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REVIEW ARTICLE

Evolution of β-lactams resistance in Gram-negative bacteria in Tunisia

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Abstract

Antimicrobial resistance is a major health problem worldwide, but marked variations in the resistance profiles of bacterial pathogens are found between countries and in different patient settings. In Tunisia, the strikingly high prevalence of resistance of bacteria to penicillins and cephalorosporins drugs including fourth generation in clinical isolates of Gram negative bacteria has been reported. During 30 years, the emerging problem of extended-spectrum β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates is substantial, and some unique enzymes have been found. Recently, evidence that Gram-negative bacteria are resistant to nearly all available antimicrobial agents, including carbapenems, have emerged.

Keywords: Antibiotic resistance, β-lactamases, Tunisia

Introduction

During the past 70 years, the use of successive generations of β-lactam antibiotics has selected consecutive generations of β-lactamase enzymes, each more potent than the last. The spread of β-lactamases has driven the development of β-lactam antibiotics for 70 years (Easton and Knowles 1982). The first analogue was benzylpenicillin, which penetrated Gram-negative bacteria poorly and was destroyed by penicillinases, but spread in *Staphylococcus aureus*. These problems were overcome in the 1960s with the development of semi-synthetic penicillins that are known to penetrate Gram-negative bacterial cell wall and those that were stable to staphylococcal penicillinase (Fisher et al. 1980). The anti-Gram-negative analogues were compromised, in turn, by the spread of plasmid-mediated β-lactamases, which drove the development of the second-, third- and fourth-generation oxyimino-cephalosporins; and of β-lactamase inhibitors (Lauretti et al. 1999).

The current rising problems of β-lactams resistance in Gram-negative bacteria include CTX-M type extended-spectrum β-lactamases (ESBLs), plasmid mediated AmpC β-lactamases, and carbapenemases in *Enterobacteriaceae*, while OXA- and metallocarbapenemases are of growing importance since the last decade (Gavin et al. 2006). The main mechanism of bacterial resistance to β-lactam class of antibiotics consists of the production of β-lactamases, which are hydrolytic enzymes with the ability to inactivate these antibiotics before they reach the penicillin-binding proteins located at the cytoplasmic membrane (Helfand et al. 2003). The ESBLs are classified as Ambler class A and functional (Bush-Jacoby-Medeiros) group 2be. They are also characterized by the ability to hydrolyze the oxyimino-β-lactam at a rate 10% of that for benzylpenicillin along with inhibition by clavulanic acid (Bush et al. 1995).

ESBL encoding genes are generally acquired by horizontal gene transfer and confer resistance to oxyimino-cephalosporins, some being mutant derivatives of established plasmid-mediated β-lactamases (TEM/SHV) or moved from environmental bacteria (CTX-M)

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(Lartigue et al. 2005). The frequency with which novel enzymes have been described in the literature reflects not only the pace of discovery and the ability to differentiate these enzymes, but also their rapid emergence and evolution under the selective pressure of antibiotic usage (Galleni et al. 1988).

Nosocomial infection is an important cause of morbidity and mortality worldwide, a problem made worse when nosocomial pathogens acquire antibiotic resistance genes. This is true in the developing countries, where antibiotic-resistant pathogens may have a higher prevalence and incidence in some African countries (Bonnet 2004). The clinical impact of antimicrobial resistance may be great or insignificant, depending on the level of resistance, the site of infection, and the availability of effective, nontoxic therapeutic alternatives. In Tunisia, the extensive use of antimicrobial agents in primary health care clinics and hospitals, and in animal husbandry has allowed the rapid emergence of these resistant bacteria (Figure 1).

This article will focus on all examples of evolving resistance and the emergence of antibiotic resistant bacteria in the hospital setting and the continued evolution of extensively drug-resistant Gram-negative bacteria. This perspective is framed by our background in clinical infectious diseases and will summarize the chronology apparition of the work performed on antimicrobial resistance in Tunisia in each class of β-lactamases (Table 1).

Class A Serine β-lactamases

In general, class A enzymes are susceptible to the commercially available β-lactamase inhibitors, although *Klebsiella pneumoniae* carbapenemase may be an important exception to this generalization (Papp-Wallace et al. 2010). The first plasmid-mediated β-lactamase was identified in *E. coli* in 1963, and was named "TEM" (Datta and Kontomichalou 1965). SHV is another common β-lactamase found primarily in *K. pneumoniae* (Castanheira et al. 2008).

