* clinical isolates from Serbia	[61]
2009-2010	Geneva	Switzerland	4	*Klebsiella pneumoniae* (n=2) *Escherichia coli* (n=1) *Proteus mirabilis* (n=1)	One autochthonous and one imported from India Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21628303.1	Molecular analysis of NDM-1-producing enterobacterial isolates from Geneva, Switzerland.	[62]
2011	Toronto	Canada	2	*Morganella morganii* (n=1) *Providencia rettgeri* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21624908.1	New Delhi metallo-β-lactamase-1: local acquisition in Ontario, Canada, and challenges in detection.	[63]
2010	Assam	India	54	*Escherichia coli* (n=30) *Citrobacter* sp. (n=12) *Klebsiella pneumoniae* (n=12)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21596721.1	Dissemination of the New Delhi metallo-β-lactamase-1 (NDM-1) among Enterobacteriaceae in a tertiary referral hospital in north India.	[64]

2011	Singapore	Singapore	12	*Klebsiella pneumoniae* (n=8) *Enterobacter cloacae* (n=2) *Escherichia coli* (n=1) *Proteus mirabilis* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21565805.1	Rapid detection of the blaNDM-1 gene by real-time PCR.	[65]
2010	Singapore	Singapore	1	*Escherichia coli* (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21552793.1	The perils of medical tourism: NDM-1-positive *Escherichia coli* causing febrile neutropenia in a medical tourist.	[66]
2009-2010	Siena	Italy	8	*Escherichia coli* (n=8)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21525229.1	Persistent carriage and infection by multidrug-resistant *Escherichia coli* ST405 producing NDM-1 carbapenemase: report on the first Italian isolates s.	[67]
2010	Marseille	France	1	*Klebsiella pneumoniae* (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21497063.1	Real-time PCR assay allows detection of the New Delhi metallo-β-lactamase (NDM-1)-encoding gene in France	[68]
2010	New Delhi	India	20	*Pseudomonas putida* (n=2) *Pseudomonas pseudoalcaligenes* (n=2) *Escherichia coli* (n=3) *Pseudomonas oryzihabitans* (n=1) *Klebsiella pneumoniae* (n=1) *Shigella boydii* (n=1) *Suttonella indologenes* (n=1) *Aeromonas caviae* (n=1) *Citrobacter freundii* (n=1) *Stenotrophomonas maltophilia* (n=1) *Vibrio cholerae* (n=2) *Achromobacter* spp. (n=2) *Kingella denitrificans* (n=1) *Pseudomonas aeruginosa* (n=1)	Autochthonous isolates	Environment	http://www.ncbi.nlm.nih.gov/pubmed/21478057.1	Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an Environment point prevalence study.	[69]

2011	Hong Kong	China	1	*Escherichia coli* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21445317.1	Complete sequencing of pNDM-HK encoding NDM-1 carbapenemase from a multidrug-resistant *Escherichia coli* strain isolated in Hong Kong.	[70]
2010	Chicago	USA	1	*Escherichia coli* (n=1)	Imported isolates from New Delhi, India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21444703.1	Characteristics of NDM-1-producing *Escherichia coli isolates* that belong to the successful and virulent clone ST131.	[71]
2010	Brussels Antwerp Antwerp Namur Namur	Belgium	5	*Escherichia coli* (n=1) *Escherichia coli* (n=1) *Klebsiella pneumoniae* (n=1) *Morganella morganii* (n=1) *Enterobacter cloacae* (n=1)	Imported isolates from Pakistan Imported isolates from Montenegro Imported isolates from Montenegro Imported isolates from Serbia/Kosovo Imported isolates from Serbia/Kosovo	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21444697.0	Emergence of NDM-1-producing Enterobacteriaceae in Belgium.	[72]
2010	Niigata	Japan	1	*Escherichia coli* (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21437680.1	Emergence of NDM-1-positive capsulated *Escherichia coli* with high resistance to serum killing in Japan.	[73]
2011	Not available	Germany	1	*Acinetobacter baumannii* (n=1)	Imported isolates from Egypt	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21427107.1	NDM-2 carbapenemase in *Acinetobacter baumannii* from Egypt.	[74]
2011	Hangzhou	China	4	*Acinetobacter baumannii* (n=4)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21398294.1	Emergence of NDM-1-producing *Acinetobacter baumannii* in China.	[75]
2011	Kolkata	India	2	*Klebsiella pneumoniae* (n=2)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21393155.1	Sepsis in neonates due to imipenem-resistant *Klebsiella pneumoniae* producing NDM-1 in India.	[76]
2011	Taipei	Taiwan	1	*Klebsiella pneumoniae* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21354091.1	Spontaneous eradication of a NDM-1 positive *Klebsiella pneumoniae* that colonized the intestine of an asymptomatic carrier.	[77]
2011	Los Angeles	USA	3	*Klebsiella pneumoniae* (n=3)	Imported isolates from Pakistan	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21325558.1	New Delhi metallo-β-lactamase (NDM-1)-producing *Klebsiella pneumoniae*: isolates report and laboratory detection strategies.	[78]
2009	Guwahati	India	3	*Escherichia coli* (n=1) *Klebsiella pneumoniae* (n=1) *Enterobacter cloacae* (n=1)	Autochthonous isolates	Human carrying isolates	http://www.ncbi.nlm.nih.gov/pubmed/21304190.1	Multidrug-resistant Enterobacteriaceae including metallo-β-lactamase producers are predominant pathogens of healthcare-associated infections in an Indian teaching	[79]

								hospital.	
2010	Brampton	Canada	1	*Klebsiella pneumoniae* (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21291614.1	New Delhi metallo-beta-lactamase, Ontario, Canada.	[80]
2010	Calgary	Canada	1	*Escherichia coli* (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21291595.1	New Delhi metallo-beta-lactamase from traveler returning to Canada.	[81]
2010	Not available	Australia	1	*Klebsiella pneumoniae* (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21258100.1	Carbapenem resistance in *Klebsiella pneumoniae* due to the New Delhi Metallo-β-lactamase.	[82]
2010	Paris	France	1	*Klebsiella pneumoniae* (n=1)	Imported isolates from Baghdad, Iraq	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21245442.1	International Transfer of NDM-1-Producing *Klebsiella pneumoniae* from Iraq to France	[83]
2010	Toronto	Canada	1	*Klebsiella pneumoniae* (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21220461.1	New Delhi metallo-β-lactamase-1 in Enterobacteriaceae: emerging resistance.	[84]
2009-2010	Graz	Austria	2	*Klebsiella pneumoniae* (n=1) *Klebsiella pneumoniae* (n=1)	Imported isolates from India Imported isolates from Kosovo	Human isolates Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21192874.1	Emergence of New Delhi metallo-β-lactamase, Austria.	[85]
2010	Winnipeg	Canada	2	*Klebsiella pneumoniae* (n=1) *Escherichia coli* (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21192866.1	New Delhi metallo-β-lactamase in *Klebsiella pneumoniae* and *Escherichia coli*, Canada.	[86]
2006-2007	New Delhi	India	9	*Escherichia coli* (n=4) *Enterobacter cloacae* (n=2) *Klebsiella pneumoniae* (n=3)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21189345.1	Early dissemination of NDM-1- and OXA-181-producing Enterobacteriaceae in Indian hospitals: report from the SENTRY Antimicrobial Surveillance Program, 2006-2007.	[87]
	Mumbai		3	*Escherichia coli* (n=1) *Klebsiella pneumoniae* (n=2)					
	Pune		3	*Klebsiella pneumoniae* (n=1) *Escherichia coli* (n=1) *Enterobacter cloacae* (n=1)					
2009	Bonn	Germany	1	*Escherichia coli* (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21189341.1	NDM-1-producing *Escherichia coli* in Germany.	[88]
2010	Tromsø	Norway	2	*Escherichia coli* (n=1) *Klebsiella pneumoniae* (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21172785.1	Identification of NDM-1-producing Enterobacteriaceae in Norway.	[89]
2009	Tochigi	Japan	1	*Escherichia coli* (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21148536.1	First isolates of New Delhi metallo-beta-lactamase 1-producing *Escherichia coli* infection in Japan.	[90]

2007-2009	Nairobi	Kenya	7	*Klebsiella pneumoniae* (n=7)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21115785.1	Detection of NDM-1-producing *Klebsiella pneumoniae* in Kenya.	[91]
2010	Muscat	Oman	2	*Klebsiella pneumoniae* (n=2)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21098539.1	NDM-1-producing *Klebsiella pneumoniae* isolated in the Sultanate of Oman.	[92]
2010	Taipei	Taiwan	1	*Klebsiella pneumoniae* (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21093828.1	First identification of a patient colonized with *Klebsiella pneumoniae* carrying blaNDM-1 in Taiwan.	[93]
2010	Paris	France	1	*Citrobacter freundii* (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/20974865.1	Extremely drug-resistant *Citrobacter freundii* isolate producing NDM-1 and other carbapenemases identified in a patient returning from India.	[94]
2010	Utrecht	Netherlands	2	*Klebsiella pneumoniae* (n=2)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/20858323.1	Carbapenem-resistant *Klebsiella pneumoniae* following foreign travel.	[95]
2009	Mumbai	India	22	*Klebsiella* spp. (n=10) *Escherichia coli* (n=9) *Enterobacter* spp. (n=2) *Morganella morganii* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/20848811.1	New Delhi Metallo-beta lactamase (NDM-1) in Enterobacteriaceae: treatment options with carbapenems compromised.	[96]
2010	Sydney	Australia	1	Escherichia coli (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/20823289.1	Emergence of metallo-β-lactamase NDM-1-producing multidrug-resistant *Escherichia coli* in Australia.	[97]
2008-2009	London	United Kingdom	37	*Klebsiella pneumoniae* (n=21) *Escherichia coli* (n=7) *Enterobacter* spp (n=5) *Citrobacter freundii* (n=2) *Morganella morganii* (n=1) *Providencia* spp. (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/20705517	Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study	[98]
	9 cities	Pakistan	25	*Klebsiella pneumoniae* (n=50)					
	Guwahati	India	3	*Enterobacteriaceae* (n=13)					
	Varanasi	India	13	*Escherichia coli* (n=10)					
2009	Mumbai	India	32		Autochthonous isolates				
	Haryana	India	26	*Klebsiella pneumoniae (n=26)*					
	Chennai	India	44	*Escherichia coli* (n=19) *Klebsiella*					

				pneumoniae (n=14) *Enterobacter cloacae* (n=7) *Proteus* spp. (n=2) *Citrobacter freundii* (n=1) *Klebsiella oxytoca* (n=1)					
2010	Chennai	India	3	*Acinetobacter baumannii* (n=3)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/20650909.1	Coexistence of blaOXA-23 with blaNDM-1 and armA in clinical isolates of *Acinetobacter baumannii* from India.	[99]
2010	Atlanta, GA	USA	3	*Klebsiella pneumoniae* (n=1) *Escherichia coli* (n=1) *Enterobacter cloacae* (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/20577157.1	Detection of Enterobacteriaceae isolates carrying metallo-beta-lactamase - United States, 2010.	[100]
2007	Örebro	Sweden	1	*Klebsiella pneumoniae* (n=1)	Imported isolates from India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/19770275.1	Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India.	[101]
2012	Various cities	China	7	*Acinetobacter* spp. (n=7)	Autochthonous isolates	Human carrying isolates	http://www.ncbi.nlm.nih.gov/pubmed/22604448.1	Epidemiological characteristics and genetic structure of blaNDM-1 in non-*baumannii* *Acinetobacter* spp. in China	[102]
2012	Beirut	Lebanon	2	*Klebsiella pneumoniae* (n=2)	imported isolates from Iraq	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22610714	Detection of carbapenem-resistant Escherichia coli and *Klebsiella pneumoniae* producing NDM-1in Lebanon.	[103]
2012	Beijing	China	1	*Acinetobacter lwoffii* (n=1)	Autochthonous isolates	Environment	http://www.ncbi.nlm.nih.gov/pubmed/22629360	Identification of New Delhi Metallo-β-lactamase 1 in *Acinetobacter lwoffii* of Food Animal Origin	[104]
2012	Chennai	India	1	*Acinetobacter baumannii* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22715297	Studies on New Delhi Metallo-Beta-Lactamse-1 producing *Acinetobacter baumannii* isolated from donor swab in a tertiary eye care centre, India and structural analysis of its antibiotic binding interactions.	[105]
2012	Providence	USA	1	*Klebsiella pneumoniae* (n=1)	Imported isolates from Vietnam	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22717513	Carbapenem-resistant Enterobacteriaceae containing New Delhi metallo-beta-lactamase in two patients - Rhode Island, March 2012.	[106]

2011	Copenhagen	Denmark	1	*Acinetobacter baumannii* (n=1)	Imported isolates from Lybia	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22743013	Patients transferred from Libya to Denmark carried OXA-48-producing *Klebsiella pneumoniae*,NDM-1-producing *Acinetobacter baumannii* and meticillin-resistant *Staphylococcus aureus*	[107]
2010	Khon Kaen	Thailand	6	*Klebsiella pneumoniae* (n=2) *Citrobacter freundii* (n=2) *Escherichia coli* (n=2)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22796889	Emergence of NDM-1- and IMP-14a-producing Enterobacteriaceae in Thailand.	[108]
2011	Hanoi	Vietnam	2	*Klebsiella pneumoniae* (n=2)	Autochthonous isolates	Environment	http://www.ncbi.nlm.nih.gov/pubmed/22840532	bla(NDM-1)-positive *Klebsiella pneumoniae* from environment, Vietnam	[109]
2012	Hunan	China	1	*Klebsiella pneumoniae* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22898285	NDM-1-producing *Klebsiella pneumoniae* in mainland China	[110]
2011	Belgrade	Serbia	1	*Klebsiella pneumoniae* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22908159	Isolation of *Klebsiella pneumoniae* Producing New Delhi Metallo-beta-lactamase-1 from Urine of Outpatient Baby Boy Receiving Antibiotic Prophylaxis.	[111]
2012	Douala	Cameroon	1	*Escherichia coli* (n=1)	Autochthonous isolates	Human carrying isolates	http://www.ncbi.nlm.nih.gov/pubmed/22932298	New Delhi Metallo-β-Lactamase 4-producing *Escherichia coli* in Cameroon.	[112]
2011	Beijing	China	41	*Acinetobacter ursingii* (n=1) *Methylobacterium* spp. (n=1) *Alcaligenes faecalis* (n=2) *Morganella morganii* (n=2) *Acinetobacter baumannii* (n=2) *Stenotrophomonas maltophilia* (n=2) *Comamonas testosteroni* (n=7) *Moraxella* spp. (n=8) *Acinetobacter lwoffii* (n=16)	Autochthonous isolates	Human carrying isolates	http://www.ncbi.nlm.nih.gov/pubmed/22955442	High Rate of New Delhi Metallo-β-Lactamase 1-Producing Bacteria Infection in China	[113]
2011	Tokyo	Japan	1	*Acinetobacter baumannii* (n=1)	Imported isolates from Chennai, India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22965842	A case of NDM-1-producing *Acinetobacter baumannii* transferred from India to Japan.	[114]
2010	Not available	Japan	1	*Klebsiella pneumoniae* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22971935	Electron microscopic structures, serum resistance, and plasmid restructuring of New Delhi metallo-β-lactamase-1 (NDM-	[115]

							1)-producing ST42 *Klebsiella pneumoniae* emerging in Japan.	
2011	Saint Pierre	France	2	*Salmonella enterica* (n=1) *Klebsiella pneumoniae* (n=1)	Imported isolates from Chennai, India Imported isolates from Mauritius	Human isolates Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22972814	Emergence of *Klebsiella pneumoniae* and *Salmonella* Metallo-Beta lactamase (NDM-1) producers in Reunion Island.	[116]
2011	Tehran	Iran	1	*Klebsiella pneumoniae* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22984942	First Report of New Delhi Metallo-Beta-Lactamase-1-Producing *Klebsiella pneumoniae* in Iran.	[117]
2011	Brampton	Canada	5	*Klebsiella pneumoniae* (n=5)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22997214	Outbreak of Carbapenem-Resistant Enterobacteriaceae Containing blaNDM-1, Ontario, Canada.	[118]
2010	Hong Kong	China	1	*Escherichia coli* (n=1)	Imported isolates from Punjab, India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/23018074	Hong Kong experiences the 'Ultimate superbug': NDM-1 Enterobacteriaceae.	[119]
2008	Dhaka	Bangladesh	3	*Klebsiella pneumoniae* (n=3)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/23019191	Occurrence and characterization of multi-drug resistant NDM-1-producing bacteria isolated between 2003 and 2010 in Bangladesh.	[120]
2010	Jerusalem	Israel	5	*Morganella morganii* (n=1) *Providencia rettgeri* (n=1) *Proteus mirabilis* (n=1) *Escherichia coli* (n=1) *Klebsiella pneumoniae* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/22951226	Emergence of New Delhi metallo-β-lactamase in Jerusalem, Israel.	[121]
2012	Beijing	China	27	*Acinetobacter pittii* (n=27)	Autochthonous isolates	Environment	http://www.ncbi.nlm.nih.gov/pubmed/23036089	Dissemination and characterization of NDM-1-producing *Acinetobacter pittii* in an intensive care unit in China	[122]
2011	Bristol	united kingdom	5	*Vibrio cholerae* (n=1) *Klebsiella pneumonia* (n=1) *Escherichia coli* (n=1) *Enterobacter cloacae* (n=1) *Citrobacter freundii* (n=1)	Imported isolates from Bihar, India	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/23063285	NDM-1 polymicrobial infections including *Vibrio cholerae*	[123]
2010	Hanoi	Vietnam	2	*Klebsiella pneumoniae* (n=1) *Escherichia coli* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/23100353	Carbapenem resistant *Escherichia coli* and *Klebsiella pneumoniae* containing New Delhi Metallo-Beta-Lactamase isolated from two patients in Vietnam.	[124]

2009-2012	Chongqing	China	1	*Enterobacter cloacae* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/23110085	Prevalence of plasmid-mediated quinolone resistance and aminoglycoside resistance determinants among carbapeneme non-susceptible *Enterobacter cloacae*.	[125]
2012	singapore	singapore	2	*Klebsiella pneumoniae* (n=2)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/23139815	Sequence of closely related plasmids encoding bla(NDM-1) in two unrelated *Klebsiella pneumoniae* isolates in Singapore.	[126]
2008-2011	Beirut	Lebanon	2	*Klebsiella pneumoniae* (n=2)	Imported isolates from Iraq	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/23142087	Underlying mechanisms of carbapenem resistance in extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* and *Escherichia coli* isolates at a tertiary care centre in Lebanon: role of OXA-48 and NDM-1carbapenemases	[127]
2012	Paris	France	1	*Pseudomonas euruginosa* (n=1)	Imported isolates from Serbia	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/23153474	Recurrent pyelonephritis due to NDM-1 metallo-beta-lactamase producing *Pseudomonas aeruginosa* in a patient returning from Serbia, France, 2012.	[128]
2010	Mumbai Bangalore Chennai	India	2 5 6	*Escherichia coli* (n=2) *Klebsiella pneumoniae* (n=3) *Enterobacter cloacae* (n=1) *Citrobacter freundii* (n=1) *Klebsiella pneumoniae* (n=4) *Escherichia coli* (n=1) *Enterobacter cloacae* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/23177221	Update on the prevalence and genetic characterization of NDM-1-producing Enterobacteriaceae in Indian hospitals during 2010.	[129]
2012	Madrid	Spain	1	*Klebsiella pneumoniae* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/23218738	New description of a NDM-1 carbapenemase producing *Klebsiella pneumoniae* carrier in Spain	[130]
2009-2012	Chongqing	China	1	*Enterobacter cloacae* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/23220359	Characterization of carbapenemases, extended spectrum β-lactamases and molecular epidemiology of carbapenem-non-susceptible *Enterobacter cloacae* in a Chinese hospital in Chongqing.	[131]
2012	Toronto	Canada	9	*Klebsiella pneumoniae* (n=9)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/23221192	Nosocomial transmission of New Delhi Metallo-β-lactamase-1-producing *Klebsiella pneumoniae* in Toronto, Canada.	[132]
2011	Rawalpindi	Pakistan	37	*Escherichia coli* (n=21) *Klebsiella pneumoniae*	Autochthonous isolates	Human carrying	http://www.ncbi.nlm.nih.gov/pubmed/23246367	Prevalence and molecular characterization of Enterobacteriaceae producing NDM-	[133]

				(n=11) *Enterobacter cloacae* (n=4) *Citrobacter freundii* (n=1)		isolates		1 carbapenemase at a military hospital in Pakistan and evaluation of two chromogenic media.	
2012	Taza	Morocco	1	*Klebsiella pneumoniae* (n=1)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/23278254	First report of a *Klebsiella pneumoniae* strain coproducing NDM-1, VIM-1 and OXA-48 carbapenemases isolated in Morocco.	[134]
2012	Chengdu	China	2	*Acinetobacter johnsonii* (n=2)	Autochthonous isolates	Environment	http://www.ncbi.nlm.nih.gov/pubmed/23288403	blaNDM-1-carrying *Acinetobacter johnsonii* detected in hospital sewage.	[135]
2010	Varanasi	India	54	*Escherichia coli* (n=30) *Klebsiella pneumonia* (n=12) *Citrobacter* spp. (n=12)	Autochthonous isolates	Human isolates	http://www.ncbi.nlm.nih.gov/pubmed/21596721	Dissemination of the New Delhi metallo-β-lactamase-1 (NDM-1) among Enterobacteriaceae in a tertiary referralhospital in north India.	[136]

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CHAPITRE II:

Développement des nouveaux outils de surveillance de la résistance aux antibiotiques

AVANT PROPOS

Au cours de la dernière décennie, l'augmentation et la diffusion rapide de bactéries résistantes aux antibiotiques et l'émergence de bactéries multi-résistantes (BMR) est devenue une préoccupation mondiale majeure et concerne non seulement des bactéries responsables d'infections nosocomiales mais également des bactéries responsables d'infections communautaires [1]. Parmi les menaces actuelles, nous pouvons citer la résistance aux Bêta-lactamines chez les bactéries à Gram négatif. L'émergence récente et la propagation dans le monde entier des gènes codant pour des carbapénèmases chez les entérobactéries, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, en particulier les carbapénémases de type VIM, IMP, KPC, OXA, et NDM-1, confirme que les gènes de résistance peuvent se propager rapidement entre bactéries, et constitue l'inquiète de la communauté scientifique [2,3]. Le suivi systématique de ces bactéries, en développant des nouveaux outils de surveillance de la résistance aux antibiotiques doit être réalisé, ceci, afin de mettre en œuvre des stratégies de contrôle de dissémination de ces gènes de résistance dans le monde. Ainsi dans cette thématique:

❖ Dans le but d'identifier et de prévenir les épidémies et la propagation des gènes de résistance, nous avons tout d'abord contribué à la mise au point d'une technique rapide utilisée en routine pour la détection phénotypique des souches bactériennes porteuses de carbapénémases chez les bactéries à Gram négatif par spectrométrie de masse (Maldi-Tof -Ms) **(Article 2)**. Nous avons utilisé à cet effet, un total de 106 souches

d'*A. baumannii,* dont 63 souches productrices de carbapénèmases et 43 souches non productrices de carbapénèmases. Après incubation des bactéries en présence d'imipénème pour un maximum de 4 h, le mélange a été centrifugé et le surnageant a été analysé par MALDI-TOF MS. Les pics représentant l'imipénème et son métabolite ont été analysés. Le résultat a été interprété comme étant positif pour la production de carbapénèmases, si le pic spécifique pour l'imipénème à 300,0 m/z disparait au cours de la période d'incubation et est remplacé par le pic du métabolite naturel à 254,0 m/z. Ce test, appliqué à une grande série de souches cliniques d'*A. baumannii,* a montré une sensibilité de 100,0% et une spécificité de 100,0%. Cette étude étant la première à démontrer que ce test, par sa rapidité et sa simplicité, peut être utilisé comme outil de routine pour l'identification et la détection en temps réel des *A. baumannii* productrices de carbapénèmases.

❖ En second lieu, nous avons utilisé l'outil MALDI-TOF pour étudier la propagation épidémiologique des souches cliniques de *K. pneumoniae* **(Article 3)**. Entre Janvier 2008 et Mars 2011, nous avons collecté 535 souches de *K. pneumoniae* à partir des hôpitaux en France et en Algérie. Ces souches que nous avons identifiées par MALDI-TOF et dont nous avons évalué le profil de sensibilité aux antibiotiques, ont permis de relever des données cliniques et épidémiologiques, enregistrées dans un fichier Excel, y compris le regroupement obtenu à partir du dendrogramme MSP, puis analysées en utilisant le logiciel statistique PASW. Le dendrogramme MSP a révélé cinq groupes distincts en fonction d'une limite arbitraire à une distance de 500. L'analyse par data mining des cinq groupes a mis en exergue que les souches de *K. pneumoniae* isolées dans

les hôpitaux algériens étaient significativement associées à des infections respiratoires et au phénotype Bêta-lactamase à Spectre Elargi (BLSE), alors que les souches des hôpitaux français étaient significativement associées à des infections urinaires et au phénotype sauvage. Par ce travail, nous avons démontré pour la première fois, que le MALDI-TOF peut être utilisé en qualité d'outil rapide de typage des isolats cliniques de *K. pneumoniae*. C'est un outil prometteur pour identifier et différencier les souches cliniques en fonction de leurs propriétés phénotypiques et leurs distributions épidémiologiques.

❖ Un troisième travail a été réalisé toujours dans la thématique de surveillance de la résistance aux antibiotiques **(Article 4)**. Son objectif était de développer un outil bioinformatique de « clustering hiérarchique », appliqué aux résultats d'antibiogramme (R, I, S) d'une série de souches cliniques de *Klebsiella pneumoniae* isolées en Algérie et en France afin de surveiller les phénotypes de résistance aux antibiotiques. Un total de 1011 souches de *K. pneumoniae,* dont 221 provenant de l'ouest Algérien et 790 provenant de Marseille, France ont été collectées entre Août 2008 et Décembre 2012, et ont été utilisées pour ce travail. Les tests de sensibilité aux antibiotiques ont été déterminés pour seize antibiotiques, puis les résultats d'antibiogramme ont été introduits dans le logiciel MultiExperiment Viewer (MeV) afin d'effectuer la classification hiérarchique en transformant les données d'antibiogramme (Résistant, Intermédiaire et Sensible) en valeurs (1, 0 et -1), respectivement. La classification hiérarchique par le logiciel MeV appliquée aux résultats d'antibiogramme des 1011 souches a permis de générer des clusters qui étaient significativement corrélés avec la classification phénotypique et

l'origine géographique des souches. De plus, l'ajout des résultats d'antibiogramme d'une souche de *K. pneumoniae* productrice d'une NDM-1 (la seule souche résistante à l'imipénème dans la collection), a généré immédiatement une nouvelle branche dans le dendrogramme. Au cours de ce travail, nous avons pu développer un outil de clustering hiérarchique simple et rapide, appliqué aux résultats d'antibiogramme, en mesure d'étudier qualitativement et quantitativement la prévalence des phénotypes connus et inconnus qui pourraient rapidement être mis en œuvre en routine dans des laboratoires de microbiologie clinique.

