

National multicenter survey of ESBL-producing *Enterobacteriaceae* and of multi-drug resistant Gram-negative non-fermenters in Belgium in 2010

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Objectives:

During a national multicenter survey conducted in Belgian acute care hospitals in 2010, the objectives were to assess and characterize:

- (1) The species distribution and diversity of extend-spectrum β -lactamases (ESBLs) genes found in *Enterobacteriaceae* isolates.
- (2) The mechanisms of β -lactam resistance to 3rd and/or 4th generation cephalosporin-resistant isolates that were referred to us as putative ESBL producers
- (3) The most prevalent mechanisms of resistance to carbapenems and other beta-lactams in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates

Materials and Methods:

Study design

All Belgian acute care hospital-affiliated laboratories were invited to participate by mailing in November 2009. Participants were asked to send a maximum of 5 non-duplicate clinical isolates of ESBL-producing Enterobacteriaceae as well as 5 non-duplicate isolates of multi-drug resistant *P. aeruginosa* and/or *A. baumannii*. These isolates were collected prospectively between 01/2010 and 05/2010 from clinical specimens and the participants were requested to fill in a short questionnaire with demographic data (age, gender), source of isolation (i.e. body site), as well as the hospitalization or ambulatory status of the patient. All isolates were referred centrally (1) for confirmation of species identification, and (2) phenotypic and genotypic characterization of ESBLs and/or carbapenemases.

Case definition

ESBL-producing *Enterobacteriaceae* were considered on the basis of decreased susceptibility to any 3rd generation cephalosporin (cefotaxime, ceftriaxone, ceftazidime) and the presence of a synergy between clavulanic acid and one or several cephalosporin substrates (cefotaxime, ceftazidime) whatever the method(s) used by the participants (manual or automated system).

The definition of Multidrug resistant (MDR) *P. aeruginosa* and *A. baumannii* referred to isolates that were resistant *in vitro* to at least 3 different classes of drugs, including broad-spectrum cephalosporins (ceftazidime), fluoroquinolones (ciprofloxacin/levofloxacin) and aminoglycosides (at least one among gentamicin, tobramycin and amikacin).

Identification and susceptibility testing

All isolates were analysed by MALDI-TOF MS (Bruker Daltonik GmbH, Leipzig, Germany) for confirmation of species identification. MICs of 15 antibiotics against confirmed ESBL-producing Enterobacteriaceae isolates and selected MDR *P. aeruginosa* and *A. baumannii* isolates (i.e. those carbapenemase- and/or ESBL-producing isolates) were determined by the reference CLSI broth dilution method (GNX2F plate, Sensititre[®], Trek Diagnostics, UK). The antimicrobial agents tested against Enterobacteriaceae isolates included: ertapenem, imipenem, meropenem, colistin, temocillin, tigecycline, gentamicin, amikacin, piperacillin-tazobactam,

cefotaxime, ceftazidime, cefepime, ciprofloxacin, levofloxacin and cotrimoxazole. The agents tested against *Pseudomonas aeruginosa* and *Acinetobacter* spp. the agents tested were: imipenem, meropenem, doripenem, colistin, polymyxin B, gentamicin, tobramycin, amikacin, piperacillin-tazobactam, ceftazidime, cefepime, aztreonam, ciprofloxacin and levofloxacin.

Phenotypic ESBL characterization

Detection of ESBL was performed centrally by double discs approximation test (Jarlier's test) on Mueller-Hinton agar using amoxicillin-clavulanate (30/10 µg) paper discs in the centre and cefotaxime (30 µg), ceftazidime (30 µg) and cefepime (30 µg) paper discs disposed 20-30 mm side to side. We used a double combination disc test (DDT) with cefotaxime (CTX) and ceftazidime (CAZ) 30 µg discs alone and in combination with clavulanic acid (CLAV) as confirmatory test. For AmpC inducible species (i.e. *Enterobacter* spp, *Citrobacter freundii*, *Morganella morganii*, *Providencia* spp. and *Serratia* spp.) the DDT assay was carried out in the presence of 400 µg of 3-amino-phenylboronic acid (APBA), a specific inhibitor of AmpC β-lactamases which was spotted on all discs. A difference of diameter ≥ 5 mm in the presence of clavulanic acid versus one or both cephalosporin indicators alone was deemed as a positive ESBL result.

In *K. oxytoca*, a bacterial species which may overproduce a chromosomal β-lactamase (K1/OXY) inhibited by clavulanic acid, and has the ability to hydrolyze partially cefotaxime and cefepime but not ceftazidime, ESBL was only inferred when a positive synergy test was observed both with CTX and CAZ.

The presence of AmpC was deduced phenotypically when a difference of diameter of ≥ 5 mm was observed for one of both cephalosporins in the presence of APBA. AmpC production was further confirmed using cloxacillin (250 mg/L) containing plates. In *K. oxytoca*, the presence of a synergy between one or both cephalosporin agents and APBA was not considered as indicating the presence of AmpC since APBA is also known to bind to and inhibit the K1/OXY β-lactamase enzyme.