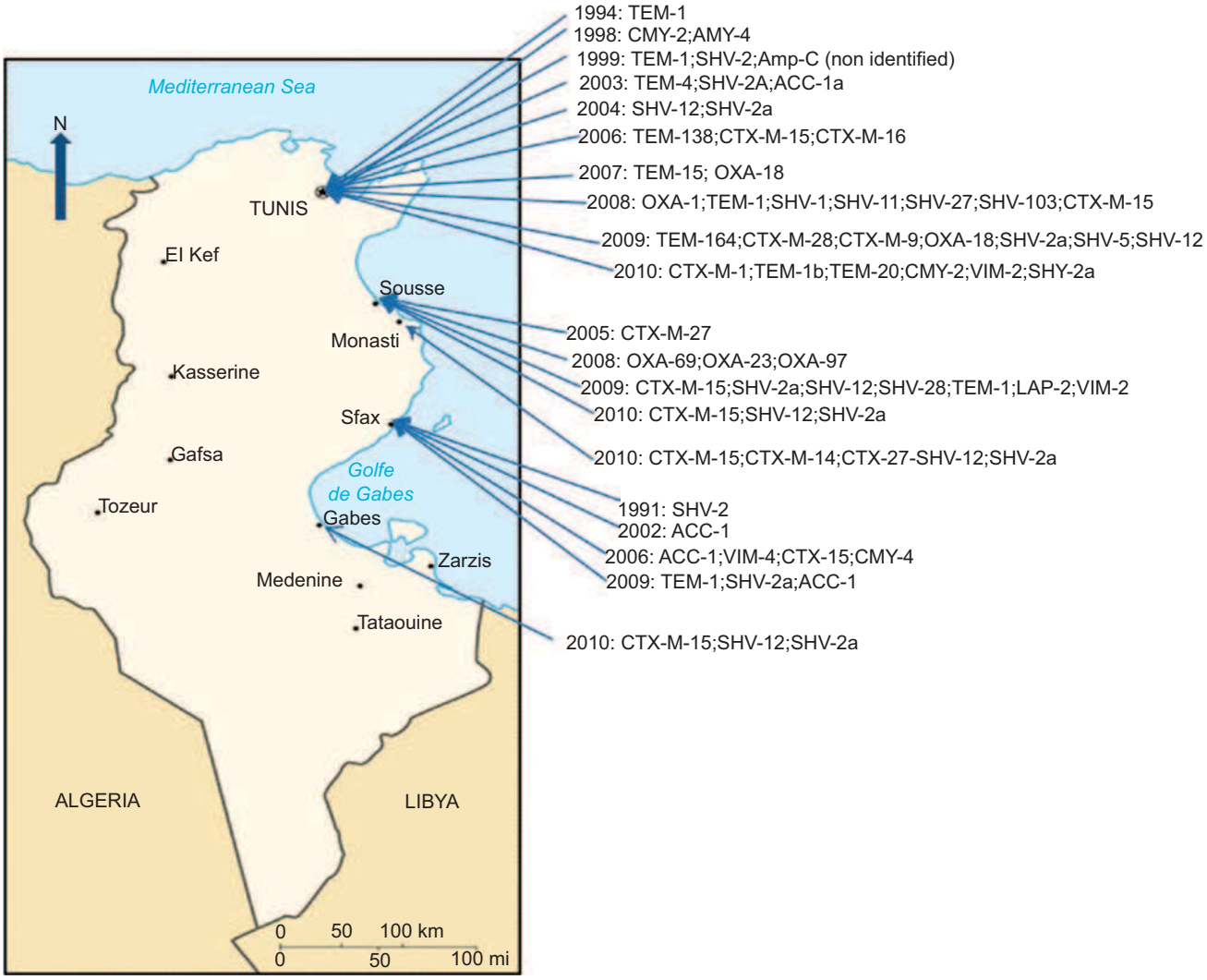

Figure 1. Map shows geographic location of the different beta-lactamases described in Tunisia. Map was obtained from http://www.misterfast.net/guide/tunisie.html

Table 1. The incidence of reported beta-lactamases in Tunisian cities.

References	Year	Journal	Species concerned	Enzymes described	Location
Hammami et al.	1991	Eur J Clin Microbiol Infect Dis	Salmonella wien	SHV-2	Sfax
Ben Hassen et al.	1994	Ann Biol Clin	Salmonella typhi	TEM-1	Tunis
Verdet et al.	1998	FEMS Microbiol Lett	Proteus mirabilis; Citrobacter freundii	CMY-2; CMY-4	Tunis
Ben Redjeb et al.	1999	Med Mal Infect	Proteus mirabilis	TEM-1; SHV-2; Amp-C (non identified)	Tunis
Rhimi et al.	2002	Pathol Biol	K. pneumoniae, P. mirabilis, S. enterica	ACC-1	Sfax
Makanera et al.	2003	J. Clin. Microbiol	Salmonella enterica	TEM-4;SHV-2a;ACC-1a	Tunis
Ben-Hamouda et al.	2004	Microb Drug Resist	Klebsiella pneumoniae	SHV-12;SHV-2a	Tunis
Bouallegue et al.	2005	J. Clin. Microbiol	Salmonella enterica	CTX-M-27	Sousse
Chouchani et al.	2006	Antimicrob. Agents Chemother	Salmonella enterica	TEM-138	Tunis
Doloy et al.	2006	Antimicrob. Agents Chemother	Klebsiella pneumoniae; Proteus mirabilis; Salmonella enterica; Escherichia coli	ACC-1	Sfax
Ktari et al.	2006	Antimicrob. Agents Chemother	Klebsiella pneumoniae	VIM-4;CTX-15;CMY-4	Sfax
Mamlouk et al.	2006	J. Clin. Microbiol	Klebsiella pneumoniae; Escherichia coli	CTX-M-15;CTX-M-16	Tunis
Lavollay et al.	2006	Antimicrob. Agents Chemother	Escherichia coli	CTX-M-15	Tunis
Chouchani et al.	2007	Diag Microbiol Infect Dis	Escherichia coli	TEM-15	Tunis
Kalai et al.	2007	Clin Microbiol Infect	Pseudomonas aeruginosa	OXA-18	Tunis
Abbassi et al.	2008	Inter. J. of Antimicrob. Agents	Klebsiella pneumoniae; Escherichia coli	OXA-1;TEM-1;SHV-1;SHV-11;SHV-27;SHV-103;CTX-M-15	Tunis
Mansour et al.	2008	Path. Biol	Acinetobacter baumannii	OXA-69	Sousse
Mansour et al.	2008	Microb. Drug. Resist	Acinetobacter baumannii	OXA-23	Sousse
Poirel et al.	2008	Antimicrob. Agents Chemother	Acinetobacter baumannii	OXA-97	Sousse
Ben Achour et al.	2009	Microb Drug Resist	Klebsiella pneumoniae	TEM-164	Tunis
Ben Achour et al.	2009	Path. Biol	Klebsiella pneumoniae	CTX-M-28	Tunis
Bourouis et al.	2009	Path. Biol	Enterobacter cloacae	CTX-M-9	Tunis
Dahmen et al.	2009	Clin Microbiol Infect	K. pneumoniae; Citrobacter freundii; Proteus mirabilis; Providencia stuartii; E. coli; Enterobacter cloacae; K. oxytoca	CTX-M-15;SHV-2a;SHV-12;SHV-28;TEM-1;LAP-2	Sousse
Kalai et al.	2009	Path. Biol	Pseudomonas aeruginosa	OXA-18;SHV-2a;SHV-5;SHV-12	Tunis
Ktari et al.	2009	Mirob. Drug Resist	Salmonella enterica	TEM-1;SHV-2a;ACC-1	Sfax
Mansour et al.	2009	Microb Drug Resist	Pseudomonas aeruginosa	SHV-2a	Sousse
Mansour et al.	2009	Diagn Microbiol Infect Dis	Pseudomonas aeruginosa	VIM-2	Sousse
Ben Slama et al.	2010	Inter J Food Microbiol	Escherichia coli	CTX-M-1;TEM-1b;TEM-20;CMY-2	Tunis
Dahmen et al.	2010	Microb Drug Resist	Klebsiella pneumoniae; Escherichia coli	CTX-M-15;SHV-12;SHV-2a	Sousse
Elhani et al.	2010	Clin Microbiol Infect	Klebsiella pneumoniae	CTX-M-15;CTX-M-14;CTX-27;SHV-12;SHV-2a	Monastir
Hammami et al.	2010	Clin. Microbiol. Infect	Pseudomonas aeruginosa	VIM-2;SHV-2a	Tunis
Cuzon et al.	2010	Int J Antimicrob Agents	Klebsiella pneumoniae	OXA-48	Djerba