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Article 2:

Rapid Detection of Carbapenem Resistance in *Acinetobacter baumannii* Using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry

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Rapid Detection of Carbapenem Resistance in *Acinetobacter baumannii* Using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry

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Abstract

Rapid detection of carbapenem-resistant *Acinetobacter baumannii* strains is critical and will benefit patient care by optimizing antibiotic therapies and preventing outbreaks. Herein we describe the development and successful application of a mass spectrometry profile generated by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) that utilized the imipenem antibiotic for the detection of carbapenem resistance in a large series of *A. baumannii* clinical isolates from France and Algeria. A total of 106 *A. baumannii* strains including 63 well-characterized carbapenemase-producing and 43 non-carbapenemase-producing strains, as well as 43 control strains (7 carbapenem-resistant and 36 carbapenem-sensitive strains) were studied. After an incubation of bacteria with imipenem for up to 4 h, the mixture was centrifuged and the supernatant analyzed by MALDI-TOF MS. The presence and absence of peaks representing imipenem and its natural metabolite was analyzed. The result was interpreted as positive for carbapenemase production if the specific peak for imipenem at 300.0 m/z disappeared during the incubation time and if the peak of the natural metabolite at 254.0 m/z increased as measured by the area under the curves leading to a ratio between the peak for imipenem and its metabolite being <0.5. This assay, which was applied to the large series of *A. baumannii* clinical isolates, showed a sensitivity of 100.0% and a specificity of 100.0%. Our study is the first to demonstrate that this quick and simple assay can be used as a routine tool as a point-of-care method for the identification of *A. baumannii* carbapenemase-producers in an effort to prevent outbreaks and the spread of uncontrollable superbugs.

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Introduction

Carbapenems, the most common of which are imipenem and meropenem, are among the drugs of choice for the treatment of nosocomial infections due to *Acinetobacter baumannii* [1]. However, their efficacies are increasingly becoming compromised because of the worldwide emergence of resistant isolates [2–4]. This resistance is principally caused by the production of carbapenemases, enzymes that are grouped into the following four classes according to their molecular structure: 1) Ambler class A β-lactamases, which are partially inhibited by clavulanic acid; 2) Ambler class B β-lactamases, which are also referred to as metallo-β-lactamases (MBL) because of the presence of a Zn^{2+} ion within the active site; 3) Ambler class C β-lactamases; and 4) Ambler class D β-lactamases, which are also referred to as oxacillinases (or OXA-type β-lactamases) and are serine-site enzymes [5,6]. For *A. baumannii*, carbapenem resistance is principally mediated by the production of oxacillinases, mainly the $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-24-like}}$ and $bla_{\text{OXA-58-like}}$ gene products [7–13]. Each of these enzymes is able to hydrolyze the amide bond of the β-lactam ring of carbapenems [6].

Currently, there is no standardized direct phenotypic method for the detection of *A. baumannii* carbapenemases in routine microbiological laboratories, although there are indirect methods that are based on the ability of some compounds to inhibit carbapenemases. For example, MBLs are susceptible *in vitro* to inhibition by EDTA, but phenotypic MBL detection using the E-test containing imipenem with or without EDTA is not reliable because there can be false positives [14]. OXA-type carbapenemases are usually susceptible to NaCl inhibition, but some do not hydrolyze oxacillin or cloxacillin [5]. In addition, NaCl-mediated *in vitro* inhibition of their activity is not always observed, and moreover, OXA-positive clinical isolates often express additional non-OXA-type carbapenemases. PCR-based methods remain the optimal tool for the identification of OXA-type carbapenemases, but the main disadvantages of such technologies include cost, the requirement for trained personal, and the inability to detect novel carbapenemase genes [15].

Thus, there is an urgent need for a rapid, sensitive, specific and inexpensive test for the detection of carbapenemase activity. The rapid detection of resistant strains is critical and will benefit patient care by hastening diagnoses, optimizing therapy with antibiotics and preventing outbreaks. Recently, it was demonstrated that the detection of carbapenemase activity in *Enterobacteriaceae* and *Pseudomonas aeruginosa* could be achieved through the detection of the ertapenem and meropenem molecules and their natural degradation products using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) [16,17]. Herein, we describe the development and successful application of a mass spectrometry profile generated by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) that utilized the imipenem antibiotic for the detection of carbapenem resistance in a large series of *A. baumannii* clinical isolates from France and Algeria.

Results

Identification of bacteria, antibiotic susceptibility testing and molecular characterization of carbapenemase encoding genes

All 106 isolates were identified as *A. baumannii* using MALDI-TOF MS, with score values above 2.2 for all strains. Results of antibiotic susceptibility testing showed that among the 106 *A. baumannii* strains, 63 were found to be resistant to imipenem (MICs >8 mg/L confirmed using Etest). All 63 strains were checked for the presence of carbapenemase encoding genes and results showed that 57 of them harbored a $bla_{\text{OXA-23}}$ gene (17 were isolated in Marseille and 40 in Algeria), 3 harbored a $bla_{\text{OXA-24}}$ gene (isolated in Algeria) and 3 a $bla_{\text{OXA-23}}$+$bla_{\text{OXA-24}}$ gene (isolated in Algeria) (Table 1).

Standardization and internal calibration of the Ultraflex I mass spectrometer with imipenem solution

Theoretical atomic masses of imipenem ($C_{12}H_{17}N_3O_4S$) and its natural metabolite ($C_{11}H_{17}N_3O_2S$) (Figure 1A) were calculated using ISIS Draw software and were at 299.35 g/mol and 255.35 g/mol, respectively. The three matrix described in methods were tested with 0.45% NaCl and then combined with imipenem. The matrix containing acetone with ethanol and TFA provided spectra with the most useful data, i.e., without additional background peaks at the imipenem and imipenem natural metabolite peaks positions. Therefore, this matrix was selected for all further tests. Firstly we established and standardize the mass spectrum of pure imipenem in order to calibrate the mass spectrometer to check for the presence and reproducibility of detection of both imipenem and its natural metabolite. The characteristic mass spectrum of pure imipenem consists of both a main peak at 300.0+/−0.2 m/z for imipenem (n = 200 experiments) and a weak peak at 254.0+/−0.1 m/z for the natural metabolite (n = 200 experiments) (Figure 1B). In order to standardize our assay we decide to include for each bacterial isolate tested a ratio calculation between area under curve of imipenem and its metabolite allowing a precise and reproducible internal control of the experiments. Finally, we have checked for autodegradation of imipenem to ensure that the compound was stable during the experiments and we show that the presence of imipenem was stable during 6 hours of incubation with a ratio

Table 1. Characterization of the 149 bacterial strains analyzed and data summary of imipenem hydrolysis assay utilizing MALDI-TOF MS.

	MALDI-TOF analysis			
Strain type: No. of isolates (Location of isolates [No.of isolates])	**Range of the ratio of the area of imipenem/metabolite based on the time of incubation**		**No. of isolates detected as carbapenemase producers (disappearance of the peak at 300 m/z or ratio between the peak for imipenem and its metabolite being <0.5) based on the time of incubation**	
	2 h	**4 h**	**2 h**	**4 h**
Carbapenem-resistant strains (70) (imipenem MIC >8 mg/L)			67	70
K. pneumoniae KPC: 1	<0.01	<0.01	1	1
K. pneumoniae NDM-1: 2	<0.01	<0.01	2	2
P. aeruginosa VIM: 2	<0.01	<0.01	2	2
P. aeruginosa IMP: 2	<0.01	<0.01	2	2
A.baumannii $bla_{\text{OXA23-like}}$: 57 (Marseille [17], Algeria [40])	<0.01–1.77	<0.01–0.23	54	57
A.baumannii $bla_{\text{OXA24-like}}$: 3 (Algeria)	<0.01	0.02–0.04	3	3
A.baumannii $bla_{\text{OXA23-like}}$+$bla_{\text{OXA24-like}}$: 3 (Algeria)	<0.01–0.48	<0.01–0.06	3	3
Carbapenem-susceptible strains (79) (imipenem MIC £2 mg/L)			0	0
K. pneumoniae ESBL: 31 (Algeria)	ND	0.64–14.84	0	0
K. pneumoniae non ESBL: 4 (Algeria)	ND	1.24–5.82	0	0
Escherichia coli ATCC 25922 (1)	1.64	1.17	0	0
A. baumannii: 43 (Marseille [1], Algeria [42])	0.61–12.86	0.97–4.96	0	0

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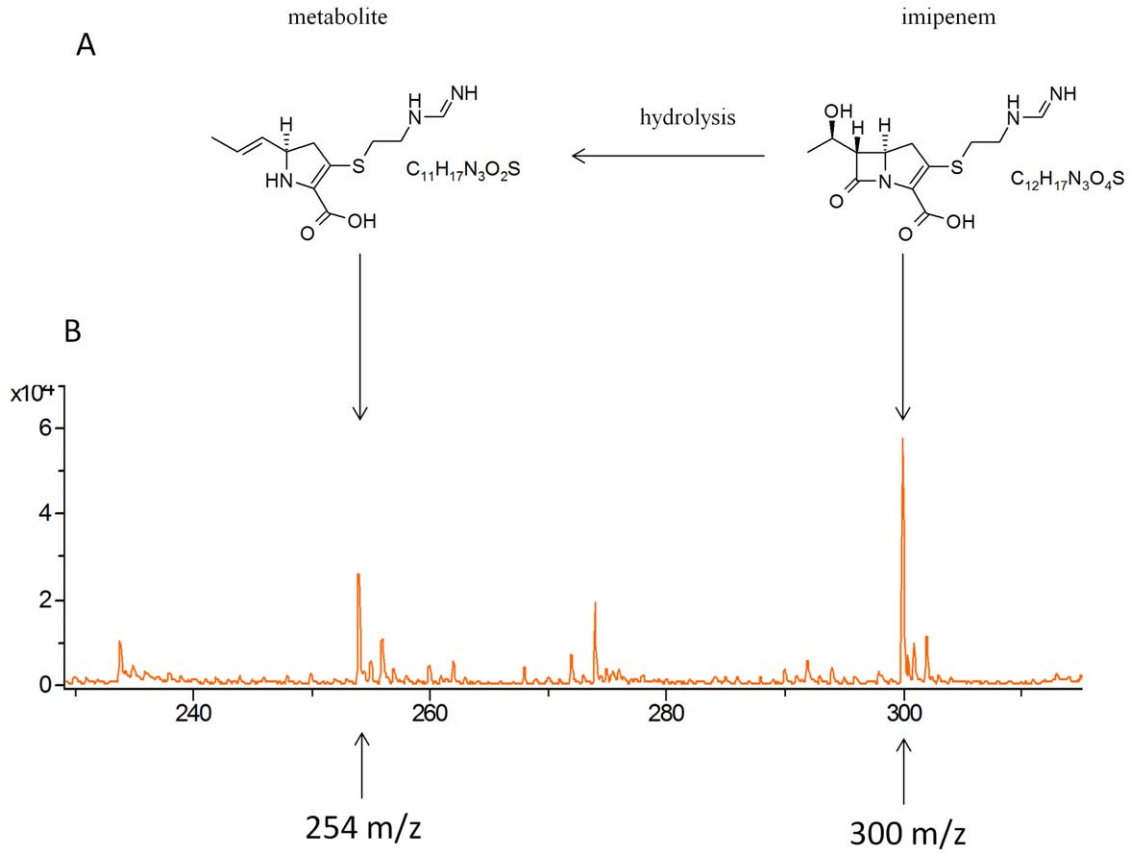

Figure 1. MALDI-TOF MS analysis of imipenem. (A) Imipenem and its natural degradation product. (B) Mass spectra of imipenem and its natural degradation product as determined using the Ultraflex mass spectrometer.
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between area under curve of imipenem and its metabolite being always >1 (Figure S1).

Imipenem hydrolysis assay

Kinetic studies of imipenem degradation were performed for the three carbapenem-resistant *K. pneumoniae* reference strains (one bla_{KPC} and two bla_{NDM-1}). These strains were incubated in the presence of imipenem at a concentration of 0.25 mg/mL for up to four hours, and the results indicate that the three strains completely degraded imipenem within 15 min. The peak at 300.0 m/z completely vanished whereas the peak at 254.0 m/z increased during the experiment. Experiments performed with the *E. coli* ATCC 25922 reference strain confirmed that this strain does not contain carbapenemase activity, as both the peak at 300.0 m/z and at 254.0 m/z were still present after 4 hours of incubation. Kinetic studies were also performed on eight *A. baumannii* strains (six strains resistant to imipenem and two strains sensitive to imipenem i.e strain SDF and strain AYE) at an imipenem concentration of 0.25 mg/mL and an incubation time of 2 hours. With these parameters, the peak at 300.0 m/z disappeared for each of the carbapenem-resistant strains (Figure 2) except one with an increase of the area under curve for the peak at 254.0 m/z. Interestingly, although the carbapenem-resistant strain for which the peak at 300.0 m/z did not completely disappear after 2 hours, the ratio of the area of the two peaks at 2 hours was <0.5, and the peak disappeared using 4 hours of incubation. Finally, for the two carbapenem-sensitive strains, the peak at 300.0 m/z was consistently present during the test both after 2 hours and 4 hours of incubation (Figure 3), and the ratio of the area of imipenem/metabolite was >0.5, confirming that these strains do not contain carbapenemase activity.

The imipenem hydrolysis assay was performed blindly twice on the 149 strains listed in Table 1 using the two criteria listed above, i.e., the disappearance of the peak at 300.0 m/z along with an increase of the peak at 254.0 m/z leading to a ratio of the area between the two peaks <0.5, to identify carbapenemase activity. These strains included 63 carbapenem-resistant *A. baumannii* strains containing either the $bla_{OXA23\text{-}like}$ and/or $bla_{OXA24\text{-}like}$ carbapenemase-encoding genes (46 from Algeria and 17 from Marseille, France), 43 carbapenem-sensitive *A. baumannii* strains (42 from Algeria and one from Marseille, France), 35 carbapenem-sensitive *K. pneumoniae* strains (31 ESBL producers with CTX-M15-, TEM- and SHV-encoding genes and four non-ESBL producers), and the eight control strains listed above (Table 1). Results showed that after 2 hours of incubation time, a carbapenemase activity was observed for all the carbapenem-resistant control strains tested, with disappearance of the peak at 300 m/z and a ratio of the area of imipenem/metabolite <0.5. Concerning the 63 carbapenem-resistant *A. baumannii* strains disappearance of the peak at 300 m/z was observed after 2 hours of incubation for 60 strains and was achieved for all strains after 4 hours of incubation (see additional examples in Figure S2). Concerning the carbapenem-sensitive controls, no carbapenemase activity was detected after 2 or 4 hours of incubation (presence of the peak at 300 m/z and ratio of the area of imipenem/metabolite >0.5). Similarly, the

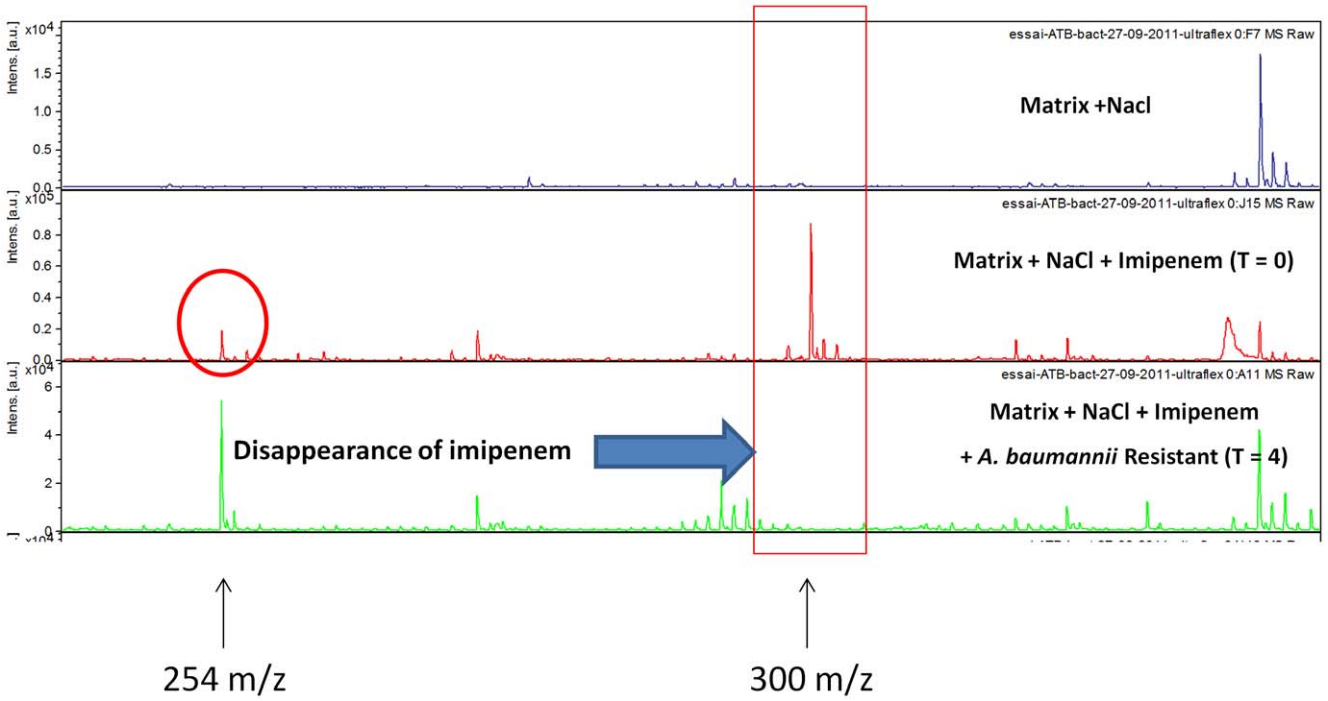

Figure 2. Mass spectra of the imipenem hydrolysis assay with a carbapenem-resistant *A. baumannii* strain. Incubation at 37°C during 4 h; NaCl 0.45%; imipenem concentration 0.25 mg/mL. Units of the *x* axis represent the mass per charge in Daltons [m/z (Da)] and that of the *y* axes, the relative intensity (a.u., arbitrary units).
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43 carbapenem-sensitive *A. baumannii* strains showed the presence of the peak at 300 m/z (see additional examples in Figure S2) and a ratio of the area of imipenem/metabolite >0.5 whatever the time of incubation tested. Thus in all experiments, disappearance of the peak at 300 m/z was correlated with a ratio <0.5 (Table S1). The overall sensitivity and specificity for the detection of

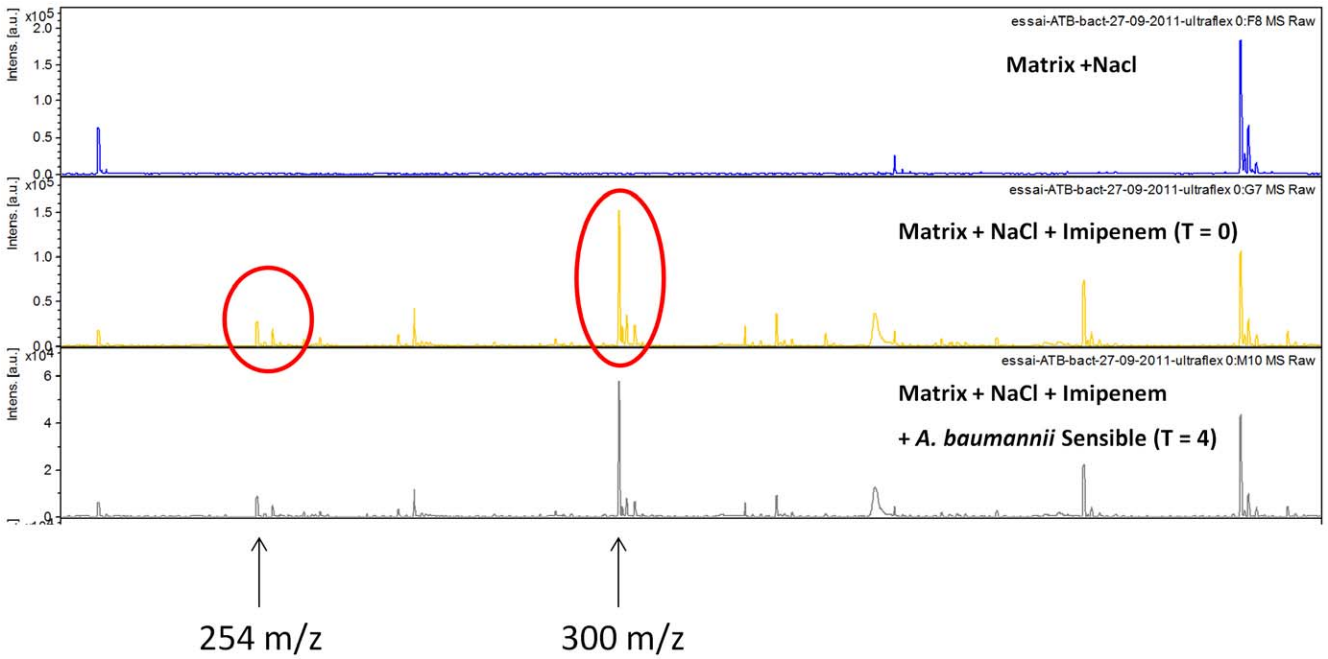

Figure 3. Mass spectra of the imipenem hydrolysis assay with a carbapenem-sensitive *A. baumannii* strain. Incubation at 37°C during 4 h; NaCl 0.45%; imipenem concentration 0.25 mg/mL Units of the *x* axis represent the mass per charge in Daltons [m/z (Da)] and that of the *y* axes, the relative intensity (a.u., arbitrary units).
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carbapenemase activity in these 106 *A. baumannii* strains were 95.2% and 100.0%, respectively after two hours of incubation with imipenem, and 100.0% and 100.0% respectively, after four hours of incubation. Thus, a concentration of 0.25 mg/L of imipenem and an incubation time of 4 hours were the optimal conditions.

Discussion

The MALDI-TOF MS technique has recently been introduced as a fast and reliable identification method that can be used for routine applications in diagnostic laboratories [18]. This rapid, simple, inexpensive and high-throughput proteomic system has been shown to be useful in bacteria and yeast identification [19]. This method has also commonly been used for proteomic research at the molecular level [20]. In our study, the MALDI-TOF MS system was used for the detection of carbapenemase activity in a large collection of *A. baumannii* strains isolated in France and Algeria. The strains were characterized according to their resistance mechanisms using phenotypic tests, including antibiotic susceptibility testing on agar plates, and also using genotypic tests, including the search for oxacillinases- and Ambler class B β-lactamase-encoding genes.

The direct detection of carbapenemase activity using similar approaches involving MALDI-TOF was recently reported in two studies of *P. aeruginosa* and *Enterobacteriaceae* [16,17], but to our knowledge, this was the first time that the direct detection of carbapenemase activity was developed using imipenem as the carbapenem compound and including a large collection of *A. baumannii* clinical isolates and other bacterial strains (a total of 149 bacterial strains). Indeed, in these two previous studies, either ertapenem [16] or meropenem [16] was used to detect carbapenemase activity for 87 bacterial isolates (including 47 carbapenem-resistant strains) and 124 bacterial isolates (including 30 carbapenem-resistant strains), respectively. However, the MALDI-TOF assay used in the current study was different from that used in these two previous reports. First, in this study, we used an Ultraflex I mass spectrometer, which has greater resolution and greater sensitivity than the Microflex mass spectrometer that was used previously [16,17]. The Ultraflex is a more reliable instrument because it contains 2 tubes for flight with a total of 2.76 meters versus only one tube of 0.95 meters for the Microflex apparatus. Moreover, the Ultraflex provides improved resolution of peaks when the positive reflectron ion mode is used between m/z 0 and 1000 Da (Data Bruker Daltonics). Finally, the Ultraflex has increased sensitivity when AnchorChip target plates covered with a hydrophobic surface with a hydrophilic center are used, which also leads to a higher concentration of the molecules to be ionized. Second, before testing clinical isolates, we evaluated different matrices to optimize the detection of imipenem and to limit the noise due to the matrix itself. Our results indicated that the HCCA matrix diluted in a mixture of acetone, ethanol and TFA was ideal to visualize the imipenem mass spectra with the Ultraflex instrument, as the background noise was low for this condition. The peak of imipenem molecule can be visualized at 300.0+/−0.2 m/z, as well as the natural degradation product at 254.0+/−0.1 m/z. The presence of the peak of the metabolite at 254 m/z even in the absence of any bacterial colonies could be explained by the fact that there is a spontaneous degradation of imipenem in sodium chloride [21]. This was also observed by Burckhardt *et al* with ertapenem (476 Da) and its natural hydrolyzed and decarboxylated ertapenem metabolite (450 Da) [16]. In order to consider this low amount of metabolite we have standardized our assay with calculation of the ratio of area under curves between imipenem and its metabolite to take this phenomenon into account. These peaks were highly reproducible and corresponded to the masses of the analytes studied, which allowed for the confirmation of their identities. Third, the method used in this study (Ultraflex machine+AnchorChip target+HCCA matrix) had the benefit that only the native imipenem molecule and its metabolite were detected without the sodium salt variants that were found with the Microflex method [16,17]. Thus, using our method, a carbapenem-sensitive strain could easily be identified by the presence of the peak at 300.0 m/z after 4 hours, and a carbapenem-resistant strain could be identified by the disappearance of this peak after 4 hours of incubation. Interestingly, we observed the same phenomenon with meropenem. However, imipenem was preferable over meropenem for this assay because it has been shown to be more sensitive and specific for the phenotypic detection of carbapenem-resistant strains. In addition, in our hands, we observed an overlapping peak between the meropenem antibiotic at 384.1 m/z and the matrix at 380.0 m/z (data not shown).

The use of this MALDI-TOF carbapenemase detection assay for the evaluation of *A. baumannii* clinical isolates was easy and efficient and demonstrated both high sensitivity and specificity. Similar sensitivity and specificity results were reported regarding the use of meropenem for carbapenemase detection in *Enterobacteriaceae* and *P. aeruginosa*. [17]. Among the 43 carbapenem-susceptible and 63 carbapenem-resistant *A. baumannii* strains tested, neither false-positive nor false-negative result was observed. Interestingly, one of the three carbapenem-resistant clinical isolates of *A. baumannii* for which the peak at 300.0 m/z did not completely disappear after 2 hours but only after 4 hours of incubation was an isolate that had acquired resistance to colistin and that is believed to have a reduced fitness [22], likely explaining the delayed disappearance of this peak, which persisted up to 2 hours. Regarding the control strains, neither false-positive nor false-negative results were noted. For all of the tested carbapenem-sensitive strains, the imipenem peak was clearly distinguishable at 300.0 m/z regardless of the incubation time tested and the ratio of the area of imipenem/metabolite was strictly >0.5. For the carbapenem-resistant strains, the peak at 300.0 m/z disappeared for all strains in all cases and the ratio of the area of imipenem/metabolite was strictly <0.5.