Genotypic ESBL/ carbapenemase characterization

ESBLs were characterized by a commercial PCR-ligase DNA microarray assay (KPC-ESBL array, Check-Points, Wageningen, The Netherlands) targeting the different *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M} (including CTX-M of group -1, -2, -9 and -8/-25) and the *bla*_{KPC} genes. In cephalosporin-resistant *E. coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Salmonella* spp. with an ESBL-negative phenotype (i.e. lack of synergy with clavulanic acid) or with negative results by ESBL check-points array, genes encoding the six major phylogenetic groups of plasmid-mediated AmpC β -lactamases were sought using a multiplex PCR assay as previously described (Bogaerts *et al. J Antimicrob Chemother.* 2009;63:1073-5). Single or multiplex end-point PCR assays targeting OXA-1/-30, OXA-2, OXA-10 groups coding genes, OXA-48 (class D carbapenemase in Enterobacteriaceae), OXA-23/-40/-58/-143 (Class D carbapenemases in *Acinetobacter* spp.), VIM/IMP/NDM (class B carbapenemases) as well as minor ESBL-coding genes (PER, VEB, GES, BEL) in *Pseudomonas* and in *Acinetobacter* spp. were carried out selectively depending on the phenotypic resistance profiles as well as on the results of other genotypic tests.

Epidemiology of resistance

The types of ESBL enzymes, their frequencies and species distributions were assessed and compared to the data obtained from the previous national surveys in 2006 and in 2008. In contrast to the previous surveys in which we only focused on ESBLs in Enterobacteriaceae isolates, we also asked the participants to send MDR *P. aeruginosa* and *A. baumannii* in order to screen for the possible presence of multi-drug resistant clones with emerging resistance mechanisms (Class A, B, and D carbapenemases and ESBLs) in these species.

Results

On the whole, 90 participants (43 from Flanders, 30 from Wallonia and 17 from Brussels) sent 433 putative ESBL-producing *Enterobacteriaceae* isolates collected between Feb. 15th and May 15th 2010. Of these, 431 isolates were viable at culture and could be tested. Additionally, 122 isolates of presumptive multi-drug resistant (MDR) *P. aeruginosa* (n=114) and *A. baumannii* (n=8) were also received from 48 and 6 hospitals, respectively. Among these, 107 isolates could be confirmed as *P. aeruginosa* and 6 as *A. baumannii* (the other culture isolates either were contaminated and did not match with the expected identification or did not grow).

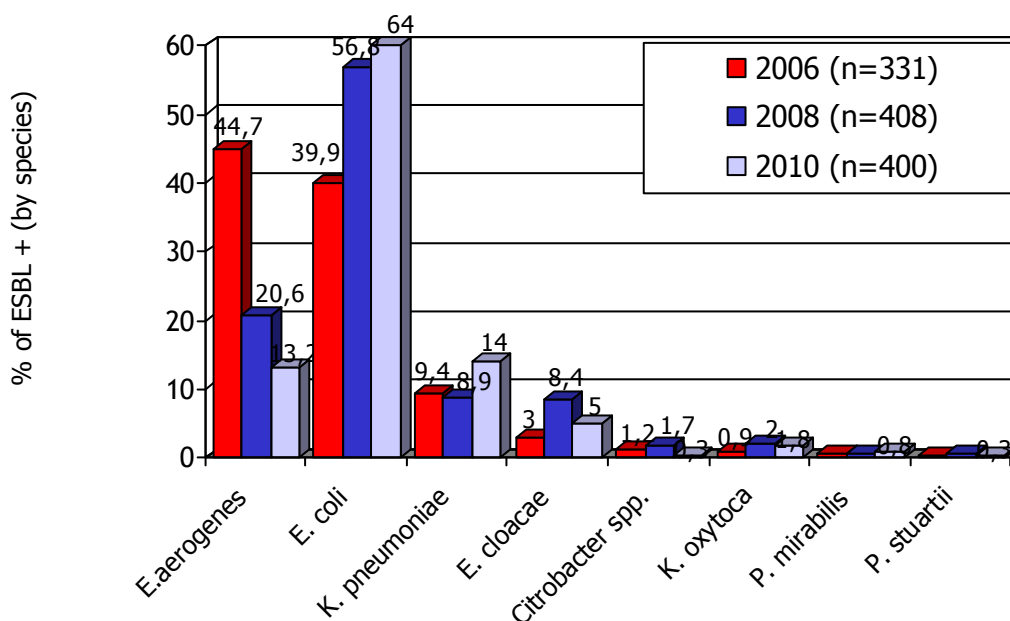
ESBL detection in Enterobacteriaceae

Overall, we could confirm the presence of an ESBL in 400 isolates. A simple disc approximation test (Jarlier's test) using cefepime, ceftazidime and cefotaxime and a central disc containing amoxicillin/clavulanate) detected 311 of the ESBL-producing *Enterobacteriaceae* isolates. For 89 of the isolates, no synergy was observed by a standard double disc approximation test but combined DDT using CAZ and CTX with or without CLAV yielded positive results for an ESBL. Interestingly, in 4 AmpC producing strains (2 *E. aerogenes*, 1 *E. cloacae*, 1 *E. coli*); a synergy with clavulanic acid could only be observed in the presence of APBA 400 µg and/or when the test was carried out on cloxacillin (250 µg/ml) containing Mueller-Hinton agar plates.