In Tunisia, the rapid emergence and spread of multi-drug resistant (MDR) *Enterobacteriaceae* was observed initially in 1980. During the 1980s and 1990s, several studies describing the emergence of *Enterobacteriaceae* phenotypically were published in local journals. The first description of SHV-type ESBL in Tunisia was SHV-2 produced by a clinical strain of *Salmonella wien* (Hammami et al. 1991). In 1994, a MDR *Salmonella typhi*, isolated from the Infectious Diseases Department of la Rabta Hospital in Tunis, detected producing TEM-1

β-lactamase carried on a plasmid of about 40 kb (Ben Hassen et al. 1994). Ten years later, genotypic investigations of ESBL-producing *K. pneumoniae* recovered in a Tunisian neonatal ward showed the spread of two epidemic strains and a high number of genetically unrelated isolates. Nucleotide sequence analysis of SHV-specific PCR products from six of these isolates identified two bla_{SHV} encoding genes corresponding to SHV derived ESBLs; SHV-12 and SHV-2a (Ben-Hamouda et al. 2004). This is inconsistent with the fact that SHV-2a and SHV-12 ESBLs are the most prevalent secondary β-lactamases among clinical isolates of *Enterobacteriaceae* worldwide (Bradford et al. 1994). Their evolutionary success is likely due to their efficient activities against penicillins and narrow- to intermediate-spectrum cephalosporins and to the fact that either the bla_{SHV} encoding gene is often carried on self-transmissible or mobilizable plasmids capable of rapid horizontal spreading among different enterobacterial species (Tenover et al. 1999).

In 2005, another article was published reporting an outbreak of *Salmonella enterica* serotype Livingstone in a neonatal ward of the maternity department of Farhat Hached Hospital, Sousse. These strains were confirmed as ESBL producers and recently identified as CTX-M-27. The $bla_{CTX\text{-}M\text{-}27}$ encoding gene was located downstream of the insertion sequence IS*Ecp1* in the same position as that known for the CTX-M-14 gene (Bouallegue et al. 2005). This first report of CTX-M group in Tunisia showed the emergence of highly resistant *Salmonella* in the environment characterized by extensive use of antimicrobial agents that may provide a setting that is conducive to nosocomial transmission.

Effectively, in 2006 more work was carried out on 62 isolates of *Enterobacteriaceae* producing CTX-M-type β-lactamases collected in different wards of Charles Nicolle Hospital in Tunis. Molecular and genetic studies on these isolates showed that the $bla_{CTX\text{-}M\text{-}15}$ encoding gene was detected in 55 isolates while $bla_{CTX\text{-}M\text{-}16}$ encoding gene was only detected in 7 isolates. The CTX-M-15 producing strains were isolated in several wards and consisted mainly of two successive clonal groups of *E. coli* and a major clonal group of *K. pneumoniae* (Mamlouk et al. 2006). During that time, the increase of consumption of cefotaxime and ceftazidime might have contributed to the emergence of ESBLs and particularly to these CTX-M-type enzymes. This is the first report of CTX-M-16 type-producing *Enterobacteriaceae* in Tunisia and in Africa (Boyd et al. 2004). Another study was published in the same year showing the prevalence of CTX-M-15 ESBL between *K. pneumoniae* clinical isolates from the hospital of Sfax. These isolates were closely related using PFGE, confirming the dissemination of $bla_{CTX\text{-}M\text{-}15}$ encoding gene among these strains (Ktari et al. 2006). A third report was released in 2006 describing the dissemination of a plasmid encoded ESBL CTX-M-15 type in *E. coli* between three countries: Tunisia, France, and Central African Republic. In this report, authors studied eighty clonally related isolates by repetitive extragenic palindromic PCR and PFGE. Incompatibility typing showed that all the plasmids transferred from the clonal strains studied, MDR phenotype resembling the MDR region located in pC15-1a, a plasmid associated with an outbreak of a CTX-M-15-producing *E. coli* strain in Canada (Lavollay et al. 2006).