Finally, this study found that the delayed degradation of imipenem varied according to carbapenemase type and not to the MIC or to the ability of the bla_{NDM-1} and bla_{KPC} carbapenemases from *K. pneumoniae* to hydrolyze imipenem faster (<30 min) than the $bla_{OXA-23-like}$- or $bla_{OXA-24-like}$ carbapenemases from *A. baumannii* (≤ 2.5 h). Our results corroborate those of Burckhardt *et al.*, who found that ertapenem was hydrolyzed in 1 h by the bla_{NDM-1} and bla_{KPC} carbapenemases and in 1.5 to 2.5 h by the bla_{IMP} and bla_{VIM} carbapenemases [16]. This difference in the time required for complete imipenem hydrolysis suggests that either the oxacillinases from *A. baumannii* have weaker carbapenemase activity/affinity for imipenem or that *A. baumannii* grows more slowly than *Enterobacteriaceae* species. The detection of carbapenem resistance using the MALDI-TOF MS method has many advantages over other techniques, such as PCR, because it can detect low-level carbapenemase activity at a low cost even when the causative enzyme is unknown. Therefore, this technique is suitable both for the rapid detection of resistance in clinical settings and for the discovery of new carbapenemases. However, one of the disadvantages of this method is that it can detect only enzymatic carbapenem resistance and not resistance due to efflux mechanisms or porin alterations in *A. baumannii* [16]. However, because this assay was able to detect specific peaks corresponding to the exact mass of the specific drug, we believe that all enzymatic

antibiotic resistance mechanisms could be rapidly detected using the method presented herein.

In conclusion, because the average turnaround time for this test was estimated to be 4 h, our study clearly demonstrates that this assay could be used in real-time for routine use in clinical microbiology laboratories as a point-of-care strategy to identify carbapenemase-producing *A. baumannii* strains, which would aid in the prevention of outbreaks and the spread of uncontrollable superbugs.

Materials and Methods

Bacterial strains and carbapenemase detection

The carbapenem-resistant and carbapenem-sensitive *A. baumannii* strains used in this study originated from the collection of the Department of Microbiology at the University Hospital of Marseille (France) and from Tlemcen, Setif, Sidi Bel Abbes, Oran and Tizi Ouzou (Algeria) (Table 1). Species were identified using the Bruker Daltonics Ultraflex MALDI-TOF MS method (Bremen, Germany), as previously described [23]. Susceptibility results for each *A. baumannii* strain were determined using the disc diffusion method. The MIC for imipenem was determined using the E-test method and was interpreted according to the guidelines recommended by the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM) (www.sfm-microbiologie.org/). For each of the *A. baumannii* strains, genes encoding the Ambler class B and D carbapenemases were identified by PCR with primers specific for the bla_{IMP}, bla_{VIM}, bla_{NDM}, $bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}24\text{-}like}$, $bla_{OXA\text{-}51\text{-}like}$ and $bla_{OXA\text{-}58\text{-}like}$ genes as previously described [24]. Four carbapenem-resistant *P. aeruginosa* strains (two bla_{VIM} and two bla_{IMP}), three carbapenem-resistant *Klebsiella pneumoniae* strains (one bla_{KPC} and two $bla_{NDM\text{-}1}$), one carbapenem-susceptible *E. coli* reference strain ATCC 25922, and 35 carbapenem-sensitive *K. pneumoniae* strains (31 ESBL producers and four non-ESBL producers) were used as controls (Table 1). For kinetic studies, the carbapenem-sensitive *A. baumannii* reference strains AYE and SDF [25] were used as well as six well characterized carbapenem-resistant strains.

MALDI-TOF MS analysis of imipenem

Commercially available imipenem that contains cilastatin (Tienam, 500 mg, MSD, Paris, France) was diluted in 0.45% NaCl. The MALDI-TOF analysis was performed using the spectra of low molecular masses ranging from 0 to 1000 Da. Due to the background peaks from the low molecular masses of the matrix, three organic matrices were tested: 1) 10 mg/ml α-cyano-4-hydroxycinnamic acid (HCCA); 2) 2,5-dihydroxybenzoic acid (DHB) diluted in acetonitrile and water (1/1) and 3) 3.3 mg/ml of α-cyano-4-hydroxycinnamic acid (HCCA) diluted in a mixture of acetone, ethanol and TFA (1/2) (all reagents were obtained from Sigma-Aldrich, Lyon, France).

One microliter of the matrix solution was mixed with one microliter of the sample, which was applied onto a target (Bruker Daltonics GmbH, Bremen, Germany; MTP AnchorChip™ 384 T F Target) and allowed to dry at room temperature. Mass spectra were acquired using an Ultraflex I mass spectrometer and the flexControl 3.0 software (Bruker Daltonics GmbH) operating in positive reflection ion mode between m/z 0 and 1000 Da. The parameters were set as follows: ion source 1: 25 kV; ion source 2: 21.5 kV; lens: 10 kV; reflector 1: 25.5 kV; reflector 2: 14.19 kV; pulsed ion extraction: 10 ns; and detection gain: 9.1×. A total of 500 shots were acquired in 5 different positions for one spectrum.

Standardization and internal calibration of the Ultraflex I mass spectrometer

The imipenem concentrations that were tested for calibration of the mass spectrometer ranged from 0.25 mg/mL to 2 mg/mL. For internal calibration of the Ultraflex mass spectrometer, we established the mass spectrum of pure imipenem that also contains a natural degradation product during time as demonstrated by Swanson *et al* [21]. The theoretical peaks of imipenem and its natural degradation product were calculated using ISIS Draw software and were used for internal calibration of the Ultraflex apparatus in a set of 200 independent experiments. Once these peaks were determined, they were used as internal calibration controls for the imipenem hydrolysis assay. Finally, stability of imipenem molecule was checked in a 6 hours incubation assay.

Imipenem hydrolysis assay

Cultures of the *A. baumannii* strains and the controls were incubated overnight on blood agar plates (bioMérieux, Lyon, France) at 37°C. Then, a 10-µl loop-sized amount of bacteria was added to 1 mL 0.45% NaCl, as previously described by Burckhardt *et al.* [16], with or without imipenem at concentrations ranging from 0.25 mg/mL to 2 mg/mL, and the cultures were incubated for up to 4 h at 37°C. The tubes were then centrifuged for 3 min at 12,000× g, and 1 µl of the clear supernatant was applied to each target spot, mixed with one microliter of matrix solution and left to dry at room temperature. After preparing and validating the analytical and technical aspects of the assay (incubation time, concentration to be used, reproducibility of the drug and metabolite peaks), all tests with clinical isolates were conducted blindly and in duplicate. Two spots of each clinical isolate were performed in all experiments.

Spectra analysis and interpretation of carbapenemase activity

For one spectrum, approximately 500 shots were totaled. The result was interpreted as positive for carbapenemase production if the specific peak for imipenem (300.0 m/z, see results section) disappeared completely during the incubation time and if the peak of the natural metabolite at 254.0 m/z (see results section) increased as measured by the area under the curves leading to a ratio between the peak for imipenem and its metabolite becoming <0.5.

Supporting Information

Figure S1 Mass spectra of pure imipenem showing the stability of the drug (presence of the specific peak at 300 m/z) during a 6 hours incubation time.
(EPS)

Figure S2 Mass spectra of 24 clinical isolates of *A. baumannii* obtained after 4 hours of incubation showing the disappearance of the peak at 300 m/z for resistant isolates (n = 15) and the persistence of this peak for susceptible isolates (n = 9). Strain numbers (S.) are those presented as * in Table S1.
(PDF)

Table S1 Area under curves (AUC) and ratio between imipenem peak and its metabolite for the 106 *Acinetobacter baumannii* clinical strains according to their location and phenotype of resistance to imipenem. R = resistant; S = susceptible. * = strains for which mass spectra at 300 m/z are provided in Figure S1.
(DOC)

Author Contributions

Conceived and designed the experiments: MD AT JMR. Performed the experiments: MK SB CF MB JMB EM. Analyzed the data: MK SB CF MB JMB JMR. Contributed reagents/materials/analysis tools: MK SB CF MB EM. Wrote the paper: MK SB CF JMB MD AT JMR. Developed the software for analysis of peaks: JMB CF SB MK.

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Article 3:

Biotyping of multidrug-resistant *Klebsiella pneumoniae* clinical isolates from France and Algeria using MALDI-TOF MS

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Biotyping of Multidrug-Resistant *Klebsiella pneumoniae* Clinical Isolates from France and Algeria Using MALDI-TOF MS

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Abstract

Background: *Klebsiella pneumoniae* is one of the most important pathogens responsible for nosocomial outbreaks worldwide. Epidemiological analyses are useful in determining the extent of an outbreak and in elucidating the sources and the spread of infections. The aim of this study was to investigate the epidemiological spread of *K. pneumoniae* strains using a MALDI-TOF MS approach.

Methods: Five hundred and thirty-five strains of *K. pneumoniae* were collected between January 2008 and March 2011 from hospitals in France and Algeria and were identified using MALDI-TOF. Antibiotic resistance patterns were investigated. Clinical and epidemiological data were recorded in an Excel file, including clustering obtained from the MSP dendrogram, and were analyzed using PASW Statistics software.

Results: Antibiotic susceptibility and phenotypic tests of the 535 isolates showed the presence of six resistance profiles distributed unequally between the two countries. The MSP dendrogram revealed five distinct clusters according to an arbitrary cut-off at the distance level of 500. Data mining analysis of the five clusters showed that *K. pneumoniae* strains isolated in Algerian hospitals were significantly associated with respiratory infections and the ESBL phenotype, whereas those from French hospitals were significantly associated with urinary tract infections and the wild-type phenotype.

Conclusions: MALDI-TOF was found to be a promising tool to identify and differentiate between *K. pneumoniae* strains according to their phenotypic properties and their epidemiological distribution. This is the first time that MALDI-TOF has been used as a rapid tool for typing *K. pneumoniae* clinical isolates.

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Introduction

Klebsiella pneumoniae are ubiquitous in nature and have two common habitats; one is the environment, including surface water, sewage, soils and plants [1], and the other is mammalian mucosal surfaces [2]. In humans, *K. pneumoniae* can be present in the intestinal tract, nasopharynx, and on the skin [3]. It is one of the most common Gram-negative bacteria encountered by clinicians worldwide as a cause of infections in humans [4] and is responsible for outbreaks due to the propagation of different clones associated with opportunistic infections in individuals with impaired immune defenses, such as diabetics, alcoholics and hospitalized patients with indwelling devices [3]. In the hospital environment, the principal reservoirs for *K. pneumoniae* transmission are blood products, contaminated medical equipment, the gastrointestinal and respiratory tracts of patients and the hands of hospital personnel [5]. The hospital-acquired infections caused by this organism mainly include pneumonia, septicemia, urinary tract infections and soft tissue infections [6].

Increased *K. pneumoniae* infections are also associated with an increase in multidrug-resistant (MDR) strains, especially those producing extended-spectrum beta-lactamases (ESBLs) [7] associated with the prior use of antibiotics, particularly the cephalosporins [8]. Furthermore, several carbapenemase-encoding genes have been described in *K. pneumoniae* species, including class A beta-lactamase KPC, class B beta-lactamases NDM, IMP and VIM, and class D beta-lactamase OXA-48 [9]. The hospital epidemiology of these infections is often complex because multiple clonal strains causing focal outbreaks may co-exist with sporadic strains that also have a reservoir in the community [10]. Infections caused by multidrug-resistant *K. pneumoniae* strains have been associated

Table 1. Origin and repartition of strains used in this study.

Hospital		Department		Sex/Source		Age	Period study	Sample origin	
Annaba	18	Intensive care unit	18	Female	8	22 to 62 years	From March 2009	Tracheal aspirate	12
				Male	10	24 to 79 years	to October 2010	Urine	4
								Blood culture	2
Sidi Bel Abbes	28	Intensive care unit	14	Female	15	11 to 66 years	From October 2009	Tracheal aspirate	9
		Surgery	7	Male	11	7 to 75 years	to February 2011	Urine	3
		Internal medicine	2	Environment	2			Environment	2
		Nephrology	3					Bedsore	2
		Trauma	1					Pus	11
		Emergency	1					Profound swab	1
Tlemcen	72	Intensive care unit	48	Female	21	24 to 70 years	From August 2008	Tracheal aspirate	28
		Surgery	5	Male	40	7 to 74 years	to January 2011	Urine	3
		Gynecology	1	Environment	11			Environment	11
		Neurology	2					Pus	11
		Pediatrics	6					Rectal swab	18
		Trauma	10					Profound swab	1
Oran	93	Intensive care unit	60	Female	36	1 day to 69 years	From April 2008	Tracheal aspirate	52
		Surgery	8	Male	52	4 to 73 years	to March 2011	Urine	13
		Neurology	10	Environment	5			Environment	5
		Pediatrics	9					Pus	13
		Trauma	6					Rectal swab	9
								Vaginal swab	1
Angers	100	Infectious diseases	100	Female	56	22 to 97 years	From January 2008	Tracheal aspirate	3
				Male	44	1 day to 97 years	to March 2011	Catheter	3
								Urine	58
								Blood culture	18
								Pus	3
								Rectal swab	9
								Vaginal swab	6
Marseille	170	Cardiology	7	Female	94	6 month to 94 years	From January 2009	Tracheal aspirate	9
		Surgery	20	Male	76	1 day to 86 years	to Jun 2009	Catheter	1
		Gastroenterology	14					Urine	108
		Geriatric medicine	3					Bedsore	1
		Gynecology	6					Blood culture	21
		Infectious diseases	6					Pus	6
		Internal medicine	14					Profound swab	6
		Nephrology	11					Subcutaneous swab	1
		Neurology	9					ND	17
		Pediatrics	7						
		Intensive care unit	32						
		Trauma	4						
		Emergency	37						
Nice	54	Infectious diseases	54	Female	21	1 day to 88 years	From January 2010	Blood culture	26
				Male	33	1 day to 88 years	to October 2010	Pus	28

Origin and repartition of 535 *Klebsiella pneumoniae* strains isolated between January 2008 and March 2011.
doi:10.1371/journal.pone.0061428.t001

with adverse clinical outcomes, including increased mortality, prolonged hospital stays and increased economic costs [11].

Therefore, epidemiological typing is useful in determining the extent of an outbreak and in investigating the sources, the reservoir and the spread of bacterial infections. Various methods, including protein profiling by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and DNA profiling by multilocus sequence typing (MLST), restriction fragment length polymorphism (RFLP) and pulsed field gel electrophoresis (PFGE), have been used for the epidemiological typing of *K. pneumoniae* isolates [12–14]. However, most of these methodologies are time consuming, laborious, require special skills and are unsuitable for use in routine clinical laboratories [15,16]. In recent years, several reports have shown the feasibility of using matrix-assisted laser desorption ionization (MALDI)-time of flight (TOF) mass spectrometry (MS) to rapidly identify microorganisms [17]. There are only a few studies that have evaluated this method as a rapid tool to classify bacterial species at the strain level [18,19]. However, there are some recent examples of the use of MALDI-TOF MS for the rapid identification and typing of a limited number of clinical strains, such as *Streptococcus pyogenes* [20] or *Klebsiella pneumoniae* [19].

Here, we report the evaluation of MALDI-TOF MS as a rapid and powerful tool for determining the epidemiological distribution of a large series of *K. pneumoniae* clinical strains of different origins from patients with various infectious syndromes and the correlation between the pathotypes, geographic locations and clonalities of these strains using MALDI-TOF MS and data-mining approaches.

Results

Clinical Data

The mean age of the infected patients was 53 years and was similar when comparing patients hospitalized in Algerian hospitals (53 years: range 12 days to 84 years) and those from French hospitals (53.1 years: range 1 day to 97 years). The male/female

Table 2. Antibiotic susceptibility testing results of *Klebsiella pneumoniae* strains.

	K. pneumoniae strains of Algeria (n = 211)						*K. pneumoniae* strains of France (n = 324)						*p* values*	Total of *K. pneumoniae* strains (n = 535)					
	S	%	I	%	R	%	S	%	I	%	R	%		S	%	I	%	R	%
AM	0	0,0	0	0,0	211	100,0	0	0,0	0	0,0	324	100,0	–	0	0,0	0	0,0	535	100,0
AMX	0	0,0	0	0,0	211	100,0	0	0,0	0	0,0	324	100,0	–	0	0,0	0	0,0	535	100,0
AMC	27	12,8	75	35,5	109	51,6	196	67,6	40	13,8	54	18,6	<0.0001	223	44,5	115	22,9	163	32,5
TIC	0	0,0	0	0,0	211	100,0	0	0,0	0	0,0	324	100,0	–	0	0,0	0	0,0	535	100,0
CF	19	9,0	0	0,0	192	91,0	228	70,4	2	0,6	94	29,0	<0.0001	247	46,2	2	0,4	286	53,4
FOX	182	86,2	25	11,8	4	1,9	259	97,0	3	1,1	5	1,9	–	441	92,2	28	5,8	9	1,9
CTX	24	11,4	0	0,0	187	88,6	238	73,4	4	1,2	82	25,3	<0.0001	262	49,0	4	0,7	269	50,3
CAZ	23	10,9	12	5,7	176	83,4	238	73,4	50	15,4	36	11,1	<0.0001	261	48,8	62	11,6	212	39,6
CRO	24	11,4	0	0,0	187	88,6	238	73,4	4	1,2	82	25,3	<0.0001	262	49,0	4	0,7	269	50,3
IMP	211	100,0	0	0,0	0	0,0	324	100,0	0	0,0	0	0,0	–	535	100,0	0	0,0	0	0,0
AN	143	67,8	8	3,8	60	28,4	199	78,3	44	17,3	11	4,3	<0.0001	342	73,5	52	11,2	71	15,3
GN	26	12,3	3	1,4	182	86,2	243	78,9	1	0,3	64	20,8	<0.0001	269	51,8	4	0,8	246	47,4
TM	21	9,9	15	7,1	175	82,9	62	68,9	0	0,0	28	31,1	<0.0001	83	27,6	15	5,0	203	67,4
CIP	60	28,4	8	3,8	143	67,8	224	72,7	2	0,6	81	26,4	<0.0001	284	54,8	10	1,9	224	43,2
CS	211	100,0	0	0,0	0	0,0	324	100,0	0	0,0	0	0,0	–	535	100,0	0	0,0	0	0,0
SXT	57	27,0	7	3,3	147	69,7	223	73,1	2	0,6	80	26,2	<0.0001	280	54,3	9	1,7	227	44,0

*the *p* values compare the percentage of resistance and sensitivity between Algerian and French strains,
S: Sensitive, **I** : Intermediate, **R** : Resistant, **AM**: Ampicillin, **AMX**: Amoxicillin, **AMC**: Amoxicillin/clavulanic acid, **TIC**: Ticarcillin, **CF**: Cefalotin, **FOX**: Cefoxitin, **CAZ**: Ceftazidime, **CTX**: Cefotaxime, **CRO**: Ceftriaxone, **IMP**: Imipenem, **GN**: Gentamicin, **AN**: Amikacin, **TM**: Tobramycin, **CIP**: Ciprofloxacin, **SXT**: Trimethoprim/Sulfamethoxazole, **CS**: Colistin.
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ratio was 1/1. Among the 535 isolates, 172 (32.1%) were retrieved from intensive care units (ICUs), 160 (29.9%) from infectious diseases wards, 40 (7.5%) from surgical wards, 38 (7.1%) from emergency wards, 22 (4.1%) from pediatric wards, 21 (3.9%) from trauma wards, 21 (3.9%) from neurology wards, 16 (3.0%) from internal medicine wards, 14 (2.6%) from nephrology wards, 14 (2.6%) from gastroenterology wards, and 17 (3.2%) from gynecology, cardiology, or geriatric medicine wards. *K. pneumoniae* strains were isolated from various clinical samples that originated as follows: 189 (35.3%) from urine, 113 (21.1%) from tracheal aspirate, 73 (13.6%) from pus, 67 (12.5%) from blood culture, 36 (6.7%) from rectal swab and 57 (10.6%) from other different clinical specimens.

Bacterial Identification

Using MALDI-TOF MS, all *K. pneumoniae* strains (100%) were identified with score values >1.9 using the Bruker Biotyper software.

Antibacterial Susceptibility Testing of *Klebsiella pneumoniae* Isolates

As shown in Table 1, the overall resistances (i.e., resistant+intermediate percentage (R+I%)) to various antibiotics were as follows: ampicillin, amoxicillin and ticarcillin (100%), amoxicillin/clavulanic acid (55.5%), cefalotin (53.8%), cefoxitin (7.7%), ceftazidime (51.2%), cefotaxime and ceftriaxone (51.0%), gentamicin (48.2%), tobramycin (72.4%), amikacin (26.4%), ciprofloxacin (45.1%), and trimethoprim/sulfamethoxazole (45.7%). All isolates were sensitive to imipenem and colistin. The *K. pneumoniae* strains from Algeria had significantly higher percentages of resistance to antibiotics compared to those from France ($p<0.0001$). The prevalence of resistance to third generation cephalosporin in Algeria was 88.7% compared with France (26.5%).

Six resistance phenotypes were found based on susceptibility to beta-lactams (Table 2). These comprised 240 (44.8%) wild-type, 7 (1.3%) inhibitor-resistant TEM penicillinase, 11 (2.0%) high-level penicillinase, 3 (0.6%) cephalosporinase, 240 (44.8%) extended-spectrum beta-lactamase (ESBL), and 34 (6.3%) ESBL associated with cephalosporinase. The difference in antibiotic resistance rate was significant between Algerian and French strains ($p<0.0001$). Overall, the percentage of non-ESBL-producing strains was higher in France (73.1%) than in Algeria (11.3%), while that of ESBL-producing strains was higher in Algeria (88.6%) compared with France (26.8%).

Data Mining Analysis of *Klebsiella pneumoniae* MSP Dendrogram

The MSP dendrogram revealed five distinct clusters according to an arbitrary cut-off at the distance level of 500 (Figure 1). Data mining analysis of the five clusters using PASW 17.0 software showed that *K. pneumoniae* strains isolated in Algerian hospitals (Tlemcen, Sidi Bel Abbes, Oran, Annaba) were significantly associated with respiratory tract infections and ESBL phenotype in the fifth cluster ($p<0.0001$), whereas *K. pneumoniae* strains isolated in Marseille hospitals were significantly associated with urinary tract infections and wild type phenotype in the first, the second and the fourth clusters ($p<0.0001$). *K. pneumoniae* strains isolated in Angers hospitals were associated with urinary tract infections and wild type phenotype in the fourth cluster ($p<0.0001$). Conversely, *K. pneumoniae* strains isolated in Nice hospital were associated with blood cultures and pus samples and wild type phenotype in the third cluster ($p<0.0001$) (Table 3). All the details of the distribution of *K. pneumoniae* strains into the five clusters according to the

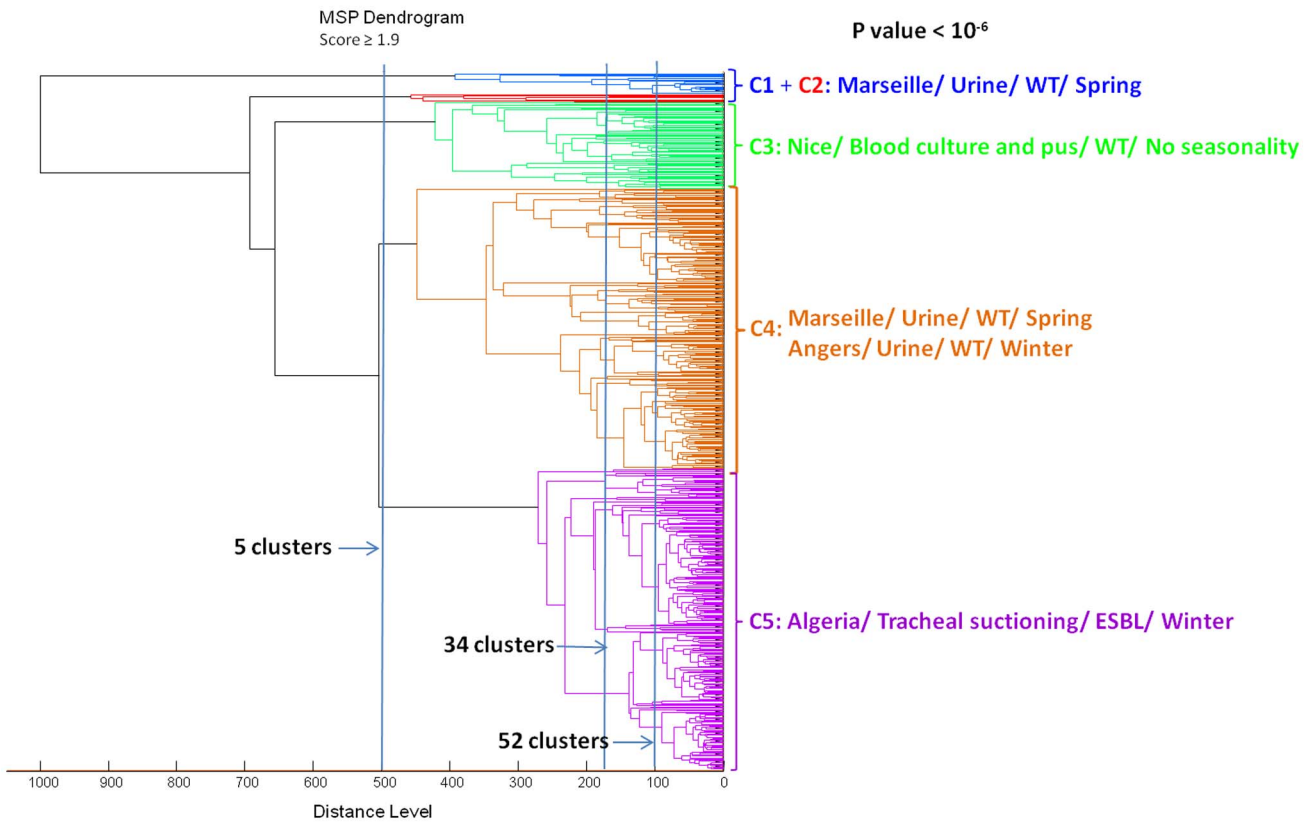

Figure 1. Geographic location of hospitals implicated in this study. 1: Tlemcen, 2: Sidi Bel Abbes, 3: Oran, 4: Annaba, 5: Marseille, 6: Nice, 7: Angers.
doi:10.1371/journal.pone.0061428.g001

dendrogram are given in supplementary Table S1. Interestingly, clustering the strains according to the arbitrary distance levels of 180 and 100 significantly clustered strains from the same hospital into the same cluster. At the distance level of 180, 34 distinct clusters were identified, and at the distance level of 100, 52 distinct clusters were identified (Table S2). For example, at the distance level of 100, one cluster containing 15 strains showed that all of them were from Marseille hospital, a second cluster contained three strains (all from Nice hospital), and a third cluster contained ten strains (eight out of ten were from Angers hospital ($p<0.0001$)).