Epidemiologic data

The ESBL producing strains originated from patients with a mean age of 66 yrs (range <1-99 yrs). In 205 patients [51%], the isolates were considered to be probably acquired outside the hospital, while in 185 [46%] they were estimated to be nosocomially-acquired. In 10 patients [3%], the origin of acquisition of the strain remained unsettled. Out of 308 hospitalized patients (on the day of isolation of the ESBL-positive strain), 160 [52%] were hospitalized in medical wards (among these 60 [37%] in a geriatric unit), 60 [19%] in a surgical unit and 59 [19%] in an ICU; the main sources of isolation of the strains were the urinary tract (61%), respiratory tract (16%), wound and pus (13%) and blood (6%). Only 4% of the ESBL producing strains originated from screening specimens (rectal swab/stools).

Fig. 1. Evolution of species distribution of ESBL-producing *Enterobacteriaceae* isolates in Belgium Hospitals (2006-2010)



In 2010, *E. coli* was the most prevalent ESBL-producing *Enterobacteriaceae* species (64%) followed by *K. pneumoniae* (14%), *E. aerogenes* (13.2%) and *E. cloacae* (5%). The other species (*K. oxytoca*, *Citrobacter freundii*, Proteus/Morganella/Providencia, *Serratia* spp and *Salmonella* spp.) altogether represented less than 4%. In comparison to the 2006 and 2008 national surveys we observed a significant increase in the proportion of *E. coli* among ESBL-producing *Enterobacteriaceae* isolates (64% in 2010 vs 56.4% in 2008 and 40% in 2006, $p < 0.001$) and a parallel marked decrease in the proportion of *E. aerogenes* (44.7% in 2006 vs 20.9% in 2008 and 13.2% in 2010; $p < 0.001$). For the first time, the proportion of *K. pneumoniae* (14%) exceeded that of *E. aerogenes* (13.2%) in 2010. The proportion of ESBLs in the other species did not significantly change between 2006 and 2010. The percentage of ESBL-producing isolates of probable community-acquired origin continued to increase in 2010 (52% in 2010 vs less than 30% in 2006; $p < 0.001$). Overall 72% of ESBL-producing isolates recovered from urine specimens were *E. coli* vs only 36% in the respiratory tract. *E. coli* alone accounted for more than 70 % of all ESBL-producers collected in a “community-acquired” setting. Also, the proportion of ESBL-positive *E. coli* from specimens of hospitalized patients in “nosocomial settings” almost doubled between 2006 and 2010 (51% of all nosocomial ESBLs in 2010 vs only 27% in 2006), illustrating the

frequent import of these isolates from the community to the hospital. It is however likely that the proportion of ESBL of “nosocomial origin” could have been overestimated because few hospitals currently systematically screen patients upon hospital admission for intestinal ESBL carriage. Further, the frequent administration of antibiotics during hospitalization may select and enrich the intestinal flora with ESBL-producing isolates that were previously acquired in the community.

Globally, these data confirm the dramatic increase of ESBL-producing *E. coli* strains of community origin and they match with those data obtained from the national epidemiologic surveillance programme in which a marked increase of the mean proportion (6% in 2009 vs 3,9% in 2006) and of the incidence (2.2/1000 hospital admissions in 2006 vs 3.0/1000 in 2009) of ESBL-producing *E. coli* was also documented over the same period (B.Jans, IPH epidemiological report 2009/1 (http://www.nsih.be/download/MREA/MREA_2009_1/MREA0901_FR_end.pdf)).

The following comments can be made concerning the 31 (7.8%) Enterobacteriaceae isolates that could not be confirmed as ESBL-producers: Although we could not formally exclude in some strains the possibility that the lack of ESBL might have been due to the loss of plasmids carrying the ESBL genes following storage or repetitive subcultures, we believe that in the majority of the cases the strains were misclassified as ESBL producers (similar findings were indeed observed in the 2006 and in the 2008 surveys). However, the proportion of false-positive ESBLs decreased from 18% in 2006 to 7.8% in 2010, illustrating the improvement in the performance of the laboratories for diagnosis/detection of ESBL. It is however possible that the shift in species distribution of ESBLs from *Enterobacter aerogenes* to *E. coli* might partly explain for this evolution, since it is well known that the detection of ESBLs (false-negative or false-positive) may be notoriously more difficult in AmpC-producing species (such as *Enterobacter* spp.) where AmpC and ESBL can coexist.

