IMPORTANCE OF MOLECULAR EPIDEMIOLOGY OF GRAM NEGATIVE BACILLI

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Abstract. Resistant Gram negative bacilli have become a major concern. They are associated with increased mortality, prolonged hospitalization and increased costs. Carbapenem-resistant Pseudomonas aeruginosa, Acinetobacter baumanii, extended spectrum beta-lactamase-producing Enterobacteriaceae have become increasingly common. A dramatic increase of CTX-M enzymes over TEM and SHV variants has recently occurred in Europe. There are considerable differences in beta-lactamase distribution between geographical areas as well as depending on the setting of infection. CTX-M type enzyme is considered to play an important role in community-acquired infections. Globalization and travel provide opportunities for the spread of resistant organisms responsible for infections that occur in hospitals as well as in community. Infections with carbapenem-resistant Enterobacteriaceae (CRE) are emerging as an important challenge in health-care settings. The potential widespread of carbapenem resistance via plasmids can lead to the spread of CRE into the community as well. Molecular epidemiology has proven very useful in detecting specific antimicrobial drug-resistance genes in a large number of organisms; it plays a key role in distinguishing between a pathogenic strain and a commensal contaminant, as well as between a relapse and a reinfection. Molecular biology typing techniques combined with epidemiological investigations also provide information about nosocomial pathogens and can be very useful for the implementation of effective infection control measures within the hospital environment. A comprehensive infection control program requires a sustained collaboration between infection control departments, clinical microbiology laboratories and hospital epidemiologist. Early detection of nosocomial drug-resistant pathogens through identification of pathogen clonality allows rapid intervention, which ensure the control of nosocomial infections.

Keywords: resistant Gram negative bacilli, molecular epidemiology, nosocomial infections control

Resistance of Gram negative bacilli (GNB) to antimicrobial agents is caused by many different genetic determinants. Initially, multidrug resistance (MDR) was not anticipated, because the appearance of multiple mutations, responsible for antimicrobial resistance was considered unlikely. It is now clear that bacteria were ready for such a challenge and had already developed the genetic tools to confer MDR.

The percentage of Enterobacteriaceae resistant to third generation cephalosporins increased between 1986 and 2003, rising nearly ten fold among Klebsiella pneumoniae and more than two-fold among Escherichia coli. The proportion of MDR Pseudomonas aeruginosa rose three-fold between 1993 and 2002 [2]. The proportion of carbapenem-resistant Acinetobacter species soared from none in 1986 to almost 20% in 2002 [1]. Infections produced by ESBL producing Enterobacteriaceae have become a global problem [3], and they were associated with increased mortality, with prolonged length of stay (LOS) and increased hospital cost [5]. Considerable heterogeneity was noted, however, among the reviewed studies [4,5] regarding study design, setting, and infection type that may have affected findings. For example, crude mortality, overall LOS, and hospital costs are typically higher in ICUs than on wards. In a review of twenty-one original studies and a meta-analysis, which included three of the original studies, Shorr found that infections caused by mixed resistant GNB, ESBL-producing Enterobacteriaceae, MDR Pseudomonas aeruginosa, and Acinetobacter species were generally associated
with increased mortality and LOS, especially in univariate analyses. Resistant Gram negative infections were associated with increased costs, although in multivariate analysis associations sometimes disappeared. [6]

Extended-spectrum beta-lactamases (ESBLs) are plasmid encoded beta-lactamases that confer resistance to penicillins, narrow- and extended-spectrum cephalosporins, and aztreonam. Organisms harboring ESBLs are also frequently resistant to many other antimicrobial drug families, including aminoglycosides, trimethoprim- sulfamethoxazole, and quinolones. ESBL-producing members of the Enterobacteriaceae family have played a leading role among nosocomially acquired MDR organisms during the past decade. Initially, the two most frequent types of ESBL were TEM and SHV types. More recently, enzymes belonging to a different type of ESBL, such as the CTX-M type (so called because they are mainly cepotaximases), are being detected with increasing frequency, particularly in Escherichia coli, an emerging cause of CA infections in many areas of the world. A shift in the distribution of different ESBLs has recently occurred in Europe, with a dramatic increase of CTX-M enzymes over TEM and SHV variants. Other non-TEM, non-SHV enzymes, such as PER, GES, IBC or certain OXA types, have also been found in some European countries. [7] Recent studies have demonstrated the clonal expansion of certain enterobacterial clones that are able to acquire multiple ESBL plasmids. These successful clones seem to have favoured the expansion of ESBLs on our continent, as exemplified by the highly virulent E. coli O25:H4-ST131, a strain that is thought to be responsible for the pandemic dissemination of the CTX-M-15 enzyme. The origin of widespread E. coli clonal complexes is still unknown, although it is likely that the resistance they exhibit against trimethoprim-sulfamethoxazole or fluoroquinolones is due to a strong selection pressure prior to ESBL acquisition. Plasmid dissemination also plays a critical role in the wide spread of ESBL in Europe.