The monthly distribution of *K. pneumoniae* strains for the January 2008 - March 2011 period was determined by PASW 17.0 software according to clusters. Strains from Angers and Algerian hospitals were significantly correlated with winter (January-March) ($p<0.0001$), whereas, strains from Marseille hospital were associated with spring (April-June) ($p<0.0001$). No seasonal variation was associated with strains from Nice hospital.

Table 3. Antibacterial resistance phenotypes of *Klebsiella pneumoniae* strains.

	Tlemcen (%)	Sidi Bel Abbes (%)	Oran (%)	Annaba (%)	Total Algeria (%)	Angers (%)	Nice (%)	Marseille (%)	Total France (%)	Total (%)
ESBL	58 (80,5)	21 (75,0)	62 (66,7)	18 (100,0)	159 (75,3)	24 (24,0)	7 (13,0)	50 (29,4)	81 (25,0)	240 (44,8)
ESBL+Case	9 (12,5)	3 (10,7)	16 (17,2)	0 (0,0)	28 (13,3)	6 (6,0)	0 (0,0)	0 (0,0)	6 (1,8)	34 (6,3)
Case	1 (1,4)	0 (0,0)	0 (0,0)	0 (0,0)	1 (0,5)	2 (2,0)	0 (0,0)	0 (0,0)	2 (0,6)	3 (0,6)
Pase High Level	1 (1,4)	1 (3,6)	2 (2,1)	0 (0,0)	4 (1,9)	0 (0,0)	7 (13,0)	0 (0,0)	7 (2,2)	11 (2,0)
Pase IRT	0 (0,0)	2 (7,1)	0 (0,0)	0 (0,0)	2 (0,9)	2 (2,0)	1 (1,8)	2 (1,2)	5 (1,5)	7 (1,3)
Wild Type	3 (4.2)	1 (3,6)	13 (14,0)	0 (0,0)	17 (8,0)	66 (66,0)	39 (72,2)	118 (69,4)	223 (68,8)	240 (44,8)
Total	72	28	93	18	211	100	54	170	324	535

ESBL: Extended-spectrum beta-lactamase, Case: Cephalosporinase, ESBL+Case: Extended-spectrum beta-lactamase associated to Cephalosporinase phenotype, Pase: Penicillinase, Pase IRT: inhibitor-resistant TEM penicillinase.
doi:10.1371/journal.pone.0061428.t003

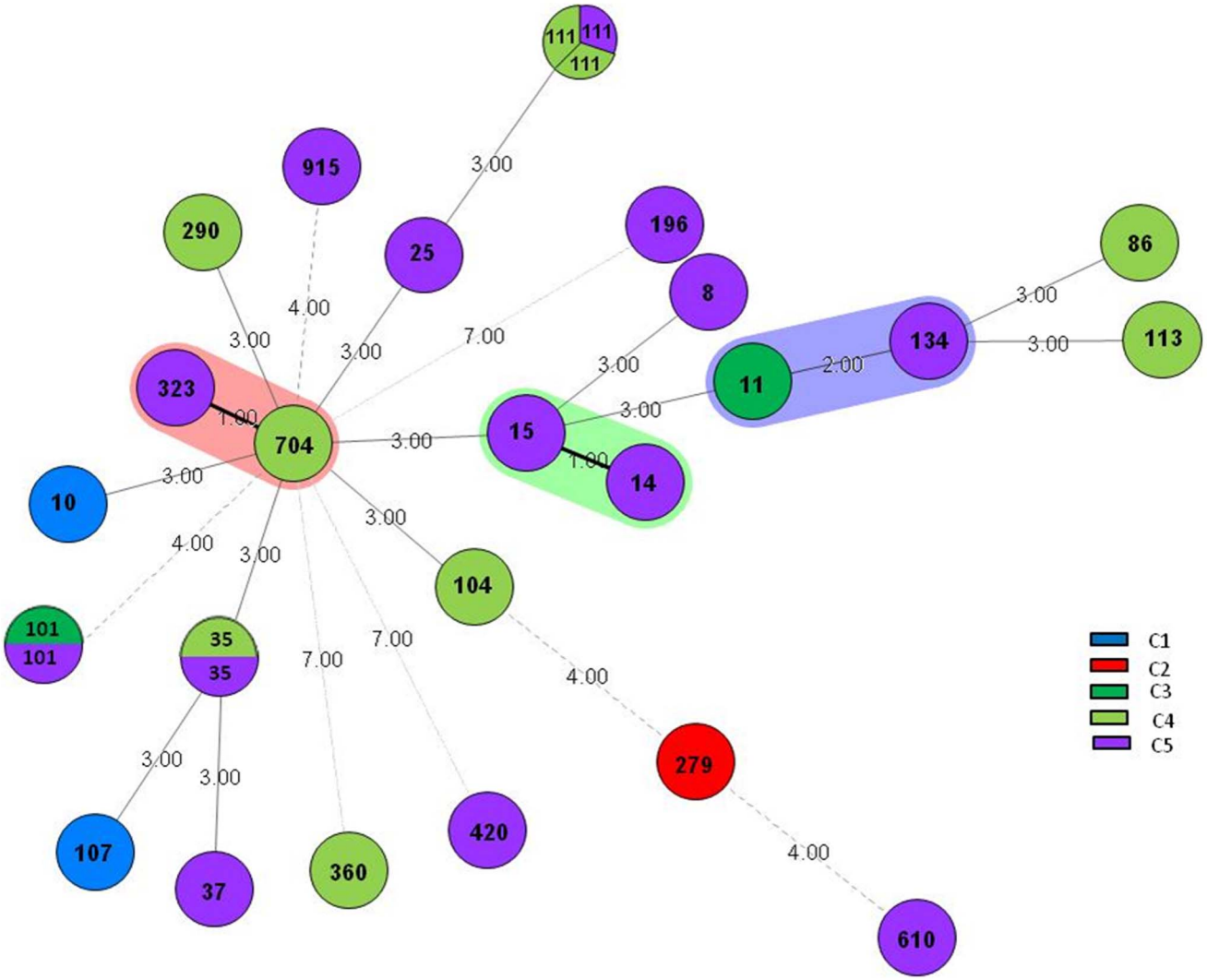

Figure 2. Mean spectra projection (MSP) dendrogram of *Klebsiella pneumoniae* strains generated by BIOTYPER software (version 2; Bruker Daltonics). ESBL: Extended-spectrum beta-lactamase, WT: wild type.
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Table 4. Data mining analysis of *Klebsiella pneumoniae* MSP dendrogram.

Clusters (No)		Geographic location (No)		Type of samples (No)		Antibiotic resistance Phenotype (No)		Association between the antibiotic resistance phenotype and type of samples (No)		Seasonality (No)	
C1+ C2	23	Marseille	22	Urine	14	WT	19	Urine and WT	13	Spring	22
C3	65	Nice	47	Blood culture Pus	22 25	WT	35	Blood culture and WT Urine and WT	17 18	Spring Summer Autumn Winter	10 17 14 6
C4	215	Marseille	117	Urine	74	WT	79	Urine and WT	56	Spring	62
		Angers	54	Urine	33	WT	34	Urine and WT	19	Winter	36
C5	232	Algeria	166	Tracheal aspirate	82	ESBL	149	Tracheal aspirate and ESBL	73	Winter	70

WT: wild type phenotype, ESBL: Extended Spectrum Beta-lactamase**.** No represents the number of strains that correspond to the significant character according to data mining analysis.
doi:10.1371/journal.pone.0061428.t004

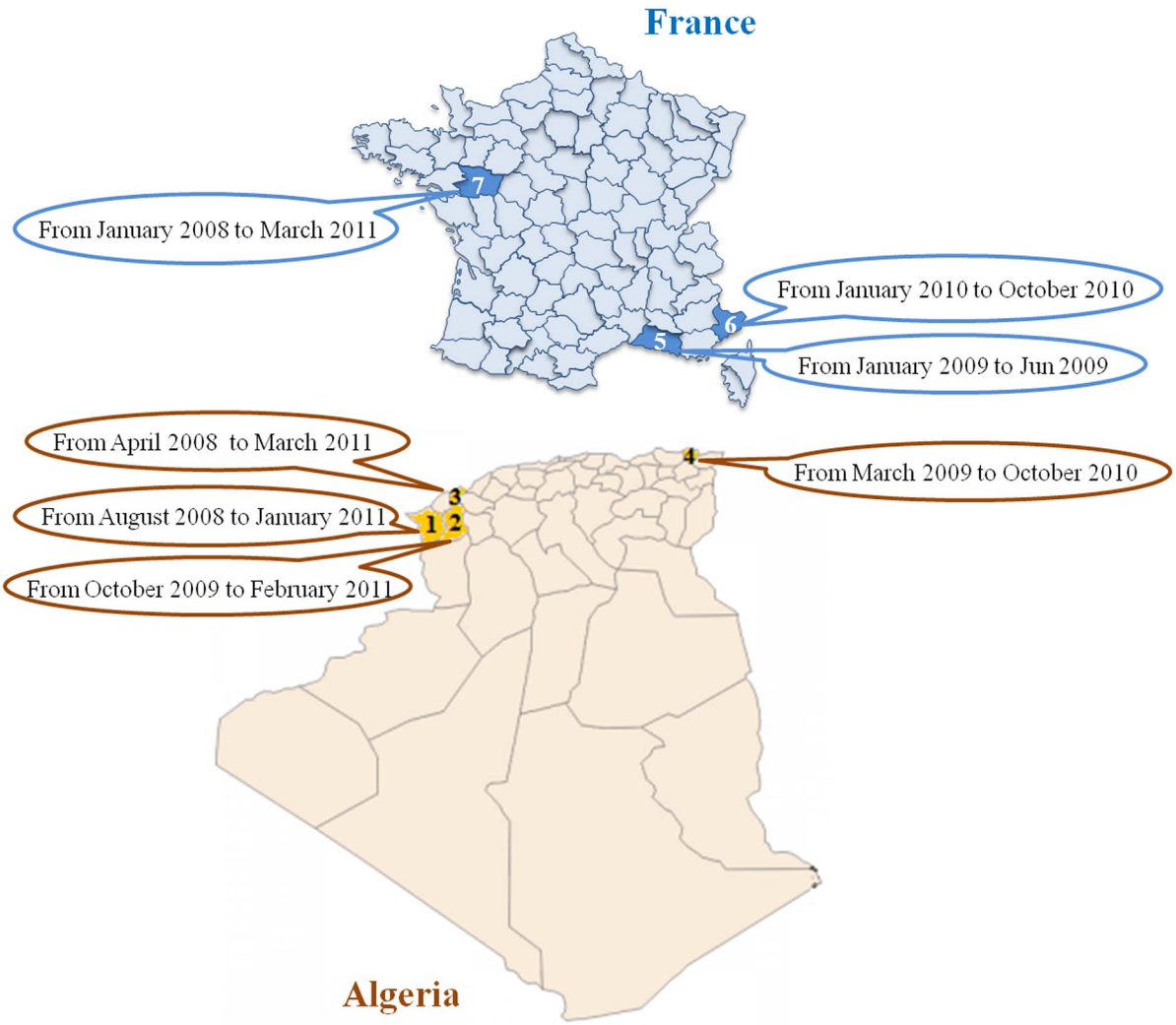

Figure 3. Minimal spanning tree (MST) of *K. pneumoniae* isolates, showing relationships between STs, compared with clusters obtained from the dendrogram generated by BIOTYPER software.
doi:10.1371/journal.pone.0061428.g003

Multilocus Sequence Typing

The MLST allelic profile of all strains distributed in the five clusters is presented in Table 4. From the 28 tested strains, we found 24 different sequence types (ST) (Figure 2). No relationship was observed between ST and the clinical and epidemiological data.

Discussion

K. pneumoniae is an important pathogen with a complex pan-genome responsible for serious nosocomial infections, especially in intensive care units (ICUs) and in wards for surgery, emergency, neurology, pediatrics, and neonatology [5]. More concerning has been the emergence and increase in the isolation rate of ESBL-producing *K. pneumoniae* worldwide [4], which frequently possess resistance factors to other classes of antibiotics, notably aminoglycosides, fluoroquinolones and trimethoprim/sulfamethoxazole [21]. In our study, we confirmed that there is an increase in the number of multidrug-resistant *K. pneumoniae* strains with a considerable variation between the two countries studied. The antibiotic resistance rate in France (26.8%) was lower than that in Algeria (88.6%). In comparing our results with those of the Mystic study (1997–2003) [7] and another surveillance trial study (2004–2009) [22], we notice a worldwide north-south gradient evolution of ESBL production rate in *K. pneumoniae* strains with 12.3–12.8% in North America, 16.7% in Northern Europe, 24.4% in Southern Europe, 33.8% in the Middle East, 28.2–35.6% in Asia-Pacific, 45.5–51.9% in South America and 54.9% in Africa.

The high infection occurrence of *K. pneumoniae*, both ESBL-producing and non-producing strains, in Algerian and French hospitals pushed us to examine the epidemiology of these strains, which are considered to have a complex pan-genome containing plastic genome repertoires that differentiate strains according to their geographical locations, pathotypes, ecotypes and resistance phenotypes [23]. MALDI-TOF MS was successfully used as a tool for biotyping because we found specific clusters that were significantly associated with particular phenotypes from different clinical and geographical sources and from different seasons. This result is seemingly supported by Trevino et al., with a series of only 13 clinical isolates of *K. pneumoniae* [24]. By increasing the number of strains collected, our dendrogram became more refined, not only by country but also by hospital. This result could be of clinical importance for unknown pathogenic isolates, whose geographical sources could be detected rapidly using MALDI-TOF MS.

Table 5. MLST allelic profile of *Klebsiella pneumoniae* clinical isolates distributed in the five clusters.

Clusters	Isolates	Hospitals	Sample origin	Antibiotic resistance phenotype	gapA	infB	mdh	pgi	phoE	rpoB	tonB	ST
C1	KpM 44	Marseille	Urine	WT	2	1	2	17	27	1	39	**ST107**
	KpM 71	Marseille	Pus	WT	4	1	7	1	9	1	12	**ST10**
C2	KpnA1211	Annaba	Tracheal aspirate	ESBL	3	3	1	1	1	1	4	**ST11**
	KpM 29	Marseille	Blood culture	ESBL	2	6	1	5	4	1	6	**ST101**
C3	29 Kp	Nice	Pus	WT	2	2	1	47	1	4	43	**ST279**
	KpM 154	Marseille	Urine	WT	2	1	5	1	17	4	42	**ST111**
	KpnA 1323	Annaba	Tracheal aspirate	ESBL	2	1	1	1	9	1	20	**ST704**
	16 KP	Nice	Pus	WT	2	1	5	1	17	4	42	**ST111**
	16 KH	Nice	Blood culture	WT	14	1	2	1	21	1	23	**ST113**
	KpA4	Angers	Vaginal swab	WT	16	24	21	53	47	17	67	**ST360**
	KpA63	Angers	Tracheal aspirate	WT	9	4	2	1	1	1	27	**ST86**
	Kpn8	Oran	Rectal swab	Pase High Level	2	1	1	37	10	1	86	**ST290**
	14 KP	Nice	Pus	WT	4	5	1	29	1	4	42	**ST610**
	10 KP	Nice	Pus	ESBL	2	1	5	1	17	4	42	**ST111**
C4	Kpn9	Oran	Tracheal aspirate	WT	2	3	1	1	2	1	43	**ST104**
	KpM 166	Marseille	ND	ESBL	1	1	1	1	1	1	1	**ST15**
	KpM 161	Marseille	ND	ESBL	17	19	39	39	51	72	72	**ST196**
	KpA86	Angers	Urine	WT	3	1	2	1	1	1	4	**ST134**
	KpA75	Angers	Blood culture	WT	2	6	1	5	4	1	6	**ST101**
	Okp46	Oran	Tracheal aspirate	ESBL+Case	2	1	2	1	10	1	19	**ST35**
	Okp45	Oran	Tracheal aspirate	ESBL	1	6	1	1	1	1	1	**ST14**
	Kp98	Tlemcen	Rectal swab	ESBL	2	1	1	1	10	4	13	**ST25**
C5	KpM 170	Marseille	Blood culture	ESBL	2	1	2	1	10	1	19	**ST35**
	Kp90	Tlemcen	Pus	ESBL	2	10	13	1	12	1	186	**ST915**
	Skp25	Sidi Bel Abbes	Pus	ESBL	2	1	1	1	9	1	93	**ST323**
	Skp22	Sidi Bel Abbes	Bedsore	ESBL	2	1	65	2	5	1	36	**ST420**
	KpnA 932	Annaba	Tracheal aspirate	ESBL	2	9	2	1	13	1	16	**ST37**
	KpnA 576	Annaba	Urine	ESBL	4	1	1	1	1	5	6	**ST8**

ESBL: Extended-spectrum beta-lactamase, ESBL+Case: Extended-spectrum beta-lactamase associated to Cephalosporinase phenotype, Pase: Penicillinase phenotype, WT: Wild Type phenotype.

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It is recognized that the distribution of bacteria may be related to geographical patterns, such as climatic zones and movement of human populations, and can provide information about pathogen evolution and transmission [25,26]. The spatial distribution of bacterial pathogens can be considered at different levels; in a hospital setting, this may indicate nosocomial transmission, but in a community setting, the simultaneous appearance of an identical phenotype in widely dispersed locations may be a warning of an outbreak [15]. Our data also suggested that rates of *K. pneumoniae* infections varied seasonally and were significantly associated with periods of isolation that were due to changes in temperature and humidity. Several characteristics of this species that were previously described support our findings, which show that temperature and dew point were both linearly predictive of increasing rates of *K. pneumoniae* clinical isolates [27,28]. Furthermore, most of the MALDI-TOF MS spectra are composed of well-conserved proteins with housekeeping functions that are minimally affected by environmental conditions and thus are considered to be optimal for the proteomic typing of bacteria, which is considerably different from genetic typing [29,30].

By comparing the clusters obtained from the MSP dendrogram (Figure 1) with the ST distribution in the minimal spanning tree (MST) of *K. pneumoniae* strains (Figure 2), we noted that no correlation was observed for these two types of analysis. Moreover, no relationship was observed between ST, clinical and epidemiological data. These results suggest that the two approaches, comprising the MSP and MLST analyses, highlight two different bacterial aspects. Indeed, MLST analysis based on the conserved bacterial genes (the house-keeping genes) classifies bacteria according to their core genome, which represents less than 10% of the genome [31], while 90% of the genome is composed of ''accessory genes'' (mobile genetic elements) that can be lost or acquired by lateral gene transfer (LGT), and that is mainly responsible for the bacterial phenotype [23,32]. Therefore, this core genome does not represent the majority of expressed proteins, in contrast to the MSP dendrogram analysis, which is based on the functional and expressed proteins of whole cells that is more

representative of the global phenotype [33]. Therefore, these two approaches could be complementary to the extent that the MST approach, based on the analysis of the core genome, allows us to classify bacteria according to their conserved genes independent of their ''accessory genes,'' which can affect phenotypes and bacterial classification.

Many researchers have used molecular methods, particularly PFGE and MLST, to distinguish *K. pneumoniae* clinical isolates in order to understand transmission patterns and to aid in the management of these infections [34]. In 2008, a comparative study between these two methods found that PFGE is appropriate to discriminate among epidemiologically unrelated strains and appears more suitable for short-term epidemiology, while MLST is appropriate for strain phylogeny and large-scale epidemiology [35]. Furthermore, these molecular methods are costly and time-consuming to obtain results and introduce delays when attempting to limit the spread of *K. pneumoniae* clones [36]. For instance, only a few hours are required to obtain results by MALDI-TOF MS, whereas several days are necessary to collect MLST data [37]. In addition, the cost of MALDI-TOF MS instrumentation is comparable to that of a sequencing machine, but running costs and consumables are considerably lower than for these methods [38]. Barbuddhe et al. have used MALDI-TOF MS to accurately identify different *Listeria* species and correctly classified all *L. monocytogenes* serotypes in agreement with PFGE [38], and recently Wang et al. identified and classified *Streptococcus pyogenes* into clusters with MALDI-TOF MS [20].

Thus, MALDI-TOF MS combined with a statistical classification strategy is appropriate for studies of local epidemiology and global population structure when compared to a local database from clinical strains. It is a powerful epidemiological method and is sufficiently reproducible and sensitive enough to rapidly survey the evolution of existing or emerging phenotypes with reduced financial and human costs [39].

Conclusion

We believe that our preliminary results should be expanded and confirmed with more strains obtained from different countries. To the best of our knowledge, this is the first study that analyzes the epidemiology of a large series of *K. pneumoniae* clinical isolates using MALDI-TOF MS application. We suggest the creation of a local database that is updated regularly to survey for the presence of abnormal phenotypes at the strain level. If a particular phenotype is detected, a real time genome sequencing approach could then be performed to investigate the origin and specific features of the strain. We believe that this methodology could be used routinely in clinical microbiology laboratories as a surveillance tool for hospital epidemiology studies to prevent outbreaks and dissemination of pathogens in hospital settings.

Materials and Methods

Bacterial Strains

A total of 535 non redundant clinical strains of *K. pneumoniae*, isolated from different clinical samples, were collected during a period of 39 weeks, between January 2008 and March 2011. All of these were collected from hospitals in France and Algeria (Figure 3): Marseille hospitals, France (n = 170 strains); Angers hospital, France (n = 100 strains); Nice hospital, France (n = 54 strains), Tlemcen hospital, Algeria (n = 73 strains); Oran hospital, Algeria (n = 92 strains); Sidi Bel Abbes hospital, Algeria (n = 28 strains) and Annaba hospital, Algeria (n = 18 strains). The clinical sources of the different strains are noted in Table 5.

Klebsiella pneumoniae Identification using MALDI-TOF MS

Isolates were plated on Trypticase Soy Agar (BioMerieux) and incubated for 24 h at 37°C. One single colony from each isolate was deposited on a MALDI-TOF MTP 384 target plate (Bruker Daltonics, Bremen, Germany) in four replicates to minimize random effects. Two microliters of matrix solution (saturated α-cyano-4-hydroxycinnamic acid, 50% acetonitrile, 2.5% trifluoroacetic acid) were then added and allowed to co-crystallize with the sample. Analysis was performed in a MALDI-TOF MS spectrometer (337 nm) (Autoflex; Bruker Daltonics) with FLEX control software (Bruker Daltonics). Ions were accelerated in the positive ion mode with an accelerating voltage of 20 kV. The pulsed extraction of ions was optimized for 1000 Da. The software employed, Bruker Biotyper 2.0 (Bruker Daltonics), automatically acquired spectra with fuzzy control of the laser intensity and analyzed them by standard pattern matching against the spectra of 2881 species used as reference data. After comparing the unknown spectra with all reference spectra in the database, the log scores were ranked. Values of >1.9 were required for secure identification at the species level, and values between 1.9 and 1.7 were required for secure identification at the genus level.

Clustering of MALDI-TOF Spectra

A consensus spectrum was produced, and an MSP dendrogram was constructed using the correlation distance measure with the average linkage algorithm setting of the Biotyper 2.0 software. Clusters were then detailed and analyzed according to arbitrary distance levels at 500, 180 and 100.

Antibiotic Susceptibility and Synergy Testing

Antibiotic susceptibility testing was performed using the disk diffusion method on Mueller-Hinton medium as per the guidelines of the French Society of Microbiology (www.sfm.asso.fr). *E. coli* ATCC 25922 was used as a quality control strain. The antimicrobial disks tested were as follows: ampicillin (10 μg), amoxicillin (25 μg), amoxicillin/clavulanic acid (20/10 μg), ticarcillin (75 μg), cefalotin (30 μg), cefoxitin (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg), imipenem (10 μg), gentamicin (15 μg), amikacin (30 μg), tobramycin (10 μg), ciprofloxacin (5 μg), trimethoprim/sulfamethoxazole (1,25/23,75 μg), and colistin (50 μg). ESBL production was detected using the method of combined antibiotic disks as previously described [40].

Antibiotic Resistance Phenotypic Classification of *K. pneumoniae* Strains based on Beta-Lactamine Compounds

We considered a wild type phenotype as a strain that confer resistance to aminopenicillins, carboxypenicillins and to ureidopenicillins. The high level penicillinase phenotype was presented by a high penicillinase activity responsible for resistance to aminopenicillins and their inhibitors, to carboxypenicillins, to ureidopenicillins, and to first generation cephalosporins (1 GC). The inhibitor-resistant TEM penicillinase phenotype included resistance to aminopenicillins, carboxypenicillins, and ureidopenicillins. It was distinguished by resistance to aminopenicillins and carbocxypenicillins associated to the beta-lactam inhibitors. 1 GC generally retain their activity. The cephalosporinase phenotype corresponded to a marked resistance to penicillins, 1 GC, 2 GC, and to at least one 3 GC. The extended spectrum B-lactamase phenotype includes resistance to penicillins and cephalosporins except cephamycins. The resistance to 3 GC and 4 GC was more or less pronounced depending on the enzymes and the strains.

Statistical Analysis

Clinical and epidemiological data were recorded in an Excel file (Microsoft, Redmond, WA, USA), including the clustering obtained using the MSP dendrogram generated by the Biotyper software (version 2; Bruker Daltonics), and were analyzed using PASW Statistics software version 17.0 (SPSS Inc., Chicago, IL, USA). Dependent variable series were analyzed using Expert Modeler, which automatically generates the best-fitting model. The chi-square analysis was used also to compare proportions using the same software, and P values <0.05 were considered to be statistically significant. Statistical analyses were conducted using Epi Info version 6 (Centers for Disease Control and Prevention, Atlanta, GA, USA).

Multilocus Sequence Typing

Randomly, 28 strains distributed in the five clusters were selected to perform the full MLST as described previously using seven housekeeping genes, including *gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB* and *tonB* [34]. The MLST database for *K. pneumoniae* can be found at http://www.pasteur.fr. Based on the allelic profiles, the evolutionary relationship between isolates was assessed by the minimal spanning tree (MST) algorithm in Bionumerics (Applied Maths, NV St-Martens-Latem, Belgium). A stringent definition of 6/7 shared alleles was used to define clonal complexes (single locus variants only).

Supporting Information

Table S1 Distribution of *Klebsiella pneumoniae* strains according to the dendrogram generated by BIOTYPER software (version 2, Bruker Daltonics) at the distance level of 500.
(DOCX)

Table S2 Comparison of the clusters obtained according to different cut-offs at 500, 180 and 100 of the dendrogram generated by BIOTYPER software (version 2, Bruker Daltonics).
(DOCX)

Author Contributions

Conceived and designed the experiments: JMR LL MD MK. Performed the experiments: MB SMD HR. Analyzed the data: MB SMD MD MK HR LL JMR. Contributed reagents/materials/analysis tools: MB SMD HR LL. Wrote the paper: MB SMD HR JMR.