In this collection, 12 of the 31 non-ESBL producing isolates were indeed found to be chromosomal AmpC-producing species (6 *Enterobacter cloacae*, 5 *Enterobacter aerogenes*, 1 *Citrobacter freundii*), 13 were *E. coli* (5 with CMY-2 plasmidic cephalosporinase, 4 with chromosomal AmpC overproduction, 4 with TEM-1 penicillinase combined with porin deficiency and 1 with OXA-30 penicillinase), 4 were chromosomal K1/-OXY penicillinase *K. oxytoca* overproducers, and 2 were *P. mirabilis* isolates which produced an AmpC enzyme

of CMY-2 type. All the above referred mechanisms of resistance may mimic the presence of an ESBL and are difficult to differentiate from each other by phenotypic tests. In particular, the presence of ESBLs in natural AmpC-producing species has previously been shown to be very challenging both for manual (disc diffusion) methods or automated systems.

ESBL characterization in Enterobacteriaceae

Overall, *bla*_{ESBL} coding genes of the CTX-M type were the most frequently found (n=282; [70.5%]) followed by TEM (n=81 [20.3%]) and by SHV types (n=52; [13%]). Fifteen isolates (4%) simultaneously carried two different ESBLs.

The distribution of ESBL-coding genes among the different *Enterobacteriaceae* species is shown in Table 1. The CTX-M type predominated, globally accounting for two-thirds of all ESBLs found but it was most often found in *E. coli* (88% of the isolates), in *K. pneumoniae* (66% of the isolates) and in *E. cloacae* (70% of the isolates). Within the CTX-M family, most ESBLs were of CTX-M-1 group (223 [79.1%]), followed by CTX-M-9 group (46 [16.3%]) and CTX-M-2 group (13 [4.6%]). In comparison to the previous surveys, the prevalence of CTX-M did increase to reach 67% of all ESBLs in 2010 vs 52.5% in 2008 and 22.5% in 2006; ($p < 10^{-6}$). This increase was essentially due to the rising number of CTX-M-1 group in *E. coli* and in *K. pneumoniae*. More specifically, DNA sequencing of a subset of isolates collected in 2006 and in 2008 revealed that over 80% of the CTX-M-1 group positive strains belonged to CTX-M-15 (H. Rodriguez *et al.*, *J Antimicrob Chemother.* 2011; 66:37-47). In 57% of the isolates, this resistance mechanism was association with TEM-1 and OXA-1/-30 coding genes (data not shown). As a matter, the gene encoding CTX-M-15 is well known to be co-localized on the same plasmid as TEM-1 and OXA-1/-30 coding genes (NB: Although OXA-1/-30 is not considered as a true ESBL, this enzyme hydrolyzes cefepime slightly and it is insensitive to β -lactamase inhibitors, hence explaining that isolates carrying the OXA-1/-30 gene are usually resistant to amoxicillin/clavulanate and to piperacillin/tazobactam).

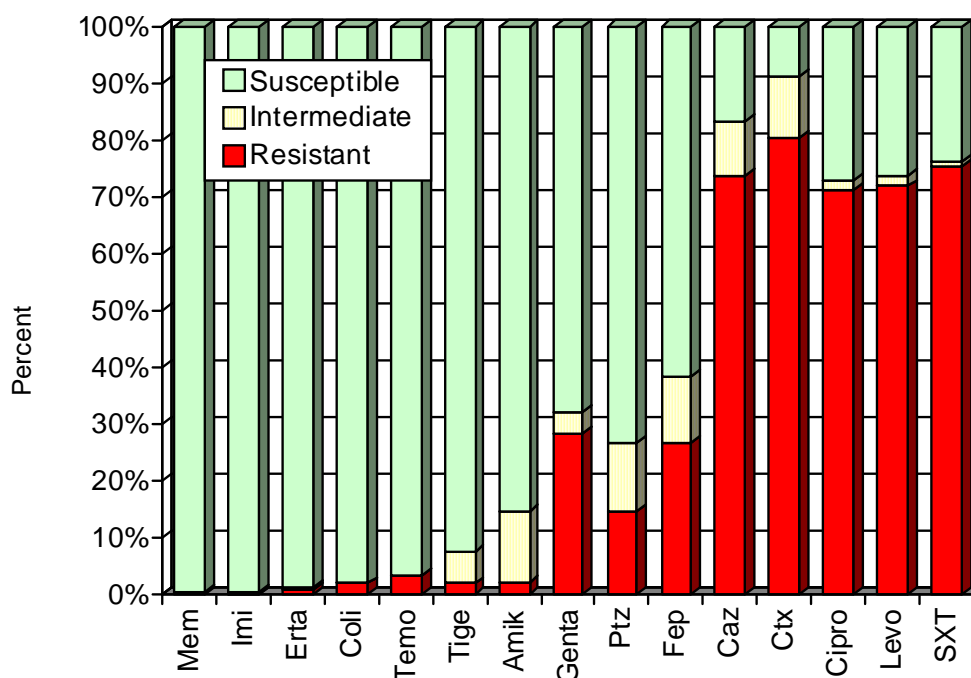
Table 1. Identification and species distribution of the ESBL enzymes