There is a new recognition that many of the drug-resistant GNB infections that occur in hospitals but also in community are caused by strains that are globally distributed. For example, in Western Europe, strains of CTX-M-producing E. coli serogroup O25, belonging to multilocus sequence type (MLST) ST131 are increasingly isolated from both community and HA infections. This increase in geographic prevalence of clonal groups of drug-resistant GNB has been suggested to be due to increased travel to certain regions of the world where these infections are common. Others have suggested that these organisms are spread by contaminated food products due to globalization of food trade. Although it is recognized that saprophytes can harbor drug resistance genes, the extent to which they contribute to human drug-resistant infections is not evident. The observation that drug resistance genes with 100% identical nucleic acid sequences are found in different species of pathogenic GNB isolated from food sources all over the world suggests that they have common sources and are dispersed by the international food trade (8). A clonal group of E. coli strains belonging to ST69 that cause urinary tract infections (UTI), harboring the class 1 integron gene cassette configuration dfrA17-aaaD5, has been isolated from clinical sources and food sources from all over the world (9).

Romania has recently joined the European Union and it has become more intimately linked to international travel as well as food trade. It is not known what the distribution of drug-resistance determinants of GNB is and if the increasing drug-resistant infections observed in Romania are associated with the introduction of international clonal groups of drug-resistant GNB strains or due to the selection of distinct local resistant strains.

There have been different efforts at national and local levels addressing the prevalence of ESBL among community isolates. A study performed in Turkey showed a prevalence of 21% ESBL producers among E. coli causing CA urinary tract infection (UTI) during 2004 and 2005 (10). This percentage was higher than the 5.2% observed in a Spanish multicentre study covering 15 microbiology laboratories in 2006 (11). Moreover, the rate of CA bacteremias caused by ESBL-producing E. coli was 6.5% in Spain, whereas it ranged from 12.9% to 26.8% for K. pneumoniae in studies performed in Spain and the United Kingdom (12-14).

All published studies have confirmed that in most northern European countries, the prevalence of ESBL isolates is still low compared to southern and eastern European countries. Unfortunately, not all publications indicate precise frequency rates, since most of them were designed to establish the molecular epidemiology of circulating ESBLs, but not to ascertain the prevalence of these isolates. The occurrence and distribution of ESBLs in Eastern countries differs from that in other countries. The prevalence of ESBLs is over 10% in Hungary, Poland, Russia and Turkey. K. pneumoniae is the most frequent ESBL-producing species in Hungary and Russia, and an increase in the percentage of ESBL producers among K. pneumoniae isolates has been reported from Poland, Turkey, Bulgaria, Hungary and Russia [15-20].

CTX-M-3, SHV-2 and SHV-5 are usually widely spread in hospitals in eastern European countries. In Poland, the proportion of ESBL producers in hospitals (11.1%) varied for different species from
2.5% for *E. coli*, 40.4% for *K. pneumoniae* and 70.8% for *Serratia marcescens*, the latter two having a higher prevalence due to outbreak situations. ESBL types were dominated by CTX-Ms (82%, CTX-M-3) and SHV types (17%, SHV-2, SHV-5, and SHV-12), while TEM-like enzymes (<1%, TEM-19 and TEM-48) were found only sporadically. In contrast to other countries, CTX-M-15 was rarely recovered in Poland [15]. The current scenario in Poland differs from that in the late 1990s, when there was a dominance of TEM ESBLs and spread of CTX-M-3 producers all over the country [21, 22]. In Bulgaria, hospital outbreaks caused by CTX-M-3, CTX-M-15 and SHV-12 are described, often with an involvement of *S. marcescens* in addition to *K. pneumoniae* [20-22]. In Hungary, a recent eruptive and extensive spread of highly ciprofloxacin resistant CTX-M-15 *K. pneumoniae* epidemic clones has been detected [16]. Nosocomial outbreaks involving SHV-2a-producing *K. pneumoniae* are also frequent [18]. In Turkey, CTX-M-15 is widely distributed [19], and epidemic strains of *K. pneumoniae* isolates producing the carbapenemase OXA-48 and the ESBLs SHV-12 or CTX-M-15 have emerged [23].