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Article 4:

Hierarchical clustering as a rapid tool for surveillance of emerging antibiotic resistance phenotypes in *Klebsiella pneumoniae* strains

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Hierarchical clustering as a rapid tool for surveillance of emerging antibiotic-resistance phenotypes in *Klebsiella pneumoniae* strains

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Antimicrobial resistance is on the rise, and its early detection and surveillance are critical to implement effective control measures. The aim of this study was to develop a rapid hierarchical clustering bioinformatic tool for application on antibiotic susceptibility testing (AST) results (resistant, intermediate, sensitive) of a series of *Klebsiella pneumoniae* clinical isolates from Algeria and from France for surveillance of antibiotic-resistance phenotypes. A total of 1011 *K. pneumoniae* strains were collected from August 2008 to December 2012: 221 clinical isolates from western Algeria and 790 clinical isolates from Marseille, France. AST against a panel of 16 antibiotics was done for all isolates. Results of AST were introduced into MultiExperiment Viewer (MeV) software to perform hierarchical clustering, with resistant, intermediate and sensitive being translated to 1, 0 and −1 values, respectively. Hierarchical clustering results were compared to standard resistance phenotypes to evaluate the accuracy of the method. Based on the AST results, the 221 *K. pneumoniae* strains from Algeria could be separated into six phenotype groups as regards their resistance to β-lactam compounds: extended spectrum β-lactamase (ESBL) (68.3 %), ESBL associated with cephalosporinase (13.1 %), cephalosporinase (0.9 %), penicillinase (3.6 %) and wild-type (14.0 %). Hierarchical clustering by the MeV software applied to the AST results for all 1011 isolates generated clusters that were significantly representative of phenotypic classification and geographical origin, in less than 1 min. Moreover, adding to the dataset the AST results of a *K. pneumoniae* NDM-1 positive strain, the only strain resistant to imipenem in the series, immediately generated a new branch in the dendrogram. We have developed a rapid and simple hierarchical clustering tool for application on AST results that was able to survey qualitatively and quantitatively the prevalence of known and unknown phenotypes. This tool could be easily implemented in routine clinical microbiology laboratories.

INTRODUCTION

Concern is growing about increasing antimicrobial resistance worldwide (Giske et al., 2010). International spread of antimicrobial-resistant micro-organisms suggests that such resistance should be regarded as a global problem that requires a common strategy and justifies specific surveillance involving majur financial and intellectual resources throughout the world (Monnet, 2000; Bush *et al.*, 2011). Such surveillance is conducted in many countries and is now recognized as a priority for scientific societies, public health officers and legislators in order to detect outbreaks in their early stages (PAHO, 2000; Giske et al., 2010). Outbreaks generally begin in small areas and subsequently spread widely. In order to detect an outbreak in its early stages, observation and counting of data remain critical (Hashimoto *et al.*, 2000). Nevertheless, poor data management,

Abbreviations: ESBL, extended-spectrum β-lactamase; MeV, MultiExperiment Viewer.

Details of the hierarchical clustering algorithm are given as supplementary data, available with the online version of this paper.

especially in low-income countries, prevents routine monitoring and reliable data collection to evaluate the prevalence of antimicrobial resistance and to survey multidrug-resistant bacteria (Bush *et al.*, 2011; WHO, 2011). Many guidelines worldwide have investigated the detection of outbreaks through surveillance systems such as the European Surveillance of Antibiotic Resistance (ESAR), the European Antimicrobial Resistance Surveillance System (EARSS) and the WHO Collaborative Center for Surveillance of Antimicrobial Resistance (Giske et al., 2010; Cornaglia *et al.*, 2004). However, such surveillance is usually not done in a real-time manner and depends on microbiological expertise that is not useful to immediately alert clinicians if a multidrug-resistant strain is detected to avoid the spread of strains and occurrence of an outbreak (Bush *et al.*, 2011; WHO, 2011).

This may have significant and dramatic consequences such as the establishment of endemic strains, e.g. carbapenem (KPC)-producing *Klebsiella pneumoniae* recently reported throughout Greece and Israel (Giakkoupi *et al.*, 2011; Adler & Carmeli, 2011). The most important questions regarding increasing resistance concern the potential rise in morbidity, mortality and costs (de Kraker *et al.*, 2011). For instance, in Europe in 2007, the number of infections by multidrug-resistant bacteria was 400 000, and there were 25 000 attributable deaths (Bush *et al.*, 2011).

The real-time detection of a new, rare or emerging resistant phenotype may be achieved only if the data from antibiotic susceptibility testing are evaluated automatically in a rapid and simple way. Such methods should be easy to perform and able to be adapted in any laboratory without molecular techniques. We might be able to detect the emergence and spread of new antibiotic-resistance encoding genes as recently exemplified by the New Delhi metallo-β-lactamase (NDM-1)-encoding gene spreading worldwide in Gram-negative bacteria (Rolain *et al.*, 2010; Walsh *et al.*, 2011). *K. pneumoniae* is one of the most recent examples of such bacteria often isolated from patients with pneumonia, bloodstream and urinary tract infections, and has emerged worldwide as a multidrug-resistant bacterium (Brisse & Verhoef, 2001; Gupta *et al.*, 2003). These bacteria may acquire multiple resistances and pose specific problems including outbreaks and rapid spread and circulation between patients (Nordmann *et al.*, 2009; Podschun & Ullmann, 1998).

The aim of this study was to evaluate and monitor the prevalence of antimicrobial resistance in *K. pneumoniae* strains isolated in western Algeria and in Marseille, France, and to develop a rapid bioinformatic tool to apply to the antibiotic susceptibility testing results (resistant, intermediate, sensitive: R, I, S) for surveillance of antibiotic-resistance phenotypes, using an original approach of hierarchical clustering with MultiExperiment Viewer (MeV) software. MeV software has been previously developed to analyse complex data obtained with DNA microarrays, and to process data from array comparative genomic hybridization (aCGH) and protein–protein interaction (PPI) experiments (Howe *et al.*, 2010).

Table 1. Origin and distribution of strains from Algeria used in this study

Year	Hospital*	Pathology samples				Total
		Tracheal aspiration	Urine	Pus	Others†	
2008	Tlm	14	6	9	10	39
	Oran	2	2	2	1	7
2009	Tlm	8	2	9	11	30
	Oran	6	4	2	3	15
	SBA	4	2	5	1	12
2010	Tlm	9	3	3	3	18
	Oran	32	5	11	5	53
	SBA	6	2	9	2	19
2011	Tlm	2	0	1	2	5
	Oran	16	4	2	1	23
Total		99	30	53	39	221

*Tlm, Tlemcen; SBA, Sidi Bel Abbes.
†Bladder and gastrointestinal tubes, catheters, rectal and nasal swab.

METHODS

Ethics statement. All bacterial strains studied were taken from a collection of bacteria stored in the microbiology laboratory of the Université Abou Bekr Belkaid – Tlemcen, Algeria, and from the Marseille hospital collection. Only bacterial isolates from this collection were used, retrospectively, for epidemiological purposes and the study did not involve human participants; thus ethics approval was not necessary.

Bacterial isolates. A total of 221 non-redundant clinical strains of *K. pneumoniae* isolated from August 2008 to February 2011 in three different hospitals from western Algeria (Tlemcen, Sidi Bel Abbes and Oran Hospitals) were used in this study. The isolates were recovered from hospital clinical samples (one isolate per patient); details are given in Table 1. One carbapenem-resistant *K. pneumoniae* strain (NDM-1), previously described in our laboratory (Diene *et al.*, 2011), along with 790 *K. pneumoniae* clinical isolates collected between January and December 2012 in Marseille hospital were used retrospectively as comparative strains in MeV software analysis.

K. pneumoniae NCTC 13443 and KPC-3 U2A 2246 were used as positive control strains for PCR assays of the bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{KPC} and bla_{NDM} genes.

***K. pneumoniae* identification.** Strains were cultured on MacConkey agar plates at 37 °C for 24 h; they were first identified using the API20E system (bioMérieux) and confirmed using a MALDI-TOF-MS spectrometer (337 nm) (Autoflex, Bruker Daltonics) with the FLEX control software (Bruker Daltonics) as previously described (Seng *et al.*, 2009).

Antibiotic susceptibility. Antibiograms were determined by disc diffusion methods on Mueller–Hinton medium according to the

Table 2. Antibiotic susceptibility testing results of *K. pneumoniae* strains from Algeria

AM, Ampicillin; AMX, amoxicillin; AMC, amoxicillin/clavulanic acid; TIC, ticarcillin; CF, cefalotin; FOX, cefoxitin; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; IMP, imipenem; GN, gentamicin; AN, amikacin; TM, tobramycin; CIP, ciprofloxacin; SXT, trimethoprim/sulfamethoxazole; CS, colistin.

Hospital		AM(%)	AMX(%)	AMC(%)	TIC(%)	CF(%)	FOX(%)	CAZ(%)	CTX(%)	CRO(%)	IMP(%)	GN(%)	TM(%)	AN(%)	CIP(%)	SXT(%)	CS(%)
Tlemcen	R	92(100)	92(100)	48(52.17)	92(100)	83(90.2)	2(2.1)	71(77.17)	78(84.78)	78(84.78)	0(0)	78(84.8)	72(78.3)	21(22.8)	67(72.8)	79(85.9)	0(0)
	I	0(0)	0(0)	31(33.7)	0(0)	0(0)	10(10.9)	9(9.78)	1(1.08)	1(1.08)	0(0)	0(0)	6(6.5)	2(2.2)	4(4.4)	5(5.4)	0(0)
	S	0(0)	0(0)	13(14.13)	0(0)	9(9.78)	80(87)	12(13.04)	13(14.13)	13(14.13)	92(100)	14(15.2)	14(15.2)	69(75)	21(22.8)	8(8.7)	92(100)
Sidi Bel Abbes	R	31(100)	31(100)	22(70.97)	31(100)	25(80.64)	0(0)	20(64.5)	24(77.4)	24(77.4)	0(0)	26(83.9)	24(77.4)	4(12.9)	19(61.3)	25(80.7)	0(0)
	I	0(0)	0(0)	4(12.9)	0(0)	0(0)	3(9.7)	4(12.9)	0(0)	0(0)	0(0)	1(3.2)	2(6.5)	1(3.2)	2(6.5)	1(3.2)	0(0)
	S	0(0)	0(0)	5(16.12)	0(0)	6(19.35)	28(90.3)	7(22.6)	7(22.6)	7(22.6)	31(100)	4(12.9)	5(16.1)	26(83.9)	10(32.2)	5(16.1)	31(100)
Oran	R	98(100)	98(100)	46(44.94)	98(100)	80(81.63)	3(3.06)	75(76.53)	78(79.6)	78(79.6)	0(0)	73(74.5)	77(78.6)	22(22.4)	53(54.1)	41(41.9)	0(0)
	I	0(0)	0(0)	29(29.6)	0(0)	0(0)	13(13.26)	3(3.06)	0(0)	0(0)	0(0)	2(2)	7(7.1)	5(5.1)	3(3.1)	2(2)	0(0)
	S	0(0)	0(0)	23(23.74)	0(0)	18(18.37)	82(83.67)	20(20.40)	20(20.4)	20(20.4)	98(100)	23(23.5)	14(14.3)	71(72.5)	42(42.8)	55(56.1)	98(100)
Total	R	221(100)	221(100)	116(52.48)	221(100)	188(85.07)	5(2.26)	166(75.11)	180(81.48)	180(81.48)	0(0)	177(80.1)	173(78.3)	47(21.3)	139(62.9)	145(65.6)	0(0)
	I	0(0)	0(0)	64(28.96)	0(0)	0(0)	26(11.76)	16(7.23)	1(0.45)	1(0.45)	0(0)	3(1.4)	15(6.8)	8(3.6)	9(4.1)	8(3.6)	0(0)
	S	0(0)	0(0)	41(18.55)	0(0)	33(14.93)	187(84.61)	39(17.65)	40(18.1)	40(18.1)	221(100)	41(18.5)	33(14.9)	166(75.1)	73(33)	68(30.8)	221(100)

guidelines of the Clinical and Laboratory Standards Institute (CLSI) (http://www.clsi.org/). *Escherichia coli* ATCC 25922 was used as a control strain. Antibiotic test discs were purchased from Bio-Rad and were as follows: ampicillin (10 μg), amoxicillin (25 μg), amoxicillin/clavulanic acid (20/10 μg), ticarcillin (75 μg), cefalotin (30 μg), cefoxitin (30 μg), ceftazidime (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg), imipenem (10 μg), gentamicin (10 μg), amikacin (30 μg), tobramycin (10 μg), ciprofloxacin (5 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), colistin (50 μg). In addition, imipenem and colistin MICs were determined using Etest strips, according to the instructions of the manufacturer (bioMérieux). The presence of extended-spectrum β-lactamase (ESBL) activity was detected by the double-disc synergy test as previously described (Jarlier *et al.*, 1988). Cephalosporinase inhibition, evaluated by the disc diffusion method on Mueller–Hinton agar containing cloxacillin at 250 mg l^{-1}, was performed for strains with decreased diameter to third-generation cephalosporins without synergy picture in order to search for high-level cephalosporinase production (Giraud-Morin & Fosse, 2008).

Antibiotic-resistance phenotypic classification of *K. pneumoniae* strains based on β-lactam compounds. We considered a wild-type (WT) phenotype as a strain that showed resistance to aminopenicillins, carboxypenicillins and ureidopenicillins. The penicillinase high-level phenotype (Pase) was indicated by a high penicillinase activity responsible for resistance to aminopenicillins and their inhibitors, to carboxypenicillins, to ureidopenicillins and to first-generation cephalosporins (1GC). The resistance may extend to the 2GC. The penicillinase-inhibitor-resistant TEM (Pase IRT) phenotype included resistance to aminopenicillins, carboxypenicillins and ureidopenicillins. However, it was distinguished by resistance to aminopenicillins and carboxypenicillins associated with the β-lactam inhibitors. 1GC generally retain their activity. The ESBL phenotype includes resistance to penicillins and cephalosporins except cephamycins. However, resistance to 3GC and 4GC was more or less pronounced depending on the enzymes and the strains (MIC of <1 to 128 μg ml^{-1}). Most of the ESBL were more sensitive to β-lactam inhibitors. The cephalosporinase (Case) phenotype corresponded to a marked resistance to penicillins, 1GC, 2GC, and to at least one 3GC. Cephamycins are not active. 3GC resistance may be fully or partially restored in the presence of cloxacillin.

Table 3. Phenotypic classification based on β-lactams for the 221 *K. pneumoniae* strains from Algeria and the 790 clinical strains from Marseille, France

Phenotypic classification*	Algeria		Marseille		Total (no.)
	No.	%	No.	%	
Wild-type	31	14.0	444	56.2	475
Pase IRT	2	0.9	39	4.9	41
Pase high level	6	2.7	73	9.2	79
Case	2	0.9	4	0.5	6
ESBL	151	68.3	190	24.1	341
ESBL+Case	29	13.1	40	5.1	69
Total	221	100	790	100	1011

*Case, Cephalosporinase; ESBL+Case, extended-spectrum β-lactamase associated with cephalosporinase; Pase, penicillinase; Pase IRT, penicillinase inhibitor-resistant.

Table 4. ESBL and carbapenemase genotypes in *K. pneumoniae* strains from Algeria

Hospital*	Total	ESBL positive (%)	bla_{SHV} positive (%)	bla_{TEM} positive (%)	bla_{CTX-M} positive (%)	bla_{NDM} positive (%)	bla_{KPC} positive (%)
Tlm	92	78 (84.78)	64 (69.56)	58 (63.04)	46 (50)	0 (0)	0 (0)
Oran	98	78 (79.59)	66 (67.34)	67 (68.36)	68 (69.38)	0 (0)	0 (0)
SBA	31	24 (75)	24 (77.41)	21 (67.74)	16 (51.61)	0 (0)	0 (0)
All	221	180 (81.44)	154 (69.68)	146 (66.06)	130 (58.82)	0 (0)	0 (0)

*Tlm, Tlemcen; SBA; Sidi Bel Abbes.

PCR amplification and sequencing. A single colony of each isolate from Algeria was resuspended in 400 µl water and boiled for 15 min. After centrifugation, the resulting supernatant was used as a bacterial template DNA in PCR assays in order to detect β-lactamase-encoding genes. Primer pairs were as previously described: bla_{TEM} (Kruger *et al.*, 2004), bla_{SHV} (Yagi *et al.*, 2000), bla_{CTX-M} (Edelstein *et al.*, 2003), bla_{KPC} (Bradford *et al.*, 2004) and bla_{NDM} (Diene *et al.*, 2011). Thermocycler conditions were as follows: an initial denaturation step at 95 °C for 15 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 50 s and extension at 72 °C for

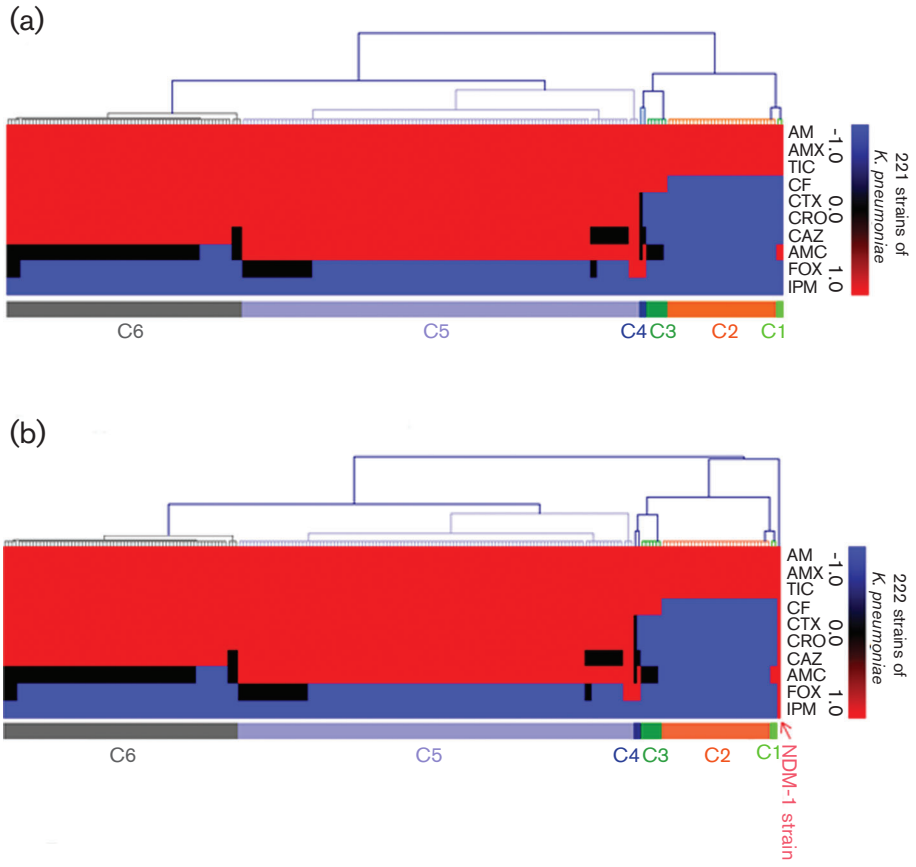

Fig. 1. Hierarchical tree of β-lactam susceptibility testing results of the 221 *K. pneumoniae* strains from Algeria and *K. pneumoniae* NDM-1 (imipenem resistant). (a) The tree was divided into six clusters: C1 containing two strains with the penicillinase IRT phenotype, C2 containing 31 strains with a wild-type phenotype, C3 containing six strains with the high-level penicillinase phenotype, C4 containing two strains with the cephalosporinase phenotype, C5 and C6 containing 113 and 67 strains respectively, both with the ESBL phenotype alone or in association with cephalosporinase. (b) The addition of the antibiotic susceptibility testing results of *K. pneumoniae* NDM-1 (imipenem resistant) to the other results led to the immediate appearance of a new cluster representing this new phenotype. See Table 2 for antibiotic abbreviations.

Table 5. Distribution of the 221 *K. pneumoniae* strains from Algeria in the clusters according to their phenotypic classification and the other classes of antibiotics

	HCL* classification					
	C1	C2	C3	C4	C5	Total
Phenotypic classification†						
Wild-type	29	2	0	0	0	31
Pase	5	3	0	0	0	8
Case	0	2	0	0	0	2
ESBL	0	0	39	88	24	151
ESBL + Case	0	0	5	19	5	29
Total	34	7	44	107	29	221
Associated resistance‡						
None	17	0	0	0	0	17
GN	0	0	0	1	0	1
TM	8	0	0	0	0	8
CIP	2	0	0	0	0	2
SXT	7	0	0	2	0	9
GN-TM	0	0	0	2	5	7
GN-SXT	0	0	0	1	0	1
TM-CIP	0	0	0	0	2	2
TM-SXT	0	1	0	0	0	1
GN-TM-CIP	0	1	0	0	14	15
GN-TM-AN	0	0	11	13	0	24
GN-TM-SXT	0	2	0	0	0	2
TM-CIP-SXT	0	0	0	0	2	2
GN-TM-CIP-SXT	0	3	0	82	1	86
GN-TM-CIP-AN	0	0	0	0	5	5
GN-TM-SXT-AN	0	0	3	0	0	3
GN-CIP-SXT-AN	0	0	4	0	0	4
GN-TM-CIP-SXT-AN	0	0	26	6	0	32

*HCL, hierarchical clustering.
†See Table 3 for details of phenotypes.
‡See Table 2 for antibiotic abbreviations.

1 min 30 s. Tubes were incubated at 72 °C for 7 min to ensure complete synthesis of the entire sequence. Positive PCR products were sequenced using BigDye terminator chemistry on an automated ABI 3730 sequencer (Applied Biosystems) based on Sanger's sequencing method (Sanger *et al.*, 1977). Data collection and analysis were performed using CodonCode Aligner 3.7.1.1 sequencing analysis software.

Hierarchical clustering. Antibiogram results (R, I, S) of the 16 antibiotics tested were recorded in an Excel file and automatically coded as follows: '1' corresponds to resistant, '0' to intermediate and '−1' to susceptible. This resulted in a 221×16 matrix. Rows represented all collected strains (221 strains) and columns represented the 16 antibiotics tested. We started by analysing β-lactam antibiotics (ten antibiotics) to correlate the hierarchical clustering with the antibiotic-resistance phenotypes based on β-lactam antibiotics then we added the other classes of antibiotics to highlight the associated resistance. Hierarchical clustering of data, based on the three values attributed, was carried out using MeV v4.6.2 software (http://www.tm4.org/). Our matrix was presented graphically by colouring each antibiotic susceptibility testing result on the basis of measured colour range: lower limit '−1' was coloured blue, upper limit '1' was coloured red and midpoint value '0' was coloured black.

A hierarchical clustering algorithm usually requires two main steps that are repeated in order to find the strains that are most similar: assigning the antibiogram result values from the same strain to its own cluster, and merging the two clusters that are closest to each other until only one big cluster results. In this study, hierarchical clustering was applied on the results of antibiotic susceptibility testing of all the strains; Pearson correlation was used as distance metric and the complete linkage method was used (details of the algorithm are given as supplementary data, available with the online version of this paper). To reduce the complexity of the tree, the algorithm produces a node height which displays the number of terminal nodes in the tree by imposing a distance threshold according to the classification. Strains on nodes which had distances below this threshold were considered as one cluster and the lower-level detail of the tree was ignored.

Statistical analyses. Statistical analyses of the antibiotic-resistance phenotypes of strains distributed in each cluster were performed by chi-squared calculation using Epi Info version 3.4.1 software (CDC, Atlanta, GA, USA) by comparing each cluster to the whole collection. Differences were considered statistically significant at $P<0.05$.

RESULTS

K. pneumoniae strains from Algeria

Using MALDI-TOF-MS, all *K. pneumoniae* strains (100 %) identified by API20E were confirmed with score values >1.9. Among the 221 isolates, 99 (44.8 %) were recovered from tracheal aspirations, 53 (24.0 %) from pus, 30 (13.6 %) from urine and 39 (17.6 %) from other different clinical samples. Details are given in Table 1.

Antibiotic susceptibility testing of *K. pneumoniae* strains from Algeria

The *K. pneumoniae* strains showed a high degree of multiresistance (Table 2). Resistance rates were as follows: ampicillin, amoxicillin and ticarcillin, 100 %; amoxicillin/clavulanic acid, 58.4 %; cefalotin, 85.07 %; ceftriaxone and cefotaxime, 81.48 %; ceftazidime, 75.11 %; gentamicin, 80.1 %; tobramycin, 78.3 %; ciprofloxacin, 62.9 %; and trimethoprim/sulfamethoxazole, 65.6 %. Conversely, cefoxitin, imipenem, amikacin and colistin were mostly effective, with 84.61 %, 100 %, 75.1 % and 100 % of susceptible bacteria, respectively. The 100 % sensitivity to imipenem (MICs $\leqslant 1$ μg ml^{-1}) and colistin (MICs $\leqslant 2$ μg ml^{-1}) for *K. pneumoniae* strains was also confirmed by Etest strips.

According to our phenotypic classification for antibiotic resistance based on β-lactams, the resistance phenotypes of the 221 collected strains from Algeria were as follows: 31 (14.0 %) had a WT phenotype, eight (3.6 %) had a Pase phenotype (six producing high-level penicillinase and two producing Pase IRT), 151 (68.3 %) had an ESBL phenotype, 29 (13.1 %) had an ESBL phenotype combined with a Case phenotype and two (0.9 %) had a Case phenotype.

Most ESBL strains showed co-resistance to aminoglycosides (71.5 %), ciprofloxacin (75.0 %) and trimethoprim/sulfamethoxazole (75.5 %) (Table 3). Conversely, among the 790 *K. pneumoniae* clinical isolates retrospectively recovered in Marseille, France, overall 444 had a WT phenotype, 112 had a Pase phenotype, 4 had a Case phenotype and 230 had an ESBL phenotype with or without Case (details are given in Table 3). Interestingly, the prevalence of ESBL-producing *K. pneumoniae* was significantly higher in Algeria (81.4 %) as compared to Marseille, France (29.2 %) ($P<10^{-6}$). Conversely, the prevalence of WT and Pase phenotypes was significantly higher in Marseille (56.2 and 14.1 %, respectively) as compared to Algeria (14.0 and 3.6 %, respectively) ($P<10^{-6}$).

Characterization of ESBL-encoding genes of Algerian strains

PCR analysis for detection of β-lactamase encoding genes in the Algerian isolates showed the presence of bla_{SHV}, bla_{TEM} and bla_{CTX-M} in 154 (85.5 %), 146 (81.1 %) and 130 (72.2 %) ESBL-producing *K. pneumoniae* strains, respectively (Table 4). Two or three ESBL-encoding genes were present in 169 (93.9 %) of 180 ESBL typable isolates. On sequence analysis, β-lactamase-encoding genes were 100 % identical to that of CTX-M-15 (GenBank accession no. AY995206), TEM-1 (JN676890), SHV-1 (JN676836), SHV-11 (JN676837), SHV-12 (FJ668801), SHV-28 (AF538324) and SHV-110 (HQ877614). No carbapenem-hydrolysing β-lactamases (NDM-1 and KPC) were detected by PCR analysis.