Species (Nr isolates)	Types of ESBL genes				
	TEM	SHV	CTX-M	CTX-M/ SHV	CTX-M/ TEM
<i>Escherichia coli</i> (256)	23	7	223	2	1
<i>Enterobacter aerogenes</i> (53)	39	12	1	1	
<i>Klebsiella pneumoniae</i> (56)	5	13	34	3	1
<i>Enterobacter cloacae</i> (20)	1	5	7	7	
<i>Klebsiella oxytoca</i> (7)	5	1	1		
<i>Proteus/Morganella/Providencia</i> (5)	4		1		
<i>Serratia marcescens</i> (1)	1				
<i>Citrobacter freundii</i> (1)	1				
<i>Salmonella</i> spp. (1)			1		
Total	79 (19.8%)	38 (9.4%)	268 (67%)	13 (3.3%)	2 (0.5%)

ESBLs of the CTX-M-9 group predominated in *E. cloacae* (either alone or in association with SHV-5/-12). CTX-M-2 group type ESBLs were almost exclusively found in *E. coli* (n=8) and in *K. pneumoniae* (n=2) from two different hospitals in the same city. The different TEM-type enzymes represented 20% of all ESBLs. The proportion of TEM-type ESBLs decreased significantly over the years (60% of all ESBLs in 2006 vs only 30% in 2008). This decrease mainly reflects the lower incidence of the TEM-24-producing Belgian epidemic clone of *E. aerogenes* over time (43% in 2006 vs 13% in 2010) and also the decrease in frequency of TEM-52 in *E. coli* (14% in 2006 vs 6% in 2010).

The proportion of SHV-type ESBLs has remained stable representing about 10% of all ESBLs. Enzymes of the SHV type were mostly encountered in *Enterobacter* spp. (SHV-4 in *E. aerogenes* and SHV-5/-12 in *E. cloacae*) and in *K. pneumoniae* (various types of SHV alleles). Less common ESBLs (sometimes referred to as “minor ESBLs”) such as GES, PER or VEB types were not found though local surveys carried out over the same period have shown that some of these (e.g. GES-5, GES-6/-7) had been occasionally detected in various Enterobacteriaceae species isolates (data not shown).

Fig. 2. Susceptibility categorization of 398 ESBL-producing isolates to 15 antibiotics (MIC by microdilution method /EUCAST interpretative criteria)



Mer= Meropenem; Imi= imipenem; Erta= Ertapenem; Coli= Colistin; Temo= Temocillin; Tige= Tigecycline; Amik= Amikacin; Genta= Gentamicin; Ptz= Piperacillin/tazobactam; Fep= Cefepime; Caz= Ceftazidime; Ctx= Cefotaxime; Cipro= Ciprofloxacin; Levo= Levofloxacin; SXT= Cotrimoxazole

Using the 2010 EUCAST interpretative guidelines, 98-99% for all ESBL producers were found susceptible to the carbapenems (MIC₉₀ of 0.06 mg/L for meropenem, 0.12 mg/L for ertapenem, and 0.5 mg/L for imipenem), to colistin (MIC₉₀ of 0.5 mg/L) and 96% of the ESBL-producing isolates were susceptible to temocillin (MIC₅₀ of 4 mg/L and MIC₉₀ of 16 mg/L). Tigecycline was on the whole active against over 90% of all ESBL-positive isolates (MIC₅₀ of 0.5 mg/L; MIC₉₀ of 1 mg/L), but this activity was clearly dependent on the species (15-20% resistance levels in *Klebsiella* and *Enterobacter* spp. vs no resistance in *E. coli*) (cf. Table 2). As shown in Figure 2, most isolates were resistant or of intermediate susceptibility to third generation cephalosporins (92% I/R to cefotaxime and 83% I/R to ceftazidime). Interestingly, when using the EUCAST breakpoints for cephalosporins in Enterobacteriaceae, only 4 (1.1%) of the 398 ESBL-positive isolates (2 *Klebsiella* spp., 1 *P. mirabilis*, 1 *E. coli*) were categorized as susceptible (despite the presence of an ESBL), hence highlighting the fact that the current EUCASTS breakpoint is well set to categorize genotypically confirmed ESBL-producing strains as resistant to expanded-spectrum cephalosporins.

Co-resistance to fluoroquinolones (ciprofloxacin, levofloxacin) and to cotrimoxazole was observed in 75% of the isolates. Resistance to cefepime, piperacillin-tazobactam, gentamicin and amikacin was also observed but their frequency varied largely depending of the genus or species considered (Table 2). These variations in resistance rates between species both reflect differences in the types of ESBLs involved in the different species as well as the type of associated resistance mechanisms co-localized on the same plasmids and also the clonal expansion of some isolates.