Other reports were appeared during 2007 and showed the emergence of new generation of TEM-type β-lactamase such as; TEM-138, A novel natural TEM β-lactamase with extended-spectrum activity, TEM-138, was identified in a ceftazidime- resistant clinical isolate of *S. enterica* serovar Infantis. Compared to TEM-1, TEM-138 contained the following mutations: E104K, N175I, and G238S. TEM-138 gene was located on a 50-kb transferable plasmid. Expression studies with *E. coli* revealed efficient ceftazidimase and cefotaximase activities of TEM-138 gene (Chouchani et al. 2006). In the same year, another variant of TEM-type β-lactamase corresponding to TEM-15 has been reported. In this study; authors showed that the TEM-15 gene was part of a chromosomally located *Tn*801 transposon. These isolates were resistant to β-lactams, including ureidopenicillins, ticarcillin–clavulanic acid, cefpirome, ceftazidime, and cefotaxime, but remained susceptible to imipenem and cefoxitin (Chouchani et al. 2007).

Between 2008 and 2009 CTX-M variants continued to appear. Indeed emergence of CTX-M group β-lactamases was increased; firstly in a collection of *K. pneumoniae* and *E. coli* ESBL-positive isolates recovered in Bone Marrow Transplantation Centre of Tunis (BMTCT). CTX-M-15 is the most prevalent β-lactamase detected amongst ESBL-positive *K. pneumoniae* and *E. coli* strains with a CTX-M phenotype in Tunisian hospital. In fact, the presence of the $bla_{CTX\text{-}M\text{-}15}$ encoding gene amongst unrelated strains and the unique genetic background of the $bla_{CTX\text{-}M\text{-}15}$ gene argued for genetic transit of mobile elements amongst unrelated strains. The largest plasmid found in all strains may harbour the CTX-M-15 gene (Abbassi et al. 2008). In concordance with this chronologic study, MDR *Enterobacter cloacae* isolated from the Military Hospital of Tunis produced new plasmid-encoded CTX-M-9 has been reported (Bourouis et al. 2009); this β-lactamase occurs almost exclusively in *E. coli*, and it is widely present in Spain (Govinden et al. 2007).

In addition to that, most recently, a paper was published identifying cefotaxime-resistant *K. pneumoniae* by production of CTX-M-28. These strains were isolated from the intensive care unit of the Military hospital in Tunis. CTX-M-28 gene was found located in a transferable plasmid (Ben Achour et al. 2009b). In CTX-M-28, amino acid at position 240 has an important role in the evolution of CTX-M type ESBLs. This substitution has already been reported in CTX-M-16 and is known to confer high-level resistance to ceftazidime. Another substitution known to increase the hydrolyzing activity of ceftazidime is the Pro167Ser in CTX-M-19 gene (Sturenburg et al. 2004). In the mean time, another paper was published studying qnr-positive *Enterobacteriaceae* isolates producing

ESBLs, being predominantly of the CTX-M-15 type, but also of the SHV-28 and SHV-12 types. The qnr genes were located on transferable plasmids. The qnrB2 gene was associated with sul1-type integron structures and the qnrB1 gene was associated with orf1005. This study highlighted the wide spread of Qnr-like determinants in Tunisia associated with the ESBL CTX-M-15 in human clinical isolates (Dahmen et al. 2009).

A novel variant of TEM was detected in a MDR *K. pneumoniae* strain, this phenotype was due to the production of a new TEM-164 ESBL with unusual mutations: L40V and I279T. This modification changed the profile of this ESBL and accordingly was able to hydrolyze cefotaxime and ceftazidime effectively. TEM-164 gene was located in a 50 kb conjugative plasmid (Ben Achour et al. 2009a). Other variants of SHV type were detected in *P. aeruginosa* isolated from National Bone Marrow Transplantation Center (NBMTC) (Kalai-Blagui et al. 2009). *P. aeruginosa* isolates were detected positive for ESBL and had the SHV-2a gene. These positive isolates were clonally related according to Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) results and this SHV-2a gene was found chromosomally located, and flanked by IS26 sequence immediately upstream of the gene (Mansour et al. 2009a).

In 2010, a new paper published in International Journal of Food Microbiology described the occurrence of resistant *E. coli* isolates from food samples of animal origin obtained from different supermarkets and local butcheries in Tunisia. These isolates were ESBL-positive and exhibited different PFGE patterns. Molecular and genetic studies showed that these isolates produced; CTX-M-1 type ESBLs and was associated with TEM-1b and TEM-20. The orf477 sequence was identified downstream of $bla_{\text{CTX-M-1}}$ encoding gene whereas ISEcp1 was located upstream of the same gene. *E. coli* isolates from food samples could represent a reservoir of ESBL encoding genes and integrons that could be transmitted to humans through the food chain (Ben Slama et al. 2010). Also in 2010, another article was published on ESBL producing *Enterobacteriaceae* strains collected from the intensive care unit and the urology ward of the University Hospital of Sahloul. The majority of the isolates showed high level of resistance to cefotaxime and ceftazidime. Molecular and genetic studies revealed that the majority of strains (91%) carried genes encoding CTX-M-15 while 9% and 3% were produced from SHV-12 and SHV-2a genes respectively. CTX-M-15 ESBLs accounted for the overpowering majority of ESBL types among *Enterobacteriaceae* from Tunisian hospital. This study confirms the high rate of ESBL in Tunisia and further demonstrates the worldwide spread of CTX-M-15 gene in the clinical setting (Dahmen et al. 2010).