Hierarchical clustering

Hierarchical clustering of the *K. pneumoniae* antibiotic susceptibility testing results was carried out using MeV software; strains were counted and automatically classified into clusters according to their antibiotic-resistance phenotypes in less than 1 min. Strains from Algeria were confidently separated into six clusters based on their susceptibility profile to β-lactam compounds (Fig. 1a). Each cluster was in perfect correlation with that of the β-lactamase phenotypic classification, with highly significant *P*-values (Table 5). Interestingly, adding the antibiotic susceptibility testing results of a *K. pneumoniae* strain resistant to imipenem (containing bla_{NDM-1}) led to the immediate and automatic appearance of a new cluster representing this new phenotype (Fig. 1b). In the same way, by adding susceptibility testing results for the other classes of antibiotics to those of β-lactams, strains were significantly separated into six clusters associated with specific antibiotic-resistance phenotypes based on β-lactams, aminoglycosides, fluoroquinolones and sulfonamides (Fig. 2, Table 5). In order to confirm that the hierarchical clustering was a simple and accurate method to trace isolates according to their phenotypes and geographical locations, we added to our dataset the 790 antibiotic susceptibility profiles obtained retrospectively from *K. pneumoniae* clinical isolates from Marseille, France. It should be noted that the delay from extraction of antibiotic susceptibility testing of these strains and coding R, I, S to 1, 0, −1 took only 40 min and reconstruction of the new hierarchical clustering containing these 1012 profiles took only 1 min. Analysis of this

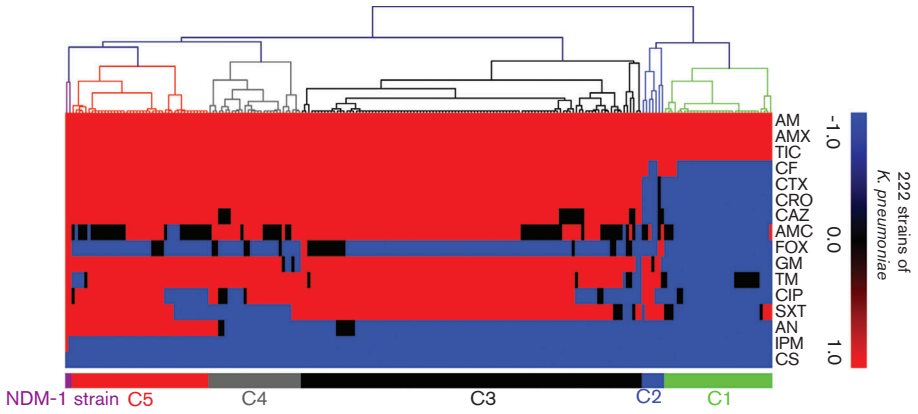

Fig. 2. Hierarchical tree of antibiotic susceptibility testing results with all antibiotics of the 221 *K. pneumoniae* strains from Algeria and *K. pneumoniae* NDM-1 (imipenem resistant). The tree was divided into six clusters. C1 contained strains sensitive to non-β-lactam antibiotics and C2 contained four variable combinations of associated resistance. These two clusters (C1 and C2) were significantly associated with non-ESBL-producing strains. C3, C4 and C5 were significantly associated with ESBL-producing strains and each of them contained significant combination of associated resistance, C6 containing the NDM-1 strain resistant to all antibiotics except colistin. See Table 2 for antibiotic abbreviations.

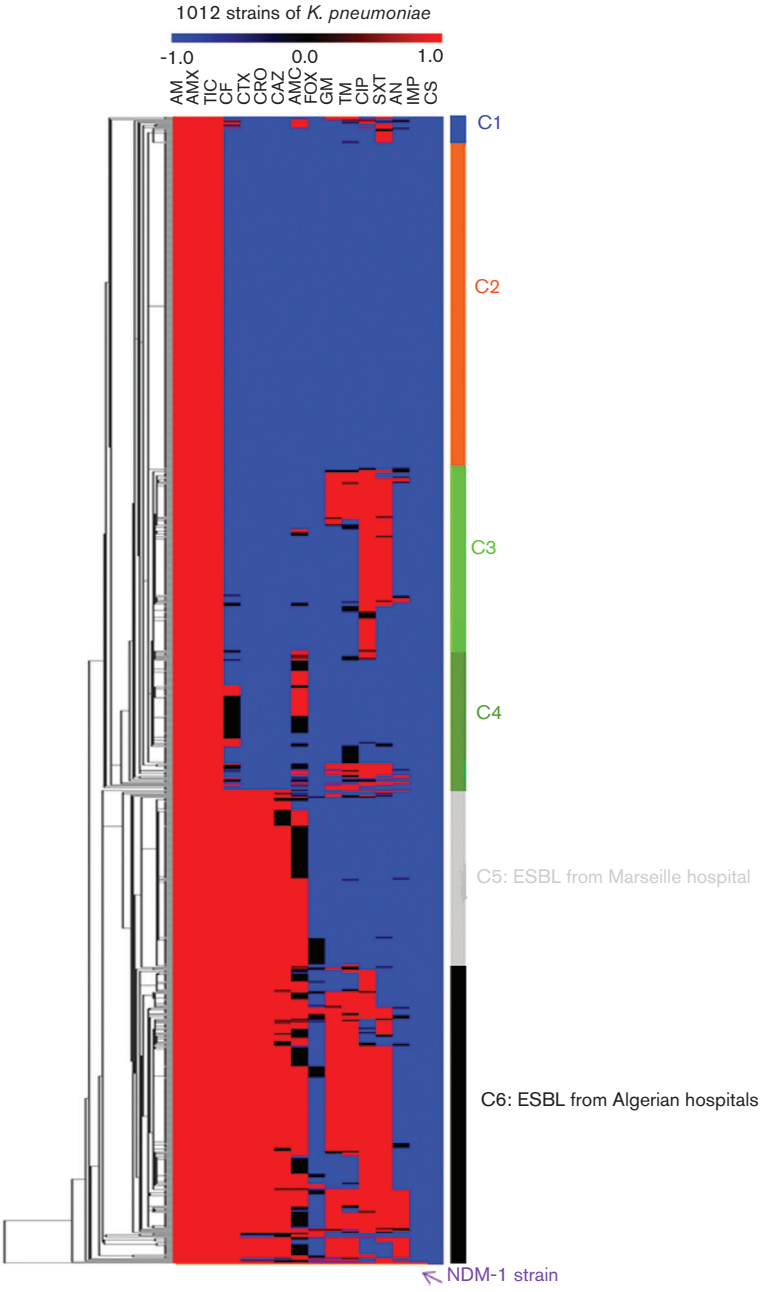

Fig. 3. Hierarchical tree of antibiotic susceptibility testing results with all antibiotics for the 1011 *K. pneumoniae* strains from Algeria and Marseille, France, and *K. pneumoniae* NDM-1 (imipenem resistant). The tree was divided into seven clusters: C1, C2 and C3 containing wild-type strains with (C1 and C3) or without (cluster C2) associated resistance to other antibiotics; C4 containing Pase phenotype strains; C5 and C6 containing ESBL-producing strains, with cluster C5 being significantly associated with strains from Marseille and cluster C6 being significantly associated with strains from Algeria; and C7 containing the NDM-1 strain resistant to all antibiotics except colistin. See Table 2 for antibiotic abbreviations.

new clustering reveals that it contains seven clusters: C1 to C3 being WT phenotype, C4 being Pase phenotype, C5 and C6 being ESBL phenotype with or without Case, and C7 being the *K. pneumoniae* strain resistant to imipenem (containing $bla_{\text{NDM-1}}$) (Fig. 3). Interestingly, the NDM-1 profile was alone in the clustering, as in Fig. 1(b). Each cluster was in perfect correlation with that of the β-lactamase phenotypic classification (Table 6). A more precise analysis of the two clusters containing ESBL profiles showed that 150 out of 230 ESBL-producing isolates from Marseille clustered in C5 along with only 4 out of 180 isolates from Algeria, whereas the remaining 80 ESBL-producing isolates from Marseille clustered in C6 with 176 ESBL strains from Algeria ($P<10^{-6}$) (Fig. 3). Details of the distribution of all phenotypes of the Algeria and Marseille strains are given in Table 7.

DISCUSSION

Since the beginning of the 21st century, *K. pneumoniae* has become a major cause of severe nosocomial infections that are difficult to treat. Outbreaks are caused by strains resistant to a wide variety of antibiotics (de Melo *et al.*, 2011). The analysis of results obtained in this study

Table 6. Phenotypic classification based on β-lactams for the 221 *K. pneumoniae* strains from Algeria and the 790 strains from Marseille in the clusters according to their phenotypic classification

	HCL classification						
Phenotypic classification*	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**Total**
Wild-type	0	475	0	0	0	0	475
Pase IRT	41	0	0	0	0	0	41
Pase high level	0	0	79	0	0	0	79
Case	0	0	0	6	0	0	6
ESBL	0	0	0	0	0	341	341
ESBL + Case	0	0	0	0	65	4	69
Total	41	475	79	6	65	345	1011

*See Table 3 for details of phenotypes.

showed a high rate of ESBL-producing *K. pneumoniae* isolates from western Algeria (81.4 %), with many different ESBL encoding genes as compared to the rate of ESBL-producing isolates both from Marseille (29.2 %) and from Europe in 2009 (EARS-Net, 2010). By comparing our results with those of the Algerian WHONET project, we found a gradual increase of ESBL production rate in *K. pneumoniae* strains, with 37.8 % in 2004 (Pasteur Institute of Algeria, 2004), 40.2 % in 2007 (Pasteur Institute of Algeria, 2008) and 46.1 % in 2008 (Pasteur Institute of Algeria, 2009), as compared to 81.4 % in 2011 in the present study. This value remains very high as compared to other countries such as France with 11.4 % (Ducki & Blech, 2004), Northern Europe with 16.7 % (Khan *et al.*, 2010), South Korea with 32 % (Kim *et al.*, 2008), South America with 51.9 % (Turner, 2005) and Tunisia with 75 % (Boutiba-Ben Boubaker *et al.*, 2002). All our ESBL-producing isolates were susceptible to imipenem, which is similar to other Algerian studies (Messai *et al.*, 2008; Sekhri-Arafa *et al.*, 2010). Nevertheless, universal susceptibility to these last-line antimicrobials in *Enterobacteriaceae* is no longer guaranteed because of the emergence of carbapenemase-encoding genes such as NDM, IMP, KPC, GES and OXA-48 that should be surveyed cautiously (Nordmann & Carrer, 2010; Cuzon *et al.*, 2011).

In this work, the cluster analysis technique was used as an exploratory method that provided a clear and succinct summary tree to look for diversity of resistance phenotype with the approach taken by Eisen *et al.* (1998). Our hierarchical clustering proved an easy and rapid tool able to group susceptibility testing results of a large series of strains in less than 1 min into clusters and to count them, according to the different specific resistance phenotype groups. Interestingly, clusters obtained using antibiotic susceptibility profiles from strains from Algeria and France allowed us to confidently and significantly separate the ESBL producers isolated in Marseille from those isolates in Algeria. This means that ESBL strains from Marseille were different from those from Algeria, likely suggesting that there are different endemic strains in these two countries. Since antibiotic susceptibility testing methods are likely similar worldwide it should be possible to share data from different countries and to use this method to trace clonal expansion of individual isolates from different countries, cities or hospitals. Moreover, we now use this method for real-time surveillance of the evolution and expansion of these clusters in Marseille hospitals in order to be able to detect specifically an outbreak and/or appearance of a new cluster. The numbers of strains in each cluster are compared every month to our local database using our epidemiological surveillance software EPIMIC, recently developed in the Clinical Microbiology Laboratory of University Hospitals in Marseille (Parola *et al.*, 2011; Kempf *et al.*, 2013). Such simple software may be useful to improve rapid detection of new resistance mechanisms, to compare resistance levels, and to share and to improve the control of antimicrobial resistance in real time. It can be combined with an in-house locally used software, such as EPIMIC, which is used to automatically count and detect an outbreak by comparing both the numbers of samples received and pathogens diagnosed to historical data as soon as they are entered; any significant increase beyond the critical threshold, defined by means of historical data plus two standard deviations, generates a signal alert (Parola *et al.*, 2011). Several systems, including VITEK 2 and SIRWEB, incorporate expert systems to control the results of susceptibility testing by applying a series of predefined rules which detect frequent or infrequent phenotypes. However, such a system is unable to classify and count different antibiotic-resistance phenotypes and cannot survey the prevalence of unknown phenotypes that may spread locally in a hospital (Joyanes *et al.*, 2001). In addition, laboratories in low-income countries generally lack these systems because of the cost, so their data cannot be shared and compared with other laboratories and countries to provide an early warning of new or unusual outbreaks of drug-resistant bacteria (Bush *et al.*, 2011; WHO, 2011).

CONCLUSION

Antimicrobial resistance among *K. pneumoniae* strains is a major global public health problem. We believe that our hierarchical clustering approach will help microbiologists to survey the evolution of resistance phenotypes in real time to avoid rapid spread of endemic bacteria (Giakkoupi *et al.*, 2011; Adler & Carmeli, 2011). The high prevalence of resistance of *K. pneumoniae* to third-generation cephalosporins in Algeria is worrying because this will likely increase the use of carbapenem compounds,

Table 7. Distribution of the 221 *K. pneumoniae* strains from Algeria and the 790 strains from Marseille in the clusters according to their phenotypic classification and the other classes of antibiotics

	HCL classification						
	C1	C2	C3	C4	C5	C6	Total
Phenotypic classification*							
Wild-type	11‡+4§	271‡+16§	148‡+3§	14‡+8§	0	0	475
Pase IRT	1‡+a§	0	6‡	32‡+1§	0	0	41
Pase high level	6	0	4‡	62‡+6§	0	1‡	79
Case	0	0	0	0	0	4‡+2§	6
ESBL	0	0	0	0	126‡+4§	64‡+147§	341
ESBL+Case	0	0	0	0	24‡	16‡+29§	69
Total	23	287	161	123	154	263	1011
Associated resistance†							
None	0	271‡+16§	0	71‡+1§	144‡	3‡	503
GN	0	0	0	0	1§	0	1
TM	0	0	0	8‡+9§	1‡	2‡	19
CIP	0	0	32‡+2§	6‡	0	16‡	56
SXT	11‡+4§	0	0	1‡+2§	1‡+2§	0	21
AN	0	0	0	0	1‡	0	1
GN-TM	0	0	0	3‡	0	7§	10
GN-SXT	0	0	0	0	1[b]	0	1
GN-AN	0	0	0	0	0	0	0
TM-CIP	1‡	0	5‡	5‡	1‡	2‡+2§	16
TM-SXT	0	0	0	1§	0	1‡+a§	3
CIP-SXT	1‡	0	59‡+3§	0	0	28‡	91
CIP-AN	0	0	2‡	0	0	0	2
SXT-AN	1‡	0	0	0	0	1‡	2
GN-TM-CIP	1‡	0	4[a]	1[b]	0	1‡+14§	21
GN-TM-AN	0	0	0	0	0	11§	11
GN-TM-SXT	1‡+a§	0	0	0	0	2‡+14§	18
GN-CIP-SXT	0	0	5‡	0	0	0	5
TM-CIP-SXT	0	0	3‡	1‡	0	2‡+2§	8
TM-CIP-AN	1‡	0	0	3‡	1‡	0	5
CIP-SXT-AN	1‡	0	5‡	1‡	0	0	7
GN-TM-CIP-SXT	0	0	32‡+1‡	7‡+2§	1‡	17‡+83§	146
GN-TM-CIP-AN	0	0	1‡	0	0	5§	6
GN-TM-SXT-AN	0	0	0	0	0	1‡+3§	4
GN-CIP-SXT-AN	0	0	0	2‡	0	1‡+4§	7
TM-CIP-SXT-AN	0	0	1‡	0	0	0	1
GN-TM-CIP-SXT-AN	0	0	6‡	0	0	8‡+32§	46

*See Table 3 for details of phenotypes.
†See Table 2 for antibiotic abbreviations.
‡Strains isolated from Marseille Hospital.
§Strains isolated in Algerian hospitals.

leading to the emergence and spread of carbapenem-resistant isolates.

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CHAPITRE III:

Etude d'épidémiologie moléculaire de la résistance aux antibiotiques à partir d'isolats cliniques multi-résistants

AVANT PROPOS

Au cours de cette thèse, nous avons pu par ailleurs participer à la réalisation des études d'épidémiologie moléculaire afin d'investiguer les supports génétiques de la résistance aux antibiotiques à partir de différentes collections d'isolats cliniques de bactéries multi-résistantes. L'ensemble de ces travaux se sont portés principalement sur l'espèce *A. baumannii,* et l'espèce *P. aeruginosa*. Les deux étant des bactéries opportunistes émergentes qui sont fréquemment résistantes à de nombreuses classes d'antibiotiques. Ceci s'explique sans doute, comme il a bien été décrit dans la littérature, par la grande plasticité de leurs génomes, par leur capacité à échanger et à acquérir du matériel génétique exogène, mais aussi par leur capacité à évoluer dans des niches écologiques très variées [1, 2, 3].

Ainsi dans cette thématique,

❖ Nous avons décrit les premiers cas de souches cliniques d'*A. baumannii* résistantes aux carbapénèmes, responsables d'infections nosocomiales, isolées sur une période de trois mois (de Mars à Mai 2012) dans l'hôpital universitaire d'Ibadan, au sud-ouest de Nigeria **(Article 5)**. En effet, trois souches sur cinq étaient multi-résistantes, y compris la résistance à l'imipénème, elles étaient sensibles seulement à colistine. La recherche moléculaire des carbapénèmases a révélé une forte prévalence du gène $bla_{\text{OXA-23}}$ (soit 60.0% des souches).

❖ De la même manière, nous avons rapporté l'émergence de carbapénèmases à partir d'une collection de souches cliniques

d'*Acinetobacter* spp, résistantes aux carbapénèmes isolées entre Octobre 2008 et Avril 2012 dans trois hôpitaux universitaire de l'ouest algérien **(Article 6)**. Sur un total de 113 *Acinetobacter* spp., 80 (70,8%) souches étaient résistantes à l'imipénème avec des CMI allant de 64 à 512 µg / ml. Le gène $bla_{\text{OXA-23-like}}$ a été détecté dans 50% (40/80) des souches, alors que le gène $bla_{\text{OXA-24-like}}$ a été détecté dans 21,2% (17/80) des souches. En outre, nous avons détecté 5 souches (6,2%) productrices de métallo-β-lactamase $bla_{\text{NDM-1-like}}$. Cette étude représente la première description de cas autochtones d'*Acinetobacter* spp. productrice de métallo-β-lactamase $bla_{\text{NDM-1}}$ en Algérie.

❖ Un autre travail a été réalisé dans le but de caractériser la résistance aux carbapénèmes de 96 souches cliniques de *Pseudomonas aeruginosa* isolées dans trois hôpitaux de l'Ouest Algérien entre Octobre 2009 et Novembre 2012 **(Article 7)**. Parmi les 96 isolats, 35 (36,45%) étaient résistantes à l'IMP (CMI ≥ 16 µg / ml), dont deux d'entre elles étaient productrices du gène $bla_{\text{VIM-2}}$. Les 33 autres souches résistantes à l'imipénème ont révélé la présence de mutations sur le gène *OprD*. D'une manière intéressante, l'analyse par MLST des souches contenant les mêmes séquences *OprD* montre qu'elles appartiennent aux mêmes groupes clonaux avec le même ST. Ce résultat nous a permis d'utiliser les mutations sur le gène *OprD* pour le typage des souches de *P. aeruginosa*. Nous avons rapporté dans cet article la première détection de $bla_{\text{VIM-2}}$ dans des souches de *P. aeruginosa* isolées dans l'ouest de l'Algérie.

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Article 5:

Emergence of multidrug-resistant *Acinetobacter baumannii* producing OXA-23 carbapenemase, Nigeria

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Letter to the Editor

Emergence of multidrug-resistant *Acinetobacter baumannii* producing OXA-23 carbapenemase, Nigeria

Acinetobacter baumannii is a nonfermentative Gram-negative bacterium commonly found in water and soil.[1] Over the last decade it has become a serious emerging community and nosocomial pathogen worldwide, known to be responsible for life-threatening infections.[2] Carbapenems are the most commonly used antibiotics for treating infections caused by *A. baumannii*, but an increase in carbapenem-resistant strains of *A. baumannii* has been reported worldwide over the last decade,[3] mainly through the production of metallo-beta-lactamases (MBLs) or oxacillinases (carbapenem-hydrolyzing class D beta-lactamases (CHDLs)).[4] Four major subgroups of acquired CHDLs have been identified in the bacterium, including OXA-23, OXA-40, OXA-58, and OXA-143 beta-lactamase groups, together with the naturally occurring OXA-51 beta-lactamase.[5] Although carbapenemase-producing *A. baumannii* has been reported in many countries worldwide in Europe, South America, North America, Australia, and Asia, there are only a few reports from Africa (Tunisia, Algeria, Egypt, Libya, South Africa, and Senegal)[3,6] (Figure 1).

Here we report the presence of carbapenemase-encoding genes in imipenem-resistant *A. baumannii* among multidrug-resistant clinical isolates collected from the University College Hospital, Ibadan, south-western Nigeria. Three out of five (60.0%) *A. baumannii* clinical isolates identified between March and May

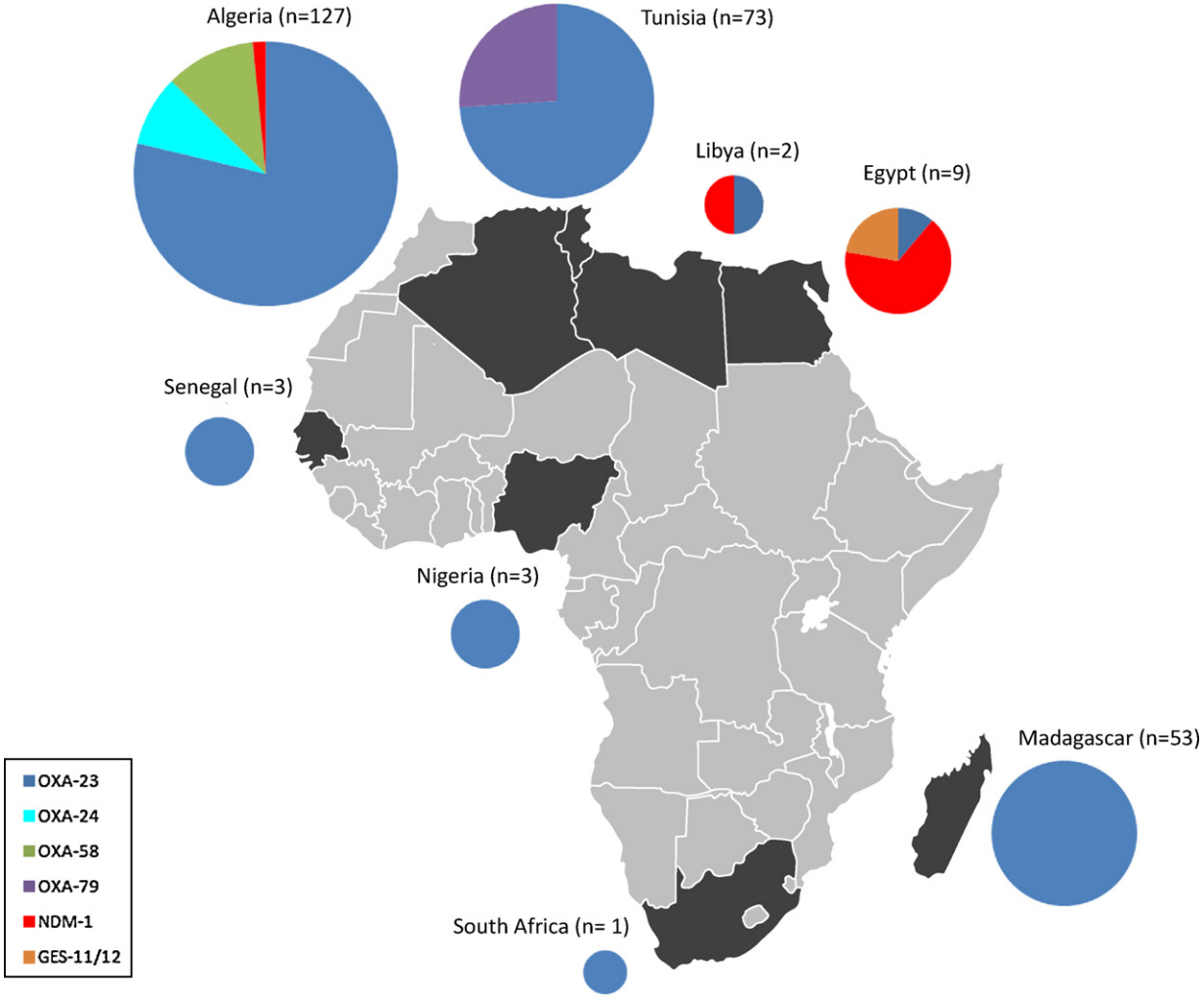

Figure 1. Emergence of carbapenemase-producing *Acinetobacter baumannii* in Africa.

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2012 using matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF) and partial sequencing of the *rpoB* gene, as previously described,[7] were multidrug-resistant, including resistance to imipenem; they were only susceptible to colistin. A modified Hodge test (MHT) using MacConkey agar and an imipenem–ethylenediaminetetraacetic acid (EDTA) combined disk diffusion test (CDDT) were performed on the carbapenem-resistant isolates and results showed that all three imipenem-resistant *A. baumannii* were positive for carbapenemase.

Real-time polymerase chain reaction (PCR) and standard PCR for the detection of $bla_{\text{OXA-23}}$, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-58}}$, $bla_{\text{NDM-1}}$, bla_{IMP}, and bla_{VIM} genes revealed that the three isolates harbored the $bla_{\text{OXA-23}}$ gene, while none of the strains harbored $bla_{\text{OXA-24}}$, $bla_{\text{OXA-58}}$, $bla_{\text{NDM-1}}$, bla_{IMP}, or bla_{VIM} genes.

In conclusion, we reemphasize the worrying recent emergence and spread of carbapenemases in clinical isolates of *A. baumannii* from Africa (Figure 1). This will certainly lead clinicians to use colistin as a last resort and lead to the emergence of pandrug-resistant *A. baumannii*, as recently demonstrated in Spain[8] and in France.[9] Because infections due to such bacteria are associated with an increased length of stay in intensive care units and increased mortality, a surveillance program in Nigerian hospitals is necessary in order to implement rapid health control policies.

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Conflict of interest: None declared.

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Article 6:

Prevalence of carbapenemase encoding genes including New Delhi Metallo-β-lactamase in *Acinetobacter* species, Algeria

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Prevalence of carbapenemase-encoding genes including New Delhi metallo-β-lactamase in *Acinetobacter* species, Algeria

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SUMMARY

Background: Nosocomial infections caused by carbapenem-resistant *Acinetobacter spp* are a global health problem. The aim of this study was to investigate the molecular epidemiology and the genetic support of carbapenem resistance in *Acinetobacter spp* clinical isolates recovered from three different hospitals in western Algeria from 2008 to 2012.

Methods: A total of 113 *Acinetobacter spp* isolates were identified by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. Antimicrobial susceptibility testing was carried out, and minimum inhibitory concentrations (MICs) were determined by the dilution method on Mueller–Hinton agar for β-lactams, aminoglycosides, fluoroquinolones, and colistin. The characterization of β-lactamases was investigated by phenotypic tests for the detection of metallo-β-lactamases and oxacillinases. Resistance genes were screened for by quantitative PCR and sequenced when positive.