Table 2. Co-resistance rates of ESBL-producing *Enterobacteriaceae* by species

Agent	Co-resistance rates (I+R%)			
	<i>E. coli</i> (n=254)*	<i>K. pneumoniae</i> (n=56)	<i>E. aerogenes</i> (n=53)	<i>E. cloacae</i> (n=20)
Amikacin	10.6	23.2	15.1	35.0
Gentamicin	28.1	60.7	9.4	60.0
Ciprofloxacin	69.7	73.2	100	40.0
Levofloxacin	72.5	71.4	96.2	40.0
Cotrimoxazole	73.2	73.2	92.5	5.0
Temocillin	3.5	0	3.8	5.0
Pipera/tazo	17.7	44.6	49.1	20.0
Cefepime	43.7	57.1	3.8	20.0
Tigecycline	0	19.6	15.0	18.9

*2 *E. coli* strains did not grow and were not available for MIC testing

Detection of resistance mechanisms other than ESBLs

AmpC cephalosporinases

12 natural chromosomal AmpC producers (6 *E. cloacae*, 5 *E. aerogenes*, 1 *C. freundii*) did overproduce their AmpC cephalosporinase but they were not found to carry an ESBL gene (negative phenotypic tests -lack of synergy between cephalosporin substrates and clavulanate in the presence of APBA).

Moreover, 10 non-natural AmpC producing species isolates (8 *E. coli*, 2 *P. mirabilis*) were found to be resistant to amoxicillin/clavulanate, to cefoxitin and displayed intermediate susceptibility or resistance to expanded-spectrum cephalosporins (cefotaxime, ceftazidime, cefepime). The presence of an ESBL could not be confirmed in these isolates by phenotypic tests nor by molecular testing using an ESBL DNA microarray (Check-Points) targeting the CTX-M, SHV and TEM ESBL genes. On the other hand, 7 of the 10 isolates (5 *E. coli*, 2 *P. mirabilis*) were AmpC positive and carried a CMY-2 gene. The 3 remaining *E. coli* isolates were probable chromosomal AmpC overproducers (negative PCR for plasmidic AmpC genes). Five *E. coli* isolates were not confirmed as ESBL by phenotypic tests and were proven as TEM-1 penicillinase producers by Check-Points DNA microarray with probable porin deficiencies (4 strains) or as OXA-1/-30 producers (1 isolate). Finally the presence of an ESBL was misidentified for 4 isolates of *K. oxytoca* that were found to overproduce a chromosomal K1/-OXY penicillinase.

Carbapenemases

The presence of carbapenemases was screened in 8 Enterobacteriaceae isolates (3 *E. coli*, 2 *E. cloacae*, 2 *E. aerogenes*, 1 *K. oxytoca*) which displayed a decreased susceptibility to meropenem (diameter zone size <23 mm and/or meropenem MICs $\geq 0,5$ mg/L).

Phenotypic tests relying on synergy between meropenem and ABPA, EDTA or dipicolinic acid (DPA) and cloxacillin as well as on molecular tests targeting various carbapenemase coding genes (KPC, GES, VIM, IMP, OXA-48) allowed the detection of the presence of an OXA-48 carbapenemase (in combination with a CTX-M-9 group ESBL) in one *E. coli* strain. This isolate had an MIC of 1 mg/L to

meropenem (zone size of 21 mm) and would still have been called as susceptible according to current MIC breakpoints of the CLSI (meropenem: $S \leq 1$; $R \geq 4$) and EUCAST (meropenem: $S \leq 2$; $R > 8$) while it would have been categorized as intermediate following interpretation of CLSI and of EUCAST disc zone breakpoints (CLSI: $S \geq 23$; $R \leq 19$; EUCAST: $S \geq 22$; $R < 16$). It has indeed been recently shown that OXA-48 carbapenemase-producing Enterobacteriaceae may display MIC values near or below the threshold of the carbapenem susceptibility breakpoint of CLSI (M100-S21) and of EUCAST. For the 7 other isolates, the genotypic tests targeting the different carbapenemase coding genes were all negative hence suggesting that the resistance to carbapenem in these isolates was probably due to the combination of AmpC and/or of ESBL production with cell wall impermeability due to porin deficiencies.

It seems however important to reemphasize here that an increasing number of sporadic carbapenemase-producing Enterobacteriaceae isolates (OXA-48 and VIM-1 like being the two most frequent types, but also NDM-1) were referred to us in 2010 by several Belgian laboratories very often in the setting of sanitary repatriation following a travel abroad. This increasing number of reports could have resulted from a better awareness by microbiologists about the risk factors and laboratory methods aiming to detect carbapenem resistance mechanisms following the large mediatization of NDM-1 and the national alert that was raised in July 2010 by the Belgian Health authorities.