The last article appeared in 2010 and described ESBL producing *K. pneumoniae* isolates collected at Mongi Slim University Hospital, Tunis. This study showed the occurrence of bla_{SHV}, $bla_{\text{CTX-M}}$, and bla_{TEM} encoding genes, demonstrated that out of 47 CTX-M type ESBLs; 43 were CTX-M-15, two CTX-M-14 and two CTX-M-27. 58 isolates were producers of SHV-12, and three were producers of SHV-2a. The MLST results showed large genetic background diversity in the SHV-12-producing isolates and dissemination of specific clones of the CTX-M-15-producing isolates within the same ward and among wards, and suggested endemicity with horizontal dissemination of the $bla_{\text{CTX-M-15}}$ and the $bla_{\text{SHV-12}}$ encoding genes (Elhani et al. 2010). Regarding the evolution of class A ESBLs in Tunisia we conclude that the ESBL patterns have now changed dramatically owing to the emergence and expansion of the relatively new CTX-M-type ESBL enzymes. Worldwide, more than 65 CTX-M β-lactamases were recognised so far and they are clustered in five groups based on their amino acid identities: CTX-M-1,-2, -8, -9, and -25 groups (Labia R. 1999; Barlow et al. 2008).

Class B Metallo-β-lactamases

Class B enzymes are Zn^{2+} dependent β-lactamases that demonstrate a hydrolytic mechanism different from that of the serine β-lactamases of classes A, C, and D (Drawz and Bonomo 2010). These enzymes hydrolyze carbapenems poorly but are able to confer resistance and are only partially inhibited by clavulanate. Metallo- β-lactamases (MBLs), like all β-lactamases, can be divided into those that are normally produced by chromosomally mediated antibiotic resistance genes and those that are encoded by transferable genes (Laraki et al. 1999). MBLs are likely evolved separately from the other Ambler classes, which have serine at their active site (Massova and Mobashery 1998; Lodise et al. 2004). Organisms produce these enzymes usually exhibit resistance to penicillins, cephalosporins, carbapenems, and the clinically available β-lactamase inhibitors (Walsh et al. 2005). Unfortunately, *P. aeruginosa*, *K. pneumoniae*, and *A. baumannii* produce class B enzymes encoded by mobile genetic elements. In contrast, *Bacillus* spp., *Chryseobacterium* spp., and *Stenotrophomonas maltophilia* harbor chromosomally encoded MBLs, but the majority of these pathogens are not frequently responsible for serious infections (Walter et al. 1996).

In Tunisia, only three studies showed the occurrence of MBLs, this shortage is due to the fact that metallo-β-lactam antibiotics were recently introduced as a therapy in Tunisian hospitals. However, the first study was published in 2006 on the emergence of clinical isolates multidrug-resistant *K. pneumoniae* producing VIM-4 MBL associated with the overproduction of CTX-M-15. These isolates were moreover highly resistant to carbapenems and were closely related as shown by PFGE. Molecular studies showed that $bla_{\text{VIM-4}}$ encoding gene was part of class 1 integron (Ktari et al. 2006). The emergence of the VIM-4 gene suggested the wide circulation of MBL-encoding genes and possesses challenges for the treatment of hospital infections due to Gram-negative bacterial infections. These data showed the outbreak of imipenem-resistant *K. pneumoniae* occurred in University hospital of

Sfax from July 2005 until July 2006. Although the spread of bla_{VIM} positive isolates was confined to this hospital units and spread at a low rate in this hospital, a strict infection control measures against such isolates should have been implemented to prevent their further dissemination. These precautions were not strongly respected, as three years later, another study presented, in addition to VIM-4 gene that previously described the detection of other MBL producers among a collection of nonrepetitive carbapenem-resistant *P. aeruginosa* from the University Hospital Sahloul. Five isolates that produced the MBL VIM-2 were clonally related according to PFGE analysis. This gene cassette was in class 1 integron and very likely to been chromosomally located (Mansour et al. 2009b). Only the MBL VIM-4 was detected in *K. pneumoniae* isolates in Sfax which located 200 km far from Sousse. Similarly, *P. aeruginosa* VIM-2 producing isolates were described as responsible for a polyclonal outbreak in a large tertiary-care center in Taiwan (Muang et al. 2007).

A 2010 study showed the diversity of VIM-2 genes associated with an occasional SHV2a gene in isolates of a persistent MDR *P. aeruginosa* strains. This study demonstrated the incidence of the MBL VIM-2 as gene cassette in class 1 integron in *P. aeruginosa* collected from different wards at Charles Nicolle hospital of Tunis. DNA sequences surrounding SHV-2a gene shared high identity with a *K. pneumoniae* plasmid sequence. Despite being clonal as shown by PFGE, the VIM-2 producing *P. aeruginosa* isolates prevalent at this hospital displayed a diversity of VIM-2 carrying integrons. Furthermore, a cloned fragment from one isolate of the collection was found to carry class 1 integron and the cassette region contained VIM-2, aacA7, and aacA4 genes. In the other isolates, the upstream region of the integron had an insertion sequence element, ISPa7 bracketed by two 17-bp inverted repeats. VIM-2 gene was found in the first position of the integron, which indicates that it was the most recently acquired gene (Hammami et al. 2010). These results are consistent with the study of Pitout and co-workers in Italy in 2007 (Pitout et al. 2007) and differs from that reported in Kenya in 2008 (Pitout et al. 2008).