Results: Among the 113 isolates, 80 (70.8%) were found to be resistant to imipenem with MICs ranging from 64 to 512 μg/ml. The $bla_{OXA-23-like}$ gene was detected in 50% (40/80) of the isolates and the $bla_{OXA-24-like}$ gene was detected in 21.2% (17/80) of the isolates. In addition, the metallo-β-lactamase $bla_{NDM-1-like}$ was detected in five isolates (6.2%).

Conclusions: This study represents the first description of autochthonous *Acinetobacter spp* producing metallo-β-lactamase $bla_{NDM-1-like}$ and oxacillinases $bla_{OXA-23-like}$ and $bla_{OXA-24-like}$ in western Algeria.

1. Introduction

Acinetobacter spp are major nosocomial pathogens. The genus currently consists of more than 40 species, including validly published species and genomic species.[1] Of these, *Acinetobacter baumannii* is the most clinically relevant *Acinetobacter* species. It has emerged as a major cause of healthcare-associated infections including pneumonia, urinary tract infection, and septicemia.[2] It has the ability to develop resistance to multiple classes of useful antimicrobial agents.[3] Closely related species, *Acinetobacter nosocomialis* (formerly named *Acinetobacter* genomic species (gen. sp.) 13TU) and *Acinetobacter pittii* (formerly named *Acinetobacter* gen. sp. 3), have also been associated with nosocomial infections and outbreaks.[4] These three clinically important species are phenotypically and genotypically difficult to differentiate, thus they are grouped together into the *Acinetobacter calcoaceticus–Acinetobacter baumannii* (ACB) complex.[1] They are so much alike that they cannot be differentiated using routine commercial systems. Genotypic methodologies can be used to differentiate them, such as the determination of specific gene sequences, including the 16sRNA, *recA*, *rpoB*, and *gyrB* genes, in combination with the technology of matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS).[5]

The efficacy of carbapenems against multidrug-resistant *Acinetobacter spp* has been undermined by the emergence of Ambler class B and class D carbapenemase-hydrolyzing β-lactamases.[6] The class D carbapenemase (oxacillinase) found in *A. baumannii* can be clustered into four distinct groups: OXA–23-like (OXA–23, OXA–27 and OXA–49), OXA–24-like (OXA–24/40, OXA–25, OXA–26 and OXA–72), OXA–58-like (OXA–58 and OXA–96), and OXA–51-like enzymes.[7] The last group constitutes a family of chromosomal enzymes typically present in *A. baumannii*.[8] The high-level carbapenem resistance due to the expression of genes encoding the class D carbapenemases, requires a strong

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promoter such as that provided by the mobile insertion sequence *IS*Aba1.[9] This is characteristic of *A. baumannii*, and most outbreaks of carbapenem-resistant *A. baumannii* associated with bla_{OXA-23} have been identified using primers based on *IS*Aba1.[6]

The new metallo-β-lactamase (MBL), New Delhi metallo-β-lactamase 1 (NDM-1), was initially reported in *Klebsiella pneumoniae* clinical isolates from a Swedish patient who had previously been hospitalized in India.[10] Recently, cases of NDM-producing *A. baumannii* have been described in several countries worldwide, including Canada, USA, Sweden, UK, Austria, Belgium, France, Netherlands, Germany, Japan, Africa, Oman, and Australia.[11,12] At present, the worldwide caseload is probably being driven by people infected or colonized in the Indian subcontinent before traveling elsewhere. However, there is already evidence of others reservoirs of infected patients in the Balkan states, the Middle East, and Israel, suggesting that the gene may become endemic worldwide, similar to the bla_{KPC} gene, which is now endemic in Greece and Israel.[13,14]

In the present study, we evaluated the prevalence of antibiotic resistance and the genetic background of carbapenem resistance in a series of 113 *A. baumannii* strains isolated in western Algeria between October 2008 and April 2012. We report five bla_{NDM-1}-positive *A. baumannii* strains recovered from autochthonous cases in the same area.

2. Materials and methods

Bacterial isolates of *Acinetobacter spp* were recovered from three different hospitals situated in north-western Algeria (Tlemcen, Oran, and Sidi Bel Abbes). All of them were isolated from the hospital environment and patients admitted to the intensive care unit (ICU) and hematology, surgery, and neurosurgery wards, during the study period of October 2008 to April 2012. They were identified using MALDI-TOF MS, which was performed with a Bruker Daltonics Microflex (Bremen, Germany) using 96-spot polished-steel targets.

Antimicrobial susceptibility was determined by disk diffusion and agar dilution methods, in accordance with the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM) 2008 guidelines. Antibiotic disks were purchased from Bio-Rad (Marnes-la-Coquette, France). The minimum inhibitory concentrations (MICs) were determined by agar dilution method in Mueller–Hinton medium (Fluka BioChemika, Spain) and E-test strips for carbapenems (imipenem, meropenem) (bioMérieux, Marcy l'Etoile, France). Isolates with MICs of imipenem >8 μg/ml and inhibition zone diameter <17 mm were investigated in this study. The double-disk synergy test (DDST) was used to detect MBL.

Strains showing non-susceptibility to carbapenems were screened for the production of acquired carbapenem-hydrolyzing class D β-lactamase: bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-58}, intrinsic β-lactamase bla_{OXA-51}, and MBL bla_{NDM-1}. Quantitative real-time PCR (CFX96, C1000 Thermal Cycler, Bio-Rad) and standard PCR were carried out to detect the encoding genes. MasterMix was prepared in accordance with the manufacturer's instructions and positive controls carrying each gene were used to determine the efficacy of the real-time PCR assay. The experimental run protocol used was as follows: denaturation program (95 °C for 15 min), amplification and quantification programs repeated 35 times (95 °C for 30 s, 60 °C for 1 min). Samples were considered positive if a threshold cycle was reached during the 35 cycles or less. Standard PCR analysis was performed for $bla_{VIM-like}$, $bla_{GIM-like}$, $bla_{IMP-like}$, $bla_{KPC-like}$, $bla_{NDM-1-like}$, $bla_{CTX-M-like}$, $bla_{SHV-like}$, $bla_{TEM-like}$, $bla_{PER-like}$, and $bla_{GES-like}$. PCR screening was also performed for aminoglycoside-modifying enzyme and fluoroquinolone resistance genes (*aac(3)-Ia*, *aac(6′)-Ib*, *aadA*, *ant(2′′)-I*, *aph(3′)-VI*, *armA*, *rmtA*, *rmtF*, *arr-2*, *qnrA*, and *qnrB*). Oligonucleotide primers and probes used are listed in the **Supplementary Material (Table S1)**. Purified PCR products were sequenced using BigDye terminator chemistry on an automated ABI 3730 sequencer (PE Applied Biosystems, Foster City, CA, USA) based on Sanger's sequencing method. Data collection and analysis were performed using CodonCode Aligner 3.7.1.1 sequencing analysis software.

3. Results

In total, 100 human isolates and 13 hospital environment isolates were collected from Tlemcen Hospital, Oran Hospital, and Sidi Bel Abbes Hospital (51, 45, and 17, respectively). Overall, 106 strains were identified as *A. baumannii*, one strain as *A. radioresistens* (from Sidi Bel Abbes Hospital), two strains as *A. nosocomialis* (from Oran Hospital), and four strains as *A. pittii* (from Tlemcen, Oran, and Sidi Bel Abbes hospitals). All the isolates were identified to the species level with a log score >2.0; the mass spectrometry-based identification scheme yielded identical results compared against the default Bruker database. A mean spectra projection (MSP) dendrogram was generated on the basis of consensus spectra obtained from each bacterium (Figure 1).

The overall susceptibility of all the strains according to the French CA-SFM breakpoints showed that most of the isolates were characterized by resistance to β-lactams (piperacillin 92.2%, piperacillin–tazobactam 88%, ticarcillin 95.9%, ticarcillin–clavulanic acid 96.2%, ceftazidime 98.6%), to fluoroquinolones (ciprofloxacin 85%, with MICs ranging from 0.125 to 0.25 μg/ml), and to aminoglycosides (amikacin 79.1%, gentamicin 56.1%, and tobramycin 38.9%, with MICs ranging from 1 to 512 μg/ml), whilst they differed in their susceptibility to imipenem (70.8%) and showed different levels of resistance with MICs ranging from 0.5 to 512 μg/ml. However, all isolates were susceptible to colistin (MIC 0.125–0.25 μg/ml) (Table 1).

Eighty imipenem-resistant strains (with MIC ranging from 64 to 512 μg/ml), including 42 (82%) imipenem-resistant *A. baumannii* from Tlemcen Hospital, 31 (69%) imipenem-resistant *Acinetobacter spp* from Oran Hospital (30 *A. baumannii* and one *A. nosocomialis*), and seven (41%) imipenem-resistant *Acinetobacter spp* from Sidi Bel Abbes Hospital (six *A. baumannii* and one *A. radioresistens*) were screened for the presence of carbapenemase-encoding genes (Table 2). Real-time PCR results showed that 40 out of 80 imipenem-resistant isolates were positive for the bla_{OXA-23} gene (31 *A. baumannii* from Tlemcen Hospital, seven *A. baumannii* and one *A. nosocomialis* from Oran Hospital, and one *A. baumannii* from Sidi Bel Abbes Hospital) and 17 isolates harbored the bla_{OXA-24} gene (four *A. baumannii* from Tlemcen Hospital, 11 *A. baumannii* from Oran Hospital, and one *A. baumannii* and one *A. radioresistens* from Sidi Bel Abbes Hospital), of which five coexisted with the OXA–23 gene (Table 3).

In addition, among all the isolates, five from Oran were positive for the MBL NDM-1. All five isolates showed positivity by DDST. The gene was sequenced and revealed 99% identity to the sequence reported in the GenBank database under accession number **JQ739157.1**. The five NDM-1-positive isolates were from autochthonous cases in five patients admitted to the ICU and hematology wards of Oran Hospital. All five bla_{NDM-1}-positive isolates were identified as *A. baumannii*. The earliest positive isolate was collected in April 2011 from a 38-year-old man hospitalized on the hematology ward who was then transferred to the ICU of Oran Hospital for a severe cranial trauma subsequent to a stair fall. He had no relevant travel history and neither did his family. Antibiotics used were ceftazidime, amikacin, and colistin, then imipenem and colistin. The patient died in July 2011. The four other patients were all men aged up to 38 years who were admitted to the same ICU as the first patient during the period April to August 2011. Unfortunately no additional clinical records were available for these patients.

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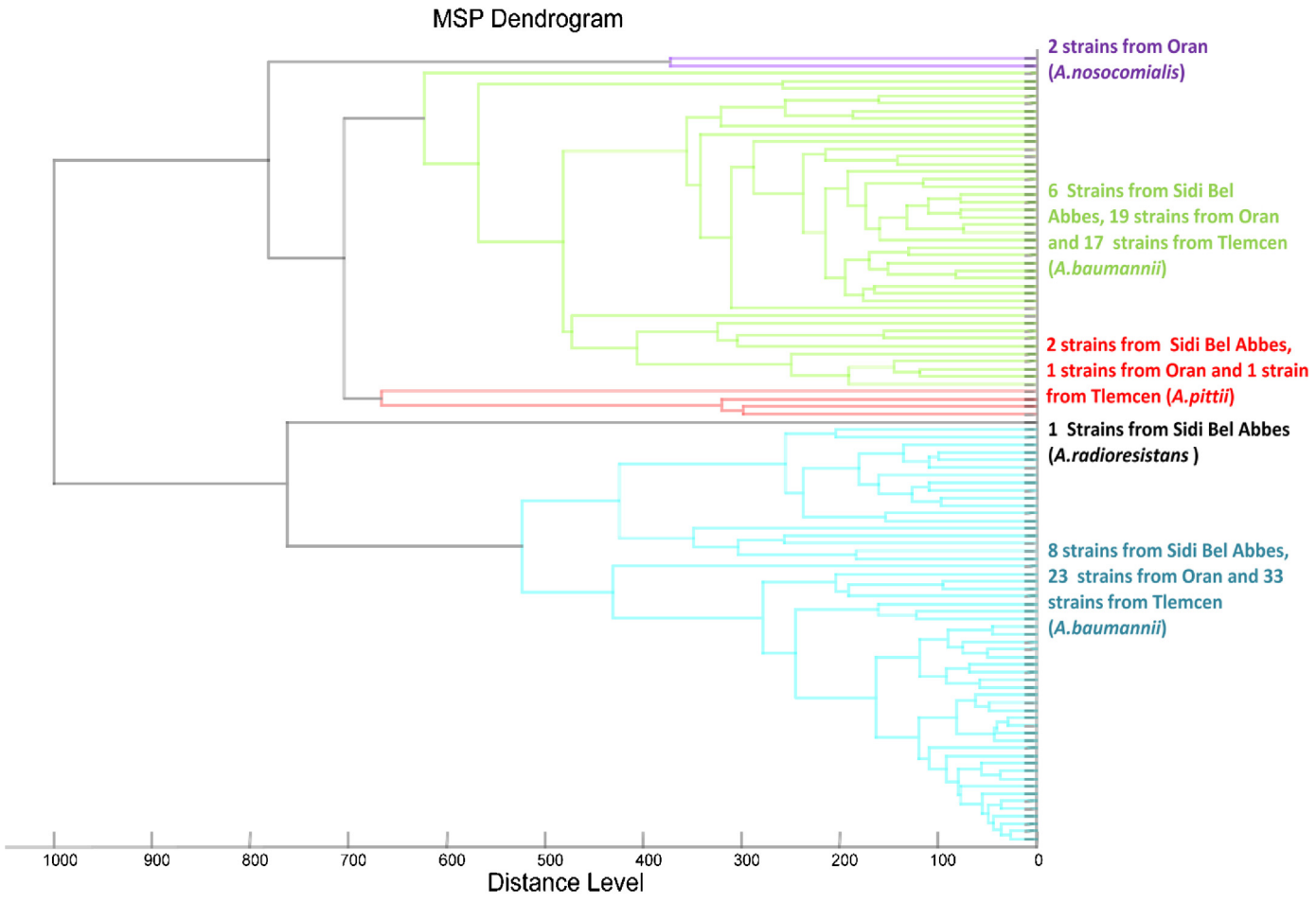

Figure 1. Mean spectra projection (MSP) dendrogram generated by BIOTYPER software (version 2; Bruker Daltonics) of *Acinetobacter spp* strains.

No $bla_{VIM-like}$, $bla_{GIM-like}$, $bla_{IMP-like}$, $bla_{CTX-M-like}$, $bla_{SHV-like}$, $bla_{TEM-like}$, $bla_{PER-like}$ $bla_{GES-like}$, $bla_{KPC-like}$, or bla_{OXA-58} were detected in the collected strains. Resistance to aminoglycosides (gentamicin, tobramycin, and amikacin) observed in almost all the isolates was due to the expression of *aac(3)-Ia* (77 isolates), *aadA* (57 isolates), *ant(2″)-I* (60 isolates), *aph(3′)* (70 isolates), and *aac(6′)-Ib* (one isolate) genes. No isolates were positive for *rmtA*, *rmtF*, *armA*, *arr-2*, or the *qnr* genes.

4. Discussion

Acinetobacter spp has recently emerged as one of the most important opportunistic nosocomial pathogens. Although *A. baumannii* is the most important species in clinical settings, the other *Acinetobacter spp*, such as *A. pittii* and *A. nosocomialis*, are also frequently isolated in hospitals and have been involved in a number of outbreaks in ICUs.[3] The analysis of the dendrogram generated by BIOTYPER software showed that the protein signatures formed five separate clusters related to each one of the species, excluding *A. baumannii* strains that form two separates clusters. This is consistent with the findings of Espinal et al., who showed that MALDI-TOF MS is able to identify and class Acinetobacter strains in separate clusters.[5]

In our study, we investigated the high prevalence of carbapenemase-encoding genes (OXA-type carbapenemase and bla_{NDM-1}) in *Acinetobacter spp*. OXA-type carbapenemase-producing

Table 1
Resistance rates for *Acinetobacter spp* isolates in this study

Antimicrobial agent	Resistance rate (%)			
	Tlemcen ($n=51$)	Oran ($n=45$)	Sidi Bel Abbes ($n=17$)	Total ($n=113$)
Piperacillin	94.1	95.5	87.0	92.2
Piperacillin–tazobactam	84.3	91.0	88.8	88.0
Ticarcillin	98.0	97.7	92.0	95.9
Ticarcillin–clavulanic acid	100.0	97.7	91.0	96.2
Ceftazidime	98.0	100.0	98.0	98.6
Imipenem	78.0	71.0	35.0	61.3
Meropenem	84.0	77.7	38.0	66.5
Gentamicin	50.9	26.6	91.0	56.1
Tobramycin	23.0	57.7	36.0	38.9
Amikacin	82.3	71.0	84.0	79.1
Ciprofloxacin	88.2	91.0	76.0	85.0
Colistin	0.0	0.0	0.0	0.0

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Table 2
Isolates of *Acinetobacter spp* in relation to the presence of carbapenemase enzymes

Carbapenemases					Hospital location	Species	No. of isolates	Samples
OXA–51	OXA–23	OXA–24	OXA–58	NDM				
+	+	+	–	–	Tlemcen	*A. baumannii*	4	Tracheal aspirate, rectal swab, urine, environment
					Oran	*A. baumannii*	1	Tracheal aspirate
					Sidi Bel Abbes	-	0	-
+	+	–	–	–	Tlemcen	*A. baumannii*	27	Tracheal aspirate, rectal swab, urine, wound environment
					Oran	*A. baumannii* ($n=6$) *A. nosocomialis* ($n=1$)	7	Wound, tracheal aspirate
					Sidi Bel Abbes	*A. baumannii*	1	Wound, environment
+	–	+	–	–	Tlemcen	-	0	-
					Oran	*A. baumannii*	10	Urine, tracheal aspirate
					Sidi Bel Abbes	*A. baumannii* ($n=1$) *A. radioresistens* ($n=1$)	2	Tracheal aspirate
+	–	–	–	–	Tlemcen	*A. baumannii* ($n=13$) *A. pittii* ($n=1$)	14	Tracheal aspirate, environment, urine
					Oran	*A. baumannii* ($n=8$) *A. pittii* ($n=1$)	9	Tracheal aspirate, urine
					Sidi Bel Abbes	*A. baumannii* ($n=5$) *A. pittii* ($n=1$)	6	Tracheal aspirate
+	–	–	–	+	Tlemcen	-	0	-
					Oran	*A. baumannii*	5	Urine
					Sidi Bel Abbes	-	0	-
–	–	–	–	–	Tlemcen	-	0	-
					Oran	*A. nosocomialis*	1	Urine
					Sidi Bel Abbes	*A. pittii*	1	Wound

Table 3
Distribution of carbapenem-encoding genes in the three hospitals

No. of imipenem-resistant	OXA–23 (%)	OXA–24 (%)	OXA–58 (%)	NDM-1 (%)
Tlemcen ($n=42$)	31 (74.0%)	4 (9.5%)	0 (0%)	0 (0%)
Oran ($n=31$)	8 (25.8%)	11 (35.4%)	0 (0%)	5 (16.0%)
Sidi Bel Abbes ($n=7$)	1 (14.0%)	2 (28.0%)	0 (0%)	0 (0%)
Total ($n=80$)	40	17	0	5

A. baumannii are increasingly reported from Europe, South America, Asia Oceania, and Africa.[11,15,16] There is a worldwide variation in the rate of carbapenem resistance of *A. baumannii* from one geographical area to another.[12] In Algeria, the dissemination of OXA–23 carbapenemases among *A. baumannii* isolates has also been reported since 2010.[15–17] In our series of isolates, the main molecular support explaining the resistance to carbapenems is the presence of bla_{OXA-23} carbapenemase-encoding genes, along with the coexistence of bla_{OXA-24}. Consequently, the isolates demonstrated high rates of co-resistance to all other classes of antimicrobial agents tested. A limited number of antimicrobial agents maintain reliable levels of activity against OXA–23-producing *A. baumannii*.[18] Neither of the non-*baumannii Acinetobacter* showed the coexistence of bla_{OXA-23} with bla_{OXA-24}, in contrast to *A. baumannii* isolates, of which five harbored both genes at the same time.

OXA–23 (formerly ARI-1) was originally reported in an *A. baumannii* detected in Scotland in 1985.[19] In a report by Opazo et al., the bla_{OXA-23} gene is reported to have originated in the chromosome of *A. radioresistens*, which might be the natural reservoir of these enzymes[20] that are currently emerging as the sources of carbapenem resistance in *A. baumannii* worldwide.[21] Although OXA–58 has previously been detected in Tlemcen and Annaba hospitals,[16,22] none of the isolates in this series were positive for this gene. The $bla_{OXA-51-like}$ gene, originally intrinsic to *A. baumannii*, was detected in all the isolates except one *A. pittii* and one *A. nosocomialis*. These $bla_{OXA-51-like}$ genes, all preceded by *IS*Aba1, may confer a high level of carbapenem resistance. They were probably located on plasmids that might have emerged between different clones of non-*baumannii Acinetobacter* species and also between *A. baumannii* clones. The plasmid-borne *IS*Aba1–$bla_{OXA-51-like}$ in non-*baumannii Acinetobacter* species not only contributes to a high level of carbapenem resistance, but also affects the accuracy of using $bla_{OXA-51-like}$ detection as a tool for differentiating *A. baumannii* from other *Acinetobacter* species.[23]

In the present study, we found five strains producing the bla_{NDM-1} gene in autochthonous cases in the ICU of Oran Hospital between April and August 2011. No bacterial isolates harboring the bla_{NDM-1} gene were detected in Algeria from the beginning of the study (2008) until this period. The global distribution of the bla_{NDM-1} gene has been extensively described.[24] It has been found in diverse isolates since it was first discovered in *K. pneumoniae* in 2008.[10] The potential presence of this gene in non-*baumannii Acinetobacter* should receive proper attention. All five of the bla_{NDM-1}-positive isolates were identified as *A. baumannii*, suggesting that this species, which has a robust survival capability, can easily acquire foreign resistance genes.[25] NDM-1-producing *A. baumannii* has already been described in two Algerian patients. The patients were hospitalized in Oran ICU and transferred to French[26] and Belgian[27] hospitals. Patient histories were confirmed as lacking any foreign travel, suggesting that NDM-producing *A. baumannii* isolates may have already spread in North Africa.[26,27] Reports describing NDM-type carbapenemase-producers isolated from patients previously hospitalized in high-prevalence countries is increasing.[28] However, the geographic origin and the time of the first appearance of this gene are unknown.[12] A recent study has suggested that the putative original source of the bla_{NDM-1} gene could be from the chromosome of plant pathogens, such as

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Pseudoxanthomonas and related bacteria widespread in the environment.[29] The spread of strains carrying the bla_{NDM-1} gene will enhance the likelihood of variants emerging. Interestingly, we have evidence that NDM-encoding genes may be widespread in *A. baumannii*, and further molecular surveys will be necessary to evaluate their distribution in that species. Many studies have constituted reports on carbapenem-resistant *A. baumannii* whose carbapenem resistance is mediated mainly by OXA-type carbapenemases. Despite being less commonly identified in *A. baumannii* than oxacillinase, NDM-1 is currently spreading worldwide and could be reported with a high frequency as a mediator of carbapenem resistance. It is thus critical to survey the presence of this gene in multidrug-resistant (MDR) *A. baumannii* isolates worldwide.

Although polymyxins such as colistin (polymyxin E) have not typically been included in regimens to treat Acinetobacter infections because of their neurotoxicity and nephrotoxicity, they are now considered as one of the last resorts against MDR Acinetobacter infections. Owing to the increasing use of colistin against Gram-negative pathogens and the high recombination rate of *Acinetobacter spp*, it is of concern that colistin resistance in *Acinetobacter spp* isolates may increase rapidly.[30]

In conclusion, the spread of NDM-1-positive *A. baumannii* isolates in the hospital setting reemphasizes the need for strict adherence to surveillance programs in order to prevent the colonization, the infection, and the dissemination of this gene in Algeria.

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Conflict of interest: None to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2013.02.024.

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Article 7:

The molecular epidemiology of carbapenem-resistant *Pseudomonas aeruginosa* clinical strains isolated from western Algeria between 2009 and 2012

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The molecular epidemiology of carbapenem-resistant *Pseudomonas aeruginosa* clinical strains isolated from western Algeria between 2009 and 2012.

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Abstract

Background. Infections caused by carbapenem-resistant *Pseudomonas aeruginosa* strains represent a major therapeutic and epidemiological problem. The aim of this study was to characterize carbapenem-resistance in 96 clinical strains of *P. aeruginosa* isolated from three hospitals in western Algeria between October 2009 and November 2012.

Materiel and Methods. Minimum Inhibitory Concentrations **(**MICs) of imipenem were determined by the Etest® method. Screening for metallo-β-lactamase (MβL) was performed using Etest® MβL strips, and a PCR was conducted to detect carbapenemase-encoding genes. The amplification of the *oprD* gene followed by a sequencing reaction was performed for all strains resistant to imipenem. The clonality of 53 *P. aeruginosa* strains was demonstrated using Multi-Locus Sequence Typing (MLST).

Results. Among the 96 isolates, 35 (36.45%) were found to be resistant to IMP (MICs ≥16 μg/mL). The $bla_{\text{VIM-2}}$ gene was detected in two strains. The remaining imipenem-resistant isolates showed the presence of OprD mutations. An MLST analysis for identical clones found identical *oprD* sequences for each ST.

Conclusions. We report the second detection of the year of $bla_{\text{VIM-2}}$ in Algerian *P. aeruginosa* strains. We also found that *oprD* mutations were the major determinant of high-level imipenem-resistance. We demonstrate that these *oprD* mutations can be used as a tool to study the clonality in *P. aeruginosa* isolates.

Introduction

Pseudomonas aeruginosa is a nosocomial pathogen that causes infections with a high mortality rate. [1] The treatment of these infections is often difficult because of the natural and acquired resistance of this organism to several antibiotics, particularly β-lactam antibiotics.

However, carbapenems remain the main antimicrobial for treating infections because *P. aeruginosa* is multidrug-resistant, but the development of carbapenem resistance through the overexpression of efflux systems (MexAB–OprM), the loss of the OprD porin, and the production of carbapenemase [2, 3] may compromise its efficacy. [4] Among the mechanisms of acquired resistance to imipenem, Class B metallo-β-lactamases (MβLs) induce resistance to all β-lactams except aztreonam. Several types of MβLs have been described worldwide in *P. aeruginosa* isolates (IMP, VIM, SPM, GIM, AIM, and NDM). Of the MβLs, VIM types are the most frequent, with countries worldwide reporting their presence. The VIM types have been identified in carbapenem-resistant isolates of *P. aeruginosa* from European countries in the Mediterranean basin (Italy, [5-8] France, [9] Greece, Spain, [10-12] Croatia, [13, 14] and Turkey [15, 16]) and from African countries (Tunisia, [17-19] Kenya, [20] and South Africa [21]). Recently, we have reported the first molecular characterization of VIM-2-producing *P. aeruginosa* clinical isolates from an intensive care unit at the University Hospital of Annaba, Algeria, [22] but no MβLs have been identified in western Algeria for *P. aeruginosa* strains to date.