In order to have a better insight of the epidemiology of carbapenem-resistant Enterobacteriaceae in Belgium, it is foreseen that a new epidemiologic and microbiologic national surveillance programme will be launched this year in collaboration with the Institute of Public Health (ISP-WIV).

Mechanisms of resistance in *P. aeruginosa*

In total, 106 putative MDR *P. aeruginosa* and 8 *A. baumannii* isolates were received from 47 centres. Thirty-seven *P. aeruginosa* isolates (35%) were phenotypically and genotypically confirmed to be MDR, resistant to carbapenem and to carry resistance genes coding for metallo-beta-lactamases (MBL) of the VIM-type (VIM-2 [n=33], VIM-4 [n=4]); Moreover, 8 *P. aeruginosa* isolates were also phenotypically confirmed to be MDR but they did not carry carbapenemase-coding genes. Among these isolates, 5 harboured an ESBL

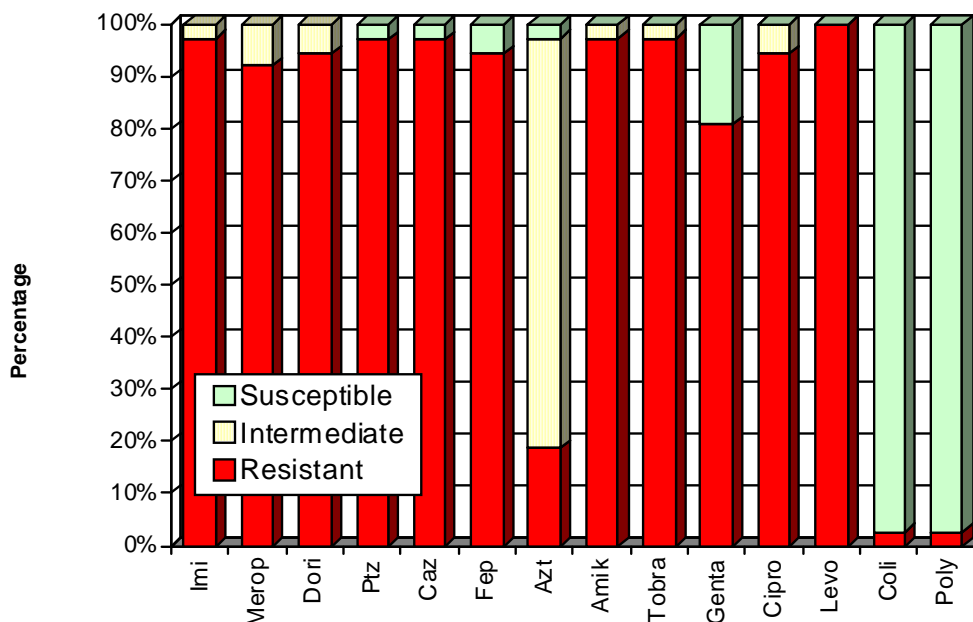
coding gene (BEL-1 [n=2], PER-1 [n=2] and VEB-1 [n=1]) and 3 carried a penicillinase coding gene (OXA-type [n=2], CARB-1,-4,-6 [n=1]).

The 37 VIM-producing *P. aeruginosa* isolates were collected from patients in 22 hospitals throughout Belgium and clustered into three major genotypes (Q, X, W) by molecular typing using PFGE. The majority of the VIM-producing isolates belonged to serotype O12 (n=23), 7 were poly-agglutinable, 5 were serotype O11 and one each was of serotype O1 and O15. Interestingly, the three epidemic genotypes did all belong to serotype O12.

Further, two of the three epidemic genotypes (types X and types Q) carried a similar *bla*_{VIM-2} gene cassette being part of a class 1 integron in addition to another gene cassette (*aacA29a*) located upstream of the *bla*_{VIM-2} cassette. The *aacA29a* gene encodes an aminoglycoside-modifying enzyme (AAC(6')-29a) which confers high level resistance to amikacin, isepamicin, kanamycin and tobramycin but not to gentamicin. The frequent co-resistance of VIM-producing *P. aeruginosa* isolates to all aminoglycosides including gentamicin results from the fact that other aminoglycoside resistance genes were also present in such isolates but were located on other genetic elements (data not shown).

In contrast to these PFGE genotypes X and Q, isolates of the W genotype carried a different class 1 integron with an *aadA5* gene that encodes the aminoglycoside-modifying enzyme (3'')₉adenylyltransferase conferring resistance to spectinomycin and to streptomycin being present downstream the *bla*_{VIM} cassette. In isolates of the W genotype, a second class 1 integron carrying an OXA-10 encoding a narrow spectrum penicillinase was also systematically found. In these strains, the observed resistance to all clinical aminoglycosides (amikacin, gentamicin, tobramycin) most probably resulted from the presence of aminoglycosides modifying enzymes that were located on other resistance genes (data not shown). The four VIM-4-producing *P.aeruginosa* isolates were all of serotype O11 but they belong to at least two different PFGE genotypes and the MBL-coding genes were carried on different integrons (all with different cassettes structures) suggesting the occurrence of horizontal transfer of the *bla*_{VIM-4} gene in different clonal lineages of *P. aeruginosa*.