Class C Serine Cephalosporinases

Class C AmpC β-lactamases are usually chromosomally encoded genes, although plasmid-borne AmpC enzymes are becoming more prevalent (Philippon et al. 2002). Bacteria expressing AmpC β-lactamase are typically resistant to penicillins, β-lactam/β-lactamase inhibitor combinations, and cephalosporins (Bush et al. 1993). Production of chromosomal AmpCs in Gram-negative bacteria is at a low level ("repressed") but can be "derepressed" by induction with certain β-lactams, particularly cefoxitin (Bethel et al. 2008). Investigation of the different mechanisms of this regulation has been the subject of intense studies early published (Jacobs at al. 1997). The selection of mutant bacterial populations that are genetically depressed for AmpC production is of significant concern; it can cause a dramatic increase in Minimum Inhibitory Concentrations (MICs) during the course of β-lactam therapy (Juan et al. 2005).

In Tunisia, the first class C β-lactamase was reported in 1998. This article characterized AmpC-type plasmid-mediated β-lactamase, CMY-4 type, isolated from MDR clinical isolates of *Proteus mirabilis*. The nucleotide sequence of the gene encoding the AmpC-type enzyme was determined. The amino acid sequence of CMY-4 gene showed a 98–99% identity with CMY-3 gene and to those of the plasmid-mediated AmpC-type β-lactamases originated from *Citrobacter freundii*. This enzyme differed from CMY-2 gene by one Arg 221 Trp substitution and from CMY-3 gene by two substitutions Glu 42 Gly and Ser 363 Asn (Verdet et al. 1998).

In 1999, another article was published describing a MDR clinical isolate of *P. mirabilis,* collected from a patient hospitalized in Charles Nicolle Hospital. The susceptibility pattern to β-lactams was similar to the usual profile mediated by the synthesis of cephalosporinases. This resistance transferred by conjugation, PCR experiments were conducted to detect the occurrence of AmpC β-lactamase encoding gene in *C. freundii* and *Enterobacter cloacae* and the results showed positive PCR products suggesting that resistance was due to the synthesis of plasmid mediated AmpC type β-lactamase (Ben Redjeb et al. 1999).

Three years earlier, another article was released showing the detection of plasmid-encoded ACC-1 cephalosporinase among clinical *Enterobacteriaceae* isolates collected from different units of the University hospital of Sfax. The investigation was conducted to examine 35 clinical strains of *Enterobacteriaceae* resistant to ceftazidime. Synergy between ceftazidime and amoxicillin/clavulanate was obtained suggesting the occurrence of ACC-1gene among isolates of *K. pneumoniae*, *P. mirabilis,* and *Salmonella*. The IEF demonstrated the simultaneous production of several β-lactamases including TEM, SHV-2, and ACC-1 genes among *S. enterica* ser. Livingstone. PCR and sequencing experiments showed the occurrence of ACC-1 gene shared high identity (99–100%) to ACC-1 gene previously described (Rhimi-Mahjoubi et al. 2002).

Interestingly, in 2003 a paper published reporting the molecular epidemiology of plasmid-encoded β-lactamases produced by 31 clinical isolates of *S. enterica* serotype Mbandaka resistant to broad-spectrum cephalosporins in Tunisia. PCR and DNA sequencing identified these genes as TEM-4 and SHV-2a. The remaining isolates were highly resistant to ceftazidime and produced a β-lactamase that focused at pI 7.8. Sequencing of PCR products showed that the plasmid mediated AmpC-type enzyme was ACC-1a. Fingerprinting analysis by repetitive-element PCR and ERIC-PCR suggested that 29 out of 31 *Salmonella* serotype Mbandaka isolates belonged to the same clonal population (Makanera et al. 2003). These studies confirmed that *S. enterica* isolates

are an important reservoir of genes encoding resistance to broad-spectrum cephalosporins in Tunisia. These multi-resistant isolates of *Salmonella* are often responsible for nosocomial outbreaks and animal-to-human transmission of disease (Winokur et al. 2001).

During 2006, two articles were published; the first work described the genetic environment of acquired ACC-1 gene in *Enterobacteriaceae* isolates. This paper investigated the genetic organization of ACC-1 gene in 14 isolates of *Enterobacteriaceae* and the results showed that in a common ancestor, IS*Ecp1* was likely to be involved in the mobilization of this gene from the *Hafnia alvei* chromosome to a plasmid. Other genetic events involving insertion sequences, transposons and *sulI*-type integrons have occurred, leading to complex genetic environments. Generally, IS*Ecp1* was never complete, and its deletion may have led to the stabilization of ACC-1 gene on different plasmids (Doloy et al. 2006). It seemed that several different DNA-mobilizing elements, such as common regions associated with integrons and insertion sequences are involved in the movement of the AmpC gene and the adjacent regions (Verdet et al. 2006). The second paper was released describing the emergence of MDR *K. pneumoniae* isolates producing CMY-4 AmpC β-lactamase, in Tunisian University hospital. The isolates were closely related as shown by PFGE, and they produced CMY-4 AmpC ESBL associated with the production of VIM-4 and CTX-M-15 genes. The coexistence of two enzymes, a MBL and non-MBL enzymes in the same strain has suggested how dangerous they are (Ktari et al. 2006).