In the absence of acquired carbapenemases, mutational inactivation of *oprD* is the main mechanism of carbapenem resistance. The outer membrane protein (OMP) OprD regulates the entry of carbapenems. [23] The loss of OprD function has been shown to play a major role in the acquired resistance to imipenem, with a lesser extent to meropenem. [24-27]

Epidemiological typing is useful in determining the relatedness of nosocomial pathogens. Many schemes for the molecular typing of *P. aeruginosa* have been

developed, including pulsed-field gel electrophoresis (PFGE), ribotyping, PCR-based fingerprinting, and Multilocus Sequence Typing (MLST). [28-30]

In this study, we evaluate the carbapenem-resistance in *P. aeruginosa* from western Algeria, we describe the inactivating mutations of *oprD,* and we report the emergence of the VIM-2 enzyme. We also determine the clonal relationships between the isolates in this region using MLST. Our results demonstrate that the *oprD* mutation can be used as a tool to study the clonality in *P. aeruginosa* isolates.

Materials and methods

Bacterial strains

A total of 96 *P. aeruginosa* clinical isolates were collected between October 2009 and November 2012 from three university hospitals in western Algeria (Tlemcen, Sidi Bel Abbes, and Oran). These strains were isolated from environmental sites and from different pathological specimens, essentially tracheal suctioning, performed on hospitalized patients in various hospital departments, mainly intensive care units. Isolates were identified using the API 20NE System (BioMerieux) and confirmed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Microflex ™, Bruker Daltonic, Bremen, Germany) with flex control software (Bruker Daltonics). [31]

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed on Mueller-Hinton agar by the standard disk diffusion method according to the recommendations of the French Society for Microbiology (CA-SFM) (www.sfm.asso.fr). Twelve antibiotics were tested, including ticarcillin, piperacillin, ticarcillin/clavulanic

acid, piperacillin/tazobactam, ceftazidime, imipenem, aztreonam, amikacin, tobramycin, gentamicin, ciprofloxacin, and colistin. The overproduction of cephalosporinase was detected on Mueller-Hinton agar supplemented with 500 mg/l cloxacillin. Minimum inhibitory concentrations (MIC_S) of imipenem were determined using an Etest® strip (AB BioMerieux, France). MβL detection was performed using Etest® MβL strips (AB BioMerieux, France), the double-disk synergy test (DDST[32]), and the combination disk test (CDT [33]).

Molecular detection of Carbapenemase

Genes encoding carbapenemases were detected by using specific primers for *bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{KPC}$ and *bla*$_{NDM}$. Sequence analyses were performed using the Big Dye® terminator chemistry on an automated ABI 3730 Sequencer (PE Applied Biosystems, Foster City, California, United States). All sequences obtained were analyzed by using BlastN and BlastP to search the NCBI database (http:/www.ncbi.nlm.nih.gov/blast). PCR amplification with primers for *aac(6′)*, *aac(3′)*, *aad*, *ant(2″)*, and *aph(3′)* was performed to further investigate the presence of genes encoding the aminoglycoside-modifying enzyme.

PCR amplification and sequencing of *oprD*

PCR amplification of *oprD* was performed on imipenem-resistant strains and susceptible strains by using specific primers. PCR products were fully sequenced as described above, and the resulting sequences were compared to the PAO1 reference strain sequence (GenBank accession no. CAA78448).

Molecular strain typing

The epidemiological relatedness of 53 strains, including 35 imipenem-resistant strains and eighteen imipenem-susceptible strains, was studied by MLST. From the 61 imipenem susceptible strains, we have taken eighteen

strains to verify their clonality. The seven genes used in the MLST analysis were *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*. The analysis was performed as previously described. [28] The types of sequences were determined by referring to the open-source software freely available on www.pasteur.fr/mlst.

Results

Ninety-six strains of *P. aeruginosa* were identified by the API20NE system and MALDI-TOF MS. These strains were isolated from different pathological specimens including tracheal suctionings (57.29%), wounds (26.04%), and urinary tracts (9.37%) and from hospitalized patients in various hospital units including intensive care (67.70%), surgery (12.5%), neurosurgery (8.33%), trauma (6.25%), emergency (3.12%), and internal medicine (2.08%). Samples from environmental sites (7.09%) were collected over identical periods and from identical hospital units. These isolates showed various resistance levels against ciprofloxacin (35.41%), ceftazidim (32.29%), ticarcillin (45.83%), ticarcillin/clavulanic acid (65.62%), piperacillin/tazobactam (33.33%), aztreonam (42.70%), and imipenem (36.45%). All isolates were sensitive to colistin.

Thirty-five isolates were resistant to imipenem (MIC $\geq$ 16 µg/ml), in which 27 of them (28.12%) were isolated from the Oran Hospital, five (5.20%) from the Sidi Bel Abbes Hospital and three (3.12%) from the Tlemcen Hospital.

Among the strains resistant to imipenem, the activity of β-lactamase is inhibited by the action of EDTA in only two out of 35 strains that produce a metallo-β-lactamase**.** These two strains were isolated from the tracheal suctioning of two patients hospitalized at two different units from the Oran Hospital. In November 2010, the first strain was isolated from a woman who was 28 years old. This young woman was hospitalized in intensive care with intrapartum eclampsia and diabetes. The second strain was isolated from a

neurosurgery unit in February 2011 from a woman who was 73 years old and hospitalized from a cerebral stroke. PCR followed by sequencing analysis revealed a $bla_{\text{VIM-2}}$ gene in the two MβL-positive isolates.

The resistance rates, as determined by the disk diffusion method, were as follows: amikacin (28.12%), gentamicin (26.04%), and tobramycin (26.04%). Of the genes examined, *aadA (1 and 13) (10.41%)* was the most frequently identified gene in the isolates displaying phenotypic resistance, followed by *aac(3′)-II* (3.12%). For the two metallo-β-lactamase producing strains, one isolate harbored *aadA1* and *aac*(3′)-II and the second isolate harbored only the *aadA1 gene*.

Because of various mutations, all carbapenem-resistant *P. aeruginosa* isolates had a modification in the amino-acid sequence of the OprD protein that was compared to that of PAO1 reference strain. Therefore, a stop codon mutation was found in different parts of the gene indicating that these strains had a significantly altered OprD protein. Based on the mutations in the *oprD* gene sequences, carbapenem-resistant *P. aeruginosa* isolates could be classified into seven *oprD* mutational groups (Figure 1). The first group (G1) had a single nucleotide deletion (C_{51}) resulting in a premature stop codon TGA_{94} (the number indicates the amino-acid position) in seventeen isolates from the hospital in Oran. For the other six groups, several polymorphism types (substitution, insertion, and deletion) led to a premature stop codon during protein processing or a frameshift mutation and were found in eighteen carbapenem-resistant *P. aeruginosa* strains isolated from the three hospitals, including G2 (TGA_{345}), G3 (TGA_{107}), G4 (TGA_{65}), G5 (TGA_{379}), G6 (TGA_{169}), and G7 (TGA_{195}) with the numbers indicating the amino-acid positions. Of the 35 carbapenem-resistant isolates with *oprD* mutations, 23 isolates (G1 and G2) had also an increased *ampC* expression confirmed by cloxacilline test. Two isolates (G2 and G5) had a mutation in *oprD* and produced an MβL VIM-2. There were no mutations in the *opr*D gene for all the imipenem-susceptible

strains that were assembled with the PAO1 *P. aeuruginosa* strain in Group 0 (G0) (Figure 1).

A total of 22 different STs were assigned to the 53 investigated *P. aeruginosa* strains including 35 imipenem-resistant strains and eighteen imipenem-susceptible strains. The MLST sequences of the studied *P. aeruginosa* isolates were aligned and clustered using CLUSTAL X and MEGA 4 (Fig. 1). Five clonal complexes were identified among the imipenem-resistant strains and were comprised of strains from the same hospital with identical STs and identical *oprD* sequences. The compositions of these groups were as follows: G1(TGA_{94}), G2 (TGA_{345}), and G5 (TGA_{379}) that were isolated from the Oran Hospital were ST244, 622, and 1076, respectively ($p < 10^{-6}$); G3 (TGA_{107}) that was isolated from the Sidi Bel Abbes Hospital was ST313 ($p < 10^{-6}$); and G4 (TGA_{65}) that was isolated from Tlemcen hospital was ST1295 ($p < 10^{-6}$). These lineages were only from intensive care units in the three different hospitals. MLST analysis revealed different STs among the two MβL-positives strains isolated from Oran: ST1406 represented G2 (TGA_{345}) and ST343 represented G6 (TGA_{169}). One strain isolated from the Sidi Bel Abbes Hospital belonged to G7 (TGA_{195}) with ST564.

Of the eighteen susceptible strains, two clones with ST381 and ST538 were detected (Figure 1), the other twelve susceptible isolates showed twelve different STs: 386, 1093, 490, 1433, 1226, 557, 1341, 493, 1175, 464, 796, and 803.

Discussion

In this study, we have investigated 96 clinical strains of *P. aeruginosa* isolated between 2009 and 2012 from three university hospitals in western Algeria. Thirty-five isolates were resistant to imipenem, including two strains producing a VIM-2 MβL. Reports of VIM class MβL isolates around the

Mediterranean basin are depicted in Figure 2. A few MβL-producing *P. aeruginosa* isolates were documented in Africa, particularly in North Africa. VIM-19 was the first carbapenemase enzyme identified from a clinical isolates of five Enterobacteriaceae (*Escherichia coli*, *Klebsiella pneumoniae* and *Providencia stuartii*) from Algeria. [34] Recently, Touati *et al.* described the first dissemination of class I integrons carrying a VIM-2 carbapenemase gene in *P. aeruginosa* clinical isolates from eastern Algeria. [22] This report has coincided with our study, thus this is the second description of VIM-2-producing *P. aeruginosa* in Algeria during the same year. This finding may reflect the current spread of MβLs in clinically relevant Gram negative strains throughout northern Africa.

In the absence of MβL, mutational inactivation of the *oprD* gene is the major determinant of resistance to carbapenem, particularly to imipenem, in *P. aeruginosa* strains. [35] Sequence analysis of the *oprD* genes of carbapenem-resistance strains, including 33 non-MβL producing and the two VIM-2-producing strains, revealed various routes of inactivation. These routes included a single nucleotide deletion resulting in a premature stop codon (Group 1) or several polymorphism types (substitution, insertion and deletion) resulting in a stop codon for the other six groups. In contrast, *oprD* genes of the eighteen carbapenem-susceptible isolates showed no mutational change that contributed to a loss of OprD function.

The present study is the first report of co-expressing VIM-2 and *oprD* porin loss in identical clinical isolates of *P. aeruginosa*. These results indicated that the mutational inactivation of the *oprD* gene was the main mechanism for imipenem resistance in *P. aeruginosa* clinical isolates as previously described in many studies. [23, 27, 36]

In this study, we investigated the clonality of carbapenem-resistant *P. aeruginosa* isolates from the three hospitals in western Algeria using MLST. Nine STs were identified among the 35 carbapenem- resistant *P. aeruginosa*

isolates and five distinct clones of carbapenem-resistance were detected, three in the Oran Hospital, one in the Sidi Bel Abbes Hospital and one in the Tlemcen Hospital. Members of an identical clone maintained an identical sequence of the *oprD* gene, illustrating the stability of these clonal complexes as previously demonstrated by Pirnay *et al.* [37]

Our study demonstrated that the epidemic population structures were nosocomial, they were found exclusively in intensive care units, and the two isolates producing MβL had a community origin with two different STs.

The clone belonging to ST244 was the most frequent in our study (which is a founder of the clonal complex CC244) corresponds to the second most prevalent Mediterranean *P. aeruginosa* clone according to a study conducted in five countries within the Mediterranean basin. [38]

Various methods have been used for the epidemiological typing of *P. aeruginosa* isolates including PCR-based typing techniques, such as ERIC-PCR, or more discriminatory techniques, such as pulsed-field gel electrophoresis (PFGE) and MLST. MLST is based on the allelic differences among housekeeping genes and the analysis of these genes provides a more realistic impression of the effect of recombination. [37] Pirnay *et al.* demonstrated that OprD-related resistance to carbapenems is mainly achieved by non-recombinational events such as point mutations. [35]. They analyzed the *oprI*, *oprL*, and *oprD* sequences and concluded that the *oprD* sequence can be used to detect an epidemic population structure of *P. aeruginosa*, [37] but they did not perform a conventional MLST. The analysis of the *oprD* sequence in imipenem-resistant strains was successfully used as a tool for biotyping in our study because we found a correlation between the ST and the *oprD* sequence from identical clones of *P. aeruginosa*. The correlation between the two types of analysis is displayed in Figure 1.

In conclusion, multiple epidemic clones of carbapenem-resistant *P. aeruginosa* isolates occurred in university hospitals from western Algeria.

Isolates from these outbreaks were associated with the major Mediterranean clone. [38] This study demonstrated that the presence of a mutational inactivated *oprD* gene is the main carbapenem resistance mechanism in *P. aeruginosa* isolates from western Algeria, followed by the acquisition of VIM-2 MβL. This finding may reflect an extensive use of imipenem for treating multi-drug-resistant Gram-negative bacteria.

This report indicated that the epidemic clones had an identical *oprD* gene sequence allowing the analysis of *oprD* gene sequences in carbapenem-resistant *P. aeruginosa* to be used as a tool to study the clonality in *P. aeruginosa* isolates. The rapid dissemination of carbapenem-resistant strains represents a major therapeutic and epidemiological threat and requires the implementation of strict hygiene procedures and regular surveillance studies.

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Transparency declarations and Competing interests

None to declare.

Figures legends.

Figure 1. Phylogenetic tree based on the MLST sequences of 53 *Pseudomonas aeruginosa* isolates aligned with the PAO1strian.

Figure 2. MßL VIM classes described in *Pseudomonas aeruginosa* strains from the Mediterranean basin.

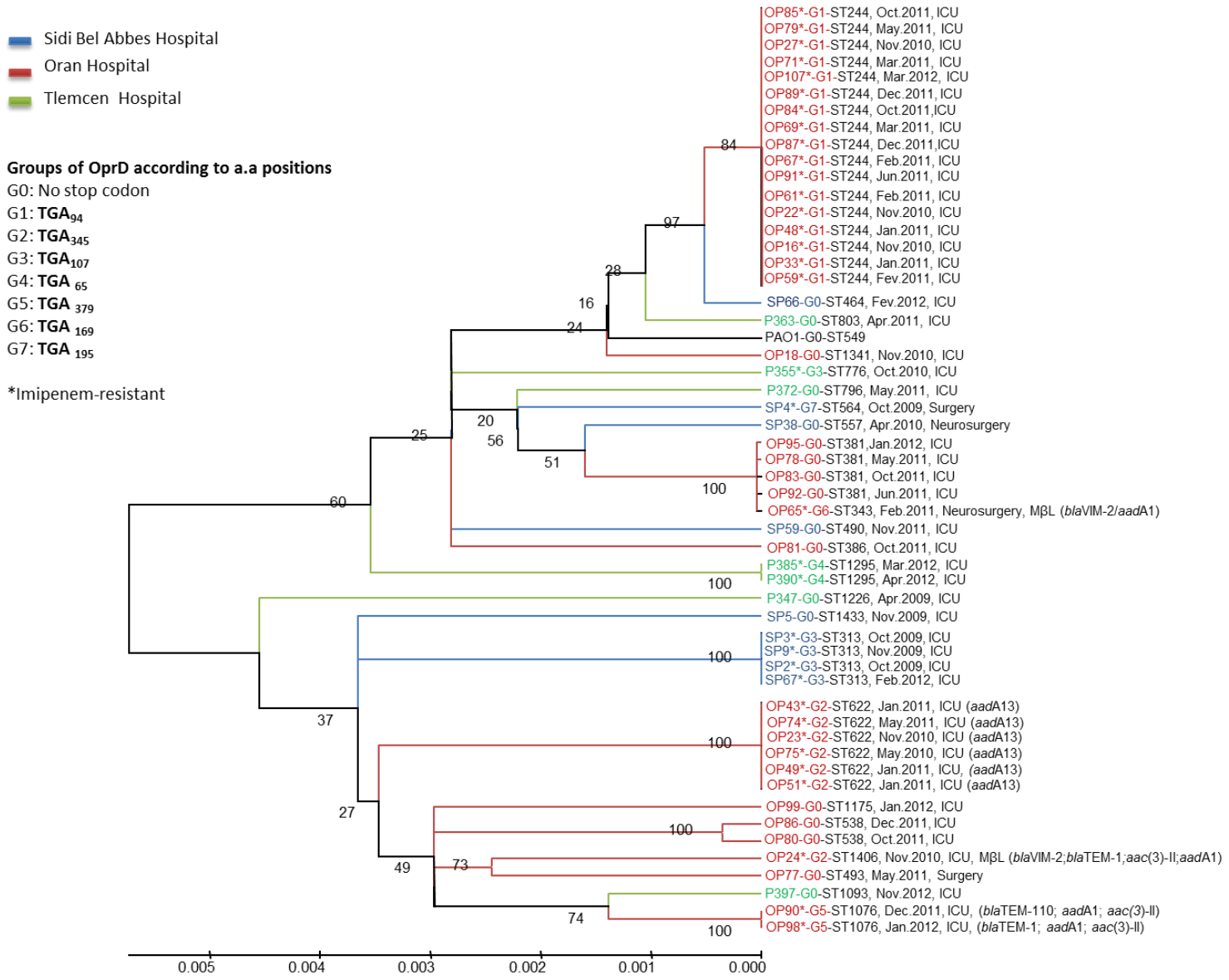

Figure 1. Phylogenetic tree based on the MLST sequences of 53 *Pseudomonas aeruginosa* isolates aligned with the PAO1 strain.

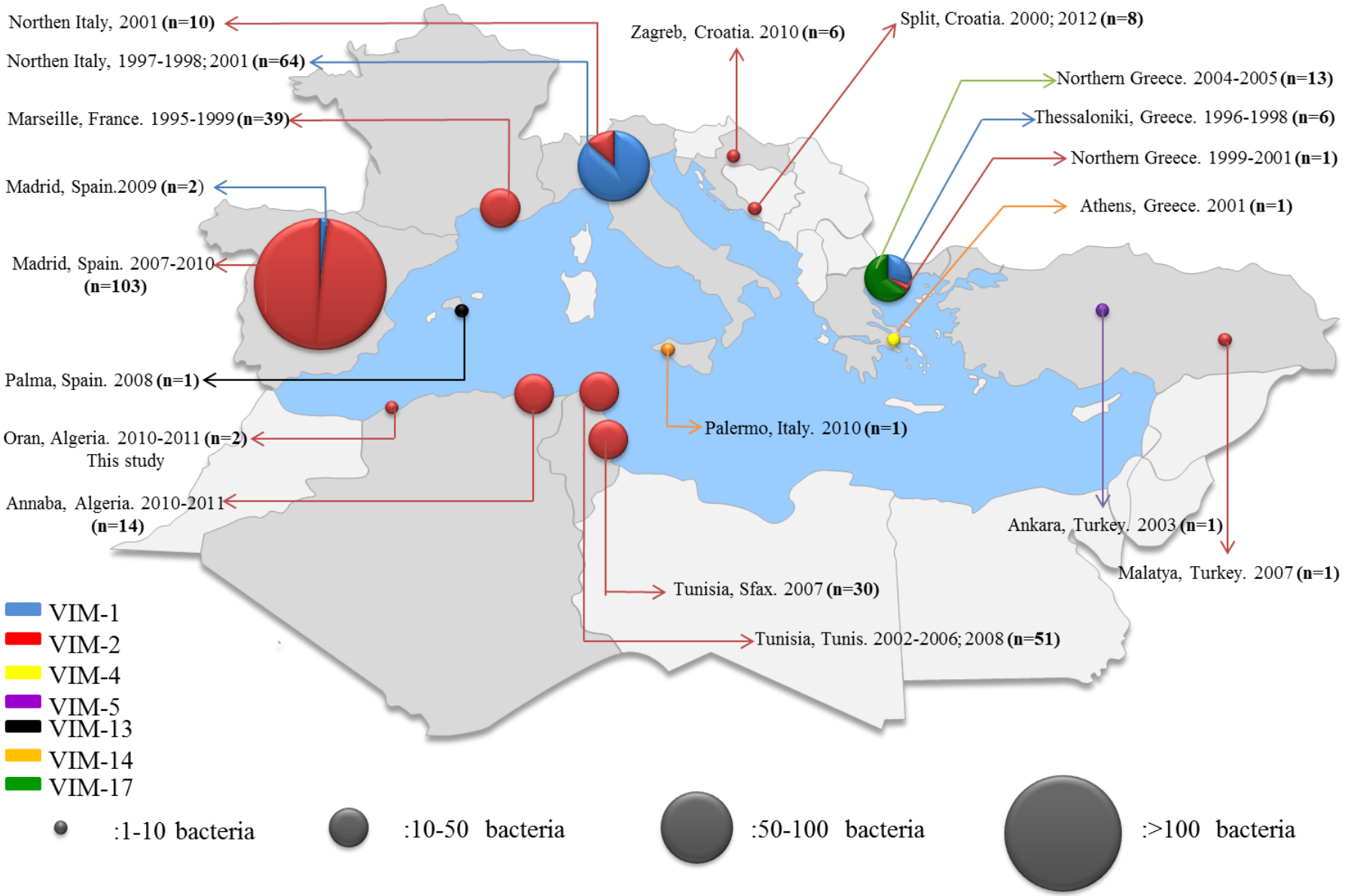

Figure 2. MßL VIM classes described in *Pseudomonas aeruginosa* strains from the Mediterranean basin.

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CONCLUSION ET PERSPECTIVES

Au terme de ce travail, nous avons confirmé que la résistance aux antibiotiques est devenue une préoccupation mondiale et constitue un problème majeur de santé publique. En effet, depuis ces dernières années, nous avons assisté à une augmentation fulgurante de la résistance aux antibiotiques, en particulier chez les bactéries à Gram négatif. Afin d'informer la communauté scientifique et médicale à travers le monde au sujet de la dissémination de la résistance aux antibiotiques, nous avons développé un outil interactif innovant de surveillance en temps réel de la diffusion des gènes de résistance dans le monde en utilisant le logiciel Google Maps. Nous avons présenté cet outil sous forme d'une e-revue de littérature, reprenant toutes les publications qui ont été rapportées sur les NDM-1(Metallo-B-lactamase) à travers le monde, qu'il s'agisse de cas autochtones, importés, ou décrits à partir de l'environnement. Les données actuelles indiquent une augmentation de la propagation des bactéries productrices de NDM-1, partout dans le monde. Nous avons décrit dans cette e-revue 950 bactéries productrices de NDM-1 à partir de différents types de prélèvements isolées dans 55 pays entre 2006 et 2012, avec la majorité des isolats provenant de l'Inde, du Pakistan et de la Chine.

Face à cette situation inquiétante, représentée par l'augmentation de la résistance aux antibiotiques, la problématique essentielle reste de

trouver les solutions à proposer pour lutter contre la diffusion de la résistance aux antibiotiques. A ce jour, peu d'antibiotiques restent actifs contre les infections causées par des bactéries multi-résistantes (BMR), on peut citer comme exemple les carbapénèmes, la colistine et la polymyxine B. De ce fait, la lutte contre ces BMR peut se faire par la prévention qui consiste entre autre, à comprendre leurs mécanismes de transmission, à trouver les déterminants de la résistance, et par la suite développer et mettre en place des outils de détection et de surveillance en temps réel.

Au cours de cette thèse, nous avons mis en œuvre des stratégies de contrôle de dissémination de la résistance aux antibiotiques grâce au développement de nouveaux outils de surveillance et des nouvelles techniques d'analyse et de criblage des phénotypes de résistance. Nous avons tout d'abord mis au point une technique rapide utilisée en routine pour la détection phénotypique des souches bactériennes porteuses de carbapénèmases chez les bactéries à Gram négatif par spectrométrie de masse (Maldi-Tof -Ms). Nous avons démontré aussi, pour la première fois, que le Maldi-Tof peut être utilisé en qualité d'outil rapide de typage protéique des isolats cliniques de *K. pneumoniae.* Nous avons développé également un outil bioinformatique simple et pratique appliqué aux résultats des tests de sensibilité aux antibiotiques afin de surveiller qualitativement et quantitativement, en temps réel, la prévalence des phénotypes de résistance connus et inconnus.

Dans un futur proche, il serait très important d'utiliser ces nouveaux outils de surveillance pour investiguer la résistance aux antibiotiques chez les BMR dans le but de parfaire et d'enrichir nos connaissances sur les déterminants génétiques de la résistance mais aussi de pouvoir arrêter cette dissémination. Nous avons actuellement commencé au laboratoire à utiliser ces outils de surveillance avec des résultats très intéressants, nous réalisons notamment une étude rétrospective sur la prévalence de la résistance aux antibiotiques des bactéries à Gram positif et à Gram négatif responsables de septicémies dans les hôpitaux de Marseille entre 2001 à 2011. Les septicémies, ayant le plus grand impact en termes de morbidité, de mortalité et de coût, en particulier pour les BMR, suite à l'utilisation accrue des procédures et dispositifs invasives et des antibiotiques.

Suite au développement des nouveaux outils de séquençage à haut débit et d'analyse bioinformatique, de nombreuses études, ayant utilisé l'approche du séquençage du génome pour étudier la résistance aux antibiotiques, ont permis de mettre en évidence les véhicules des gènes de résistance à plusieurs familles d'antibiotiques. L'analyse des génomes bactériens avec les outils disponibles actuellement nécessite des connaissances en bioinformatique, ce qui ne facilite pas la recherche et l'identification rapide des déterminants de la résistance pour les laboratoires modestes. Il serait alors intéressant de développer et de proposer de nouveaux outils d'utilisation simple permettant de comprendre le résistome d'une BMR dans certaines situations particulières.

Les études récentes démontrant des sources anciennes et/ou environnementales des gènes de résistance aux antibiotiques, devraient nous encourager à rechercher de nouveaux gènes de résistance à partir des métagénomes du sol, de l'eau, de l'environnement, et des animaux qui représentent sans doute de très importants réservoirs de gènes de résistance jamais explorés jusqu'ici.

Ce travail de thèse ayant été réalisé dans un environnement méditerranéen, entre la France et l'Algérie, nous rédigeons une revue de littérature reprenant toutes les publications qui ont été rapportées dans le bassin Méditerranéen dans le but d'étudier la résistance aux antibiotiques des bacilles à Gram négatif dans cette région, particulièrement les entérobactéries, les bactéries du genre *Pseudomonas* et *Acinetobacter*. Nous présenterons dans cette revue un aperçu sur les mécanismes biochimiques et génétiques de la résistance aux antibiotiques des bacilles à Gram négatif, avec une attention particulière sur l'épidémiologie moléculaire des gènes de résistance aux antibiotiques décrits jusqu'ici dans le bassin méditerranéen.

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