**Carbapenemase-producing organisms**

Another issue regarding resistance in GNB is the emerging occurrence of carbapenemase-producing organisms. Carbapenemases represent the most versatile family of beta-lactamases, with a broad spectrum activity unsurpassed by other beta-lactam-hydrolyzing enzymes. Although known as “carbapenemases,” many of these enzymes recognize almost all beta-lactams, hydrolyzing, at least partially, imipenem or meropenem together with other penicillin or cephalosporin antimicrobial agents. Carbapenemases represent a heterogeneous mixture of beta-lactamases belonging to molecular Ambler class A (penicillinases), class B (metalloenzymes) and class D (oxacillinases).

Resistance to broad-spectrum antimicrobials, such as the extended-spectrum cephalosporins, is a well recognized problem among *Enterobacteriaceae* [24]. Carbapenems have served as an important antimicrobial class for the treatment of these organisms and, until recently, resistance to carbapenems has been uncommon among *Enterobacteriaceae*. However, the emergence of novel beta-lactamases with direct carbapenem-hydrolyzing activity has contributed to an increased prevalence of carbapenem resistant *Enterobacteriaceae* (CRE).

One of the recent drug resistance issues among HA infections is the emergence of a class A carbapenemase beta-lactamase that confers carbapenem resistance (for long time “last line” of antibiotic agents) to *Klebsiella pneumoniae*, referred to as KPC CRE. KPC CRE are particularly problematic given the frequency with which *Enterobacteriaceae* cause infections, the high mortality associated with infections caused by CRE [25-27], and the potential for widespread transmission of carbapenem resistance via mobile genetic elements [28]. Although CRE have primarily been recognized in health care settings [29], *Enterobacteriaceae* are common causes of both health care and community infections, raising the possibility of spread of CRE into the community. These issues, combined with the limited therapeutic options available to treat patients infected with these organisms, have made CRE of epidemiologic importance world-wide.

Although initial reports described that carbapenem resistance among *Enterobacteriaceae* was due to overproduction of Amp C-mediated beta-lactamases or ESBLs in organisms with porin mutations [30], carbapenemases have now become another mechanism for carbapenem resistance among CRE. The New Delhi Metallo-β-lactamase (NDM-1) was first reported in 2009 in a Swedish patient, followed by spreading and dissemination of NDM-1 positive strains in countries all over the world, in North America (United States, Canada), Europe (Sweden, United Kingdom, Austria, Belgium, France, Netherlands, Germany), Australia, Japan, China, Africa, and Oman. [31] NDM-1 now becomes potentially a major global health threat.

**Molecular epidemiology - an emerging science.**

The ability to identify specific strains within a given species of pathogens is an important aid in the rational development of effective measures to prevent and control NIs. The efforts of both microbiologist and hospital epidemiologists are facilitated greatly by the availability of the newer molecular epidemiologic typing techniques.

Molecular techniques can be employed to detect specific antimicrobial drug-resistance genes in a large number of organisms. Another increasingly important use for these techniques is the detection of specific point mutations associated with resistance in antiviral agents.[32]

Genotyping methods facilitate:

- the identification of the source (environmental, food, or personnel) of organisms, and distinguish infectious from noninfectious strains. It is important to determine if an isolate is a pathogenic strain causing the infection or a commensal contaminant that might not be the source of the infection, since many of the species that are key HA causes of infection are also common commensal organisms.

- documentation of cross-infection among hospitalized patients

- distinguishing relapse from reinfection, since it is
important to know whether a second infection in a patient is due to reinfection by a strain distinct from that causing the initial infection or whether the infection is likely a relapse of the original infection, because the initial treatment was not effective and requires an alternative therapy.

Phenotypic methods can occasionally be useful for epidemiological purposes; however they are too inconsistent, time-consuming, and labor-intensive to be of much use in epidemiological investigations. The introduction of DNA-based typing methods has dramatically impacted on these limitations and they are now the preferred techniques for epidemiology typing. Plasmid-profiling, restriction endonuclease analysis of plasmid and genomic DNA, Southern hybridization analysis with specific DNA probes and chromosomal DNA profiling with pulsed-field electrophoresis or PCR-based methods are the most widespread molecular techniques used for typing. [33] These methods use electrical fields to separate DNA fragments or larger molecules like plasmids or whole chromosomes into unique patterns that can be visualized using ethidium bromide or by nucleic acid probe hybridization.

Related isolates have the same DNA profile, while epidemiologically unrelated isolates can have distinctive patterns, different from the epidemiologically related ones. If isolates from different patients have the same DNA profile, they probably originated from a common source.