Three years later, one more work was published showing the molecular epidemiology and studying the genetic environment of acquired $bla_{\text{ACC-1}}$ encoding gene in *S. enterica* Serotype Livingstone causing a large nosocomial outbreak in Tunisia. PFGE showed that these isolates were closely related and the antimicrobial susceptibility testing demonstrated a particular β-lactam resistance phenotype, suggested the presence of an AmpC-type enzyme. TEM-1 gene was characterized in all strains and SHV-2a gene in only two strains. The plasmid-borne ACC-1 gene mapped immediately downstream of *ISEcp1*. This *ISEcp1* insertion sequence was itself disrupted by IS26 insertion sequence. A supplementary deletion of 13 bp was observed in ISEcp1 upstream IS26 (Ktari et al. 2009). Acquisition of new resistance genes by this genus could be facilitated by the simultaneous presence in the environment of naturally resistant *Enterobacteriaceae* or Gram-negative bacilli and by traces of antimicrobial agents used in human and veterinary medicine. This resistance was mostly due to the acquisition of plasmid-mediated ESBLs such as SHV-2, TEM-4, and CTX-M-27 (Bidet et al. 2005).

The last article was appeared in 2010 year studying the prevalence of broad spectrum cephalosporin resistant *E. coli* isolates in food samples in Tunisia. In this study, CMY-2 type β-lactamase was detected in one of these isolates. Furthermore, ESBL positive *E. coli* isolates in a high percentage of food samples analyzed in this study (12.6%) is of remarkable relevance, indicating that food of animal origin represents a reservoir of this type of resistant bacteria that potentially could be transferred to humans through the food chain (Ben Slama et al. 2010).

Class D Serine Oxacillinases

Class D β-lactamases were initially categorized as "oxacillinases" because of their ability to hydrolyze oxacillin at a rate of at least 50% of that of benzylpenicillin which is in contrast to the relatively slow hydrolysis of oxacillin by classes A and C (Bush et al. 1995). Generally speaking, OXA enzymes are resistant to inhibition by clavulanate, sulbactam, and tazobactam (Mulvey et al. 2004; Poirel and Nordmann 2006). Site-directed mutagenesis studies suggest that susceptibility to inhibition by NaCl is related to the presence of a Tyr 144. Presumably, Tyr144 may facilitate binding of NaCl better than the Phe residue found in resistant oxacillinases, although the molecular mechanism remains unexplained (Girlich et al. 2004). Examples of OXA genes include those rapidly emerging in *A. baumannii* like OXA-23 and OXA-40 genes and constitutively expressed in *P. aeruginosa* like OXA-50 gene (Walther-Rasmussen and Hoiby 2006).

In Tunisia, class D β-lactamase was exceptionally encountered in clinical isolates, and the first report was very recent dated in 2007 and described the nosocomial outbreak of OXA-18 gene produced by *P. aeruginosa* isolates resistant to ceftazidime which were recovered at the NBMTC of Tunisia. The ESBLs produced by these isolates were inhibited by clavulanate, and PFGE defined two dominant genotypic groups: group A and group B. Sequencing of PCR products from representative isolates identified OXA-18 gene associated with the overproduction of SHV and TEM β-lactamases. Isolates producing OXA-18 gene belonged to genomic group A and were isolated from immunocompromised patients and from two wash-basins in the graft unit. No immunocompromised patients harbored the clonal epidemic strain upon admission. Early detection of these isolates, and information concerning their dissemination, are important, as outbreaks caused by *P. aeruginosa* are usually difficult to control because *P. aeruginosa* may like other *Enterobacteriaceae* constitute a reservoir of ESBL genes. The spread of OXA-18 producing *P. aeruginosa* strains may be enhanced by under-detection (Kalai-Blagui et al. 2007).

In 2008, three papers were reported the presence of class D β-lactamases in Tunisia. Mansour and co-workers reported the dissemination of OXA-23 gene produced by 99 clinical strains of *A. baumannii*. The carbapenem-resistant *A. baumannii* isolates were obtained from patients hospitalized at the University hospital Sahloul. Amplification and sequencing of genes encoded for such phenotype showed that these isolates produced the carbapenem-hydrolyzing oxacillinase OXA-23. All the OXA-23 positive isolates were clonally related, and the

bla$_{OXA-23}$ encoding gene was found to be chromosomally located and associated with an upstream-located insertion sequence ISAba1 located upstream of the OXA gene (Mansour et al. 2008b).

Another work published in the same year characterized the resistance mechanism to β-lactams in 26 clinical strains of *A. baumannii*. The IEF of the crude extract revealed two bands of β-lactamase activity with a pI upper than 8. None ESBL or MBL was detected and PCR experiments for AmpC, ISAbaI and OXA-69 produced products in all studied strains. Sequencing of these alleles gave high identity (99–100%) with genes described previously and PFGE analysis demonstrated clonality of isolates. These results suggested that resistance to β-lactams including imipenem is associated to the hyper production of the AmpC enzyme and expression of OXA-69 gene. Those enzymatic mechanisms are associated with the natural low permeability to β-lactams which characterizes *A. baumannii* (Mansour et al. 2008a).

The third study focused on 39 MDR *A. baumannii* isolates belonged to two distinct clones collected at the Sahloul hospital. One clone included 19 isolates produced OXA-97 gene that differed from OXA-58 gene by a single amino acid substitution and conferred the same β-lactam resistance profile as OXA-58 gene. OXA-97 gene was located on plasmids that varied in size in 18 isolates and was chromosomally located in a single isolate. Cloning and sequencing identified genetic structures surrounding the *bla*$_{OXA-97}$ encoding gene similar to those reported to be adjacent to the OXA-58 gene. In addition, the novel IS*Aba8* element was identified (Poirel et al. 2008). The emergence of OXA-23, OXA-69, and OXA-97 genes in the same hospital indicates that carbapenem resistant are in this hospital environment. These studies represent the first reporting of the nosocomial dissemination of a CHDL-producing *A. baumannii* strain in Tunisia and in Africa, after the identification of single OXA-23-producing *A. baumannii* isolates from Algeria, Libya, and South Africa (Corvec et al. 2007; Segal et al. 2005). The current worldwide emergence of multi-resistant *A. baumannii* is mostly associated with carbapenemase producers. Therefore, such carbapenemases may be considered as main targets in creating new inhibitors.