Fig. 3. Susceptibility categorization of 37 VIM-metallo-beta-lactamase producing isolates of *P. aeruginosa* to 14 antibiotics (MIC by microdilution method /EUCAST interpretative criteria).



The susceptibility of the VIM-2 or of the VIM-4-producing *Pseudomonas aeruginosa* isolates is shown in the Figure 3. As expected, all isolates carrying metallo-beta-lactamase coding genes exhibited a high resistance level to all carbapenems and also to all other beta-lactam agents except aztreonam (to which almost 80% of the isolates displayed an intermediate susceptibility level [MIC of 8-16 mg/L]). Only 20% of the isolates were susceptible to gentamicin while resistance to tobramycin and to amikacin was almost constantly observed. Further, all VIM-producing strains were also high-level resistant to the fluoroquinolones (MIC of > 32 mg/L) and the polymyxins (polymyxin B and colistin) remained as the only active agents (MIC₉₀ of 2 and 4 mg/L, respectively).

Mechanisms of resistance in *A. baumannii*

Concerning the *Acinetobacter* spp. isolates, only 2 of the 8 isolates referred as being MDR (resistant to ceftazidime, fluoroquinolones and aminoglycosides) were found to be resistant to carbapenems; both of these isolates carried an OXA-23 coding gene (one in association with a PER-1 ESBL). Interestingly, while the initial cases of OXA-carbapenemase-producing isolates of *A. baumannii* detected in Belgium from 2005 onwards were almost always associated with importation following international travel and hospitalization in countries abroad, an increasing

number of autochthonous cases have been documented since 2009 suggesting the possibility of local environmental and/or patient's reservoirs for these organisms. Three other *A. baumannii* strains (all collected at the same centre) carried a PER-1 coding gene but they were susceptible to carbapenems. Interestingly, *A. baumannii* isolates expressing ESBL of the PER-1 type have already been detected at several places in Belgium since 2003, either in the setting of sporadic cases or in association with nosocomial outbreaks (T. Naas *et al.*, *J. Antimicrob. Chemother.* 2006; 58: 178-82) .

Conclusions and perspectives

This multicenter nationwide survey confirmed the overwhelming increase of the community-acquired ESBL-producing *E. coli* isolates in 2010, especially those of CTX-M-1 group which now accounts as the most frequent ESBL both in the community and in Belgian hospitals. From the analysis of strains collected in 2006 and in 2008, it is known that around 75% of the *E. coli* and *K. pneumoniae* isolates carrying a CTX-M-1 group ESBL are of CTX-M-15 type. Meanwhile, there was a continuous decrease in the prevalence of ESBL-positive *E. aerogenes* isolates while the prevalence of ESBL remained stable in other species. For the first time in this survey, an OXA-48 carbapenemase producing *E. coli* isolate was identified. In 2010, an increasing number of carbapenemase-producing Enterobacteriaceae isolates have been referred by several hospitals to our laboratory, OXA-48, NDM-1 and VIM-1 being the most frequent. Of note, several of the transferable carbapenemases may only be expressed at low level in Enterobacteriaceae and hence may be difficult to detect by routine testing methods whether manual or automated. The next national multicenter surveys will focus more specifically on the epidemiology of carbapenem-resistance in Enterobacteriaceae in terms of proportion, prevalence, incidence, sources of importation and occurrence of possible outbreaks.

. Results of analysis focusing on *P. aeruginosa* displaying a MDR pattern by phenotypic and molecular tests did confirm the spread and expansion of at least three epidemic clones of VIM-2-producing isolates mostly of O12 serotypes carrying different integrons and associated resistance genes. These MDR *P. aeruginosa* producing VIM-2 metallo-beta-lactamase had already been detected in several Belgian hospitals since 2004, but seem to have widely disseminated since then (A. Deplano *et al.* Eurosurveillance 2007 Jan 18;12(1):E070118.2.)

Also identical gene cassettes and integrons have been occasionally found in isolates of different genetic background (genotypes) suggesting that carbapenem- and MDR resistance might increase in *P. aeruginosa* not only by clonal expansion but also by horizontal transfer of resistance genes. The number of carbapenemase and/or ESBL-producing *A. baumannii* collected during this survey is too small to allow drawing any conclusion. In parallel to this microbiological survey, a new collaborative surveillance program launched in 2009 with the Scientific Institute of Public Health aims besides ESBLs to follow the epidemiology of resistance in *P. aeruginosa* and in *Acinetobacter* spp. This twice yearly surveillance will run in parallel with the ESBL surveillance, and we would encourage laboratories to participate to these programmes in order to gain a better insight of the epidemiology of these organisms and of the associated resistance mechanisms.