The source of infection (environmental or healthcare worker) can be identified by establishing clonality of pathogens. This can also be used to distinguish infectious vs. non-infectious strains and in differentiating relapse from reinfection. A number of usual causes of HA causes of infection are common commensal organisms. Consequently it is essential to differentiate whether a certain isolate from a patient is a pathogenic strain and therefore the cause of infection or a commensal contaminant that is not likely to have caused the infection. It is also very important to establish if a second infection in a patient is caused by a distinctively different strain from the one causing the primary infection, and therefore a reinfection; or whether it is likely to be a relapse of the original infection. A relapsing infection can be indicative of an ineffective therapy and, in this case, a change in treatment might be required [34]. Molecular biology typing techniques can allow microbiologists and infection control personnel to identify specific strains from a certain species and examine the epidemiology of nosocomial pathogens. This in turn can be used to introduce effective infection control measures within the hospital environment. It is important to use molecular typing techniques in combination with a thorough epidemiologic investigation. Conversely, when the most powerful molecular techniques are used indiscriminately, without epidemiologic data, these could provide confusing information.

It is widely accepted that a comprehensive and effective infection control program includes molecular strain typing and also involves the infection control and infectious diseases departments and pharmacy that will identify cases where infection control measures are needed, implement these measures in addition to establishing an adequate therapy. [35]

A comprehensive infection control program employs active surveillance by the infection control departments as well as the clinical microbiology laboratory in order to identify clusters of infections with a common microbial phenotype (same species and same antimicrobial susceptibility profile). By using a number of molecular typing methods, isolates are characterized in the laboratory in order to establish clonality. An intervention strategy is then established by the hospital epidemiologist based on the available epidemiological and molecular data. Therefore molecular typing can shorten or prevent an epidemic as well as decrease the rate and costs incurred by NIs.

Cost-effective application of typing methods

A study group led by Hacek et al. examined the medical and economic advantages of an infection control program which includes routine determination of pathogen clonality and showed that NIs were significantly reduced and more than 2 million dollars were saved over a 2-year period. [35] The decreased number of NIs following the integration of molecular typing methods within the classic epidemiologic surveillance practices has proven cost-effective. [35-39] The cost-effectiveness is maximized through the collaboration of the laboratory where genetic typing is performed and the infection control department during an outbreak. [40-42] A hospital from Chicago, USA introduced an in-house molecular typing program used to assess pathogen clonality and integrated these techniques into their infection control program. [38-39]. The effectiveness of this program was evaluated by examining data on NIs during a 7-year period: 24 months before the implementation of the new program and the following 60 months after its implementation. After the program was introduced, the rate of infections per 1000 patient-days decreased by 13%, and the number of hospitalized patients with NIs fell by 23%. The rate of infection fell to 43% below the national average, and 50 deaths were avoided during the 60 months period. The cost for the implementation of the program was 400,000 dollars; however five times as much money was saved through the reduction of NIs. [43, 44] The lower costs achieved through
the introduction of molecular typing to the infection control program for endemic NIs is linked to the ability to enact early interventions after the identification of pathogen clonality, which could signal the beginning of an outbreak. On the other hand, if unrelated pathogens are involved (sporadic infections), the initiation of a potentially costly epidemiologic investigation is avoided. A reduction in costs was also achieved by early recognition of person-to-person dissemination of pathogens compared to traditional epidemiologic surveillance. Therefore the further spread of pathogens to other patients could be avoided. A study conducted by the Center for Disease Control and Prevention evaluated the benefits and costs of the PulseNet molecular subtyping based surveillance system. [45] The investigation, led by the state health laboratories looked into the 1997 E. coli O157:H7 outbreak in which contaminated frozen hamburger meat was recalled. If the occurrence of only 15 cases could have been avoided by the recall, the PulseNet would have recovered the entire start-up costs as well as the costs for 5 more years of operation. The cost-effectiveness of the molecular subtyping system increases even more if the resources that would have been wasted on epidemiologic investigations of sporadic cases of E. coli O157:H7 would be considered. Similarly, in the hospital setting, an early detection of an outbreak would set off an appropriate investigation and the implementation of infection control measures to limit further infections, whereas in the case of sporadic infections, costly and time-consuming outbreak investigations could be minimized. Another potential use for molecular techniques would be in the early detection of nosocomial drug-resistant pathogens. Consequently, the integration of molecular methods and microbial genotyping in the traditional infection control programs has been found to be medically useful and cost-saving.

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