More recently in 2010, Cuzon and co-workers reported a multidrug-resistant strain of *K. pneumonia* isolate from an 86-year-old male with severe chronic obstructive pulmonary disease. The antibiogram data revealed that *K. pneumoniae* HPA-1 was resistant to extended-spectrum cephalosporins, ertapenem and meropenem but was susceptible to imipenem. Amplification and sequencing of genes encoded for such resistance identified the OXA-48 gene associated with the overproduction of TEM-1 and CTX-M-15 β-lactamases. Using a series of PCR primers, two copies of the insertion sequence IS1999 were found surrounding the OXA-48 gene. Plasmid analysis identified three plasmids, one of 120 kb harboring *bla*$_{CTX-M-15}$ encoding gene, one of 90 kb harboring TEM-1 gene and a 70 kb plasmid similar in size to plasmid pA-1 harboring OXA-48 gene (Cuzon et al. 2010). This 70 kb plasmid once transferred to *E. coli*, conferred β-lactam resistance pattern with reduced susceptibility to carbapenems (Cuzon et al. 2010). This work has provided the further evidence of the spread of the OXA-48 gene outside of Turkey and again in the Mediterranean regions (Aubert et al. 2006; Carrër et al 2008). The diversity of carbapenemases in *Enterobacteriaceae* (KPC,VIM/IMP, and OXA-48) is of great concern in determining future therapeutic options for infected patients in that region. In addition, in OXA-48-producing isolates the imipenem MIC values may remain low, leading to lack of detection and thus enhancing the silent spread of this resistance determinant.

Finally, the evolution of Gram-negative bacterial resistance to antimicrobial drugs in Tunisia is summarized in Figure 2.

Conclusion

The increasing prevalence and shifting epidemiology of ESBL-producing microorganisms, particularly *Enterobacteriaceae*, render the infections caused by

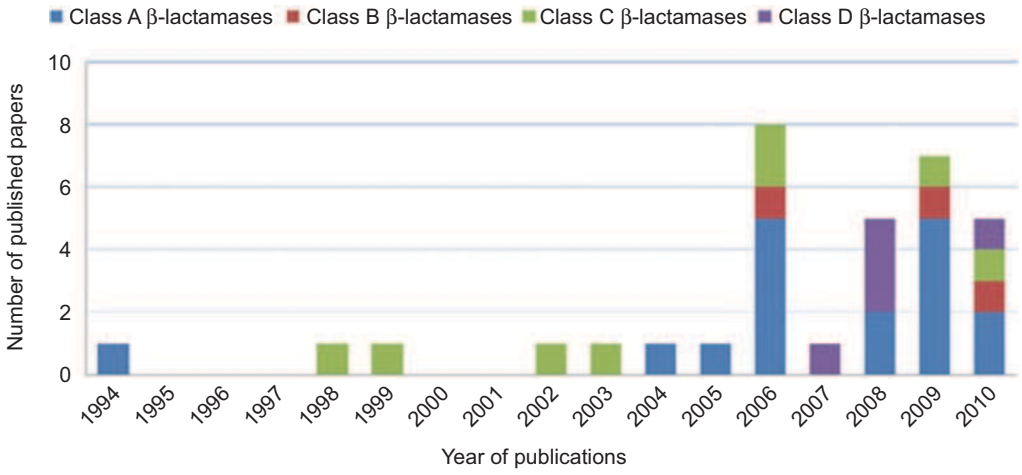

Figure 2. Chronologic order of publications showing the prevalence of antimicrobial resistance in Gram-negative bacteria in Tunisia from 1994 to 2010.

these pathogenic micro-organisms as an important public health problem. The resistance of Gram-negative bacteria to extended-spectrum β-lactams by the production of β-lactamases renders the options to treat various bacterial infections ineffective. This may be of particular importance for community-acquired infections, since options for oral antibiotic therapy against ESBL producing organisms appear to be limited. Regarding nosocomial infections caused by these organisms, carbapenems are considered the most reliable therapeutic agents. However, a trend of increasing resistance among pathogens causing nosocomial infection continues to be seen. Currently, the main task is to encourage the appropriate use of antibiotics in hospital settings. Further research is required using appropriate strategies to limit the emergence and spread of resistant bacteria, both in the community and the hospital settings, as well as to evaluate the available therapeutic agents and identify new ones.

In the new millennium, many measures to control resistance problems should be instituted in Tunisia in order to tackle this problem. Measures include restricting the use of antibiotics in trivial upper respiratory tract infections and avoiding the inappropriate use of antibiotics for surgical prophylaxis. Moreover, antibiotics should be removed from the list of non-prescriptive drugs available at drugstores. Antibiotic interventions should be implemented in many hospitals, particularly in intensive care units, to address the high prevalence of resistance among nosocomial pathogens. The Ministry of Sanitary in collaboration with the Ministry of Agriculture should prohibit the use of several antimicrobial agents that have been widely used as growth promoters or prophylactic agents in animal husbandry in Tunisia, as they may select for critical forms of resistance in human pathogens in food-producing animals.

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