encoding a putative replication protein was identified. No typical genetic element (such as an insertion sequence) that could explain the acquisition of bla_{OXA-72} was identified at each extremity.

In the Middle East, clonal outbreaks of infections caused by carbapenem-resistant *A. baumannii* strains have been reported, having occurred in the United Arab Emirates, Qatar, Iran and Iraq.[7–9] In the United Arab Emirates, resistance to carbapenems was associated with the production of OXA-23;[6] the carbapenem resistance mechanisms were not investigated in the other studies. Here we identified heterogeneity of CHDL-encoding genes as the source of carbapenem resistance among *A. baumannii* isolates from the same hospital. This is, to the best of our knowledge, the first report of such diversity of carbapenem resistance determinants in a restricted geographical area. This diversity of *A. baumannii* isolates and of CHDL genes in Bahrain may be related to the fact that this region of the Middle East has a very mixed population originating from various parts of the world.

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Transparency declarations

None to declare.

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Emergence of extended-spectrum-AmpC-expressing *Escherichia coli* isolates in Belgian hospitals

Pierre Bogaerts[1]*, Hector Rodriguez-Villalobos[2], Christine Laurent[1], Ariane Deplano[2], Marc J. Struelens[2] and Youri Glupczynski[1]

[1]*Laboratoire de Bactériologie, Cliniques Universitaires UCL de Mont-Godinne, B-5530 Yvoir, Belgium;* [2]*Laboratoire de Bactériologie, Hôpital Universitaire Erasme—ULB, B-1070 Bruxelles, Belgium*

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*Corresponding author. Tel: +32-81-42-32-41; Fax: +32-81-42-32-46; E-mail: pierre.bogaerts@uclouvain.be

Sir,

The overproduction of AmpC chromosomal cephalosporinases with broadened substrate activity has recently been reported in clinical isolates of *Escherichia coli*.[1] These so-called extended-spectrum AmpC (ESAC) β-lactamases confer reduced susceptibility to all cephalosporins, including the fourth-generation agents cefepime and cefpirome. We aimed to investigate the possible occurrence of ESAC-expressing *E. coli* isolates in Belgium.

Among the 6850 non-duplicate *E. coli* clinical isolates (one isolate/patient) that were recovered from two Belgian hospitals (Erasme Hospital, Brussels and UCL de Mont-Godinne Hospital, Yvoir) between 2004 and 2006, 83 were found to display a β-lactam resistance pattern consistent with AmpC overproduction on the basis of resistance to amoxicillin, amoxicillin/clavulanic acid, cefazolin and cefoxitin by the disc diffusion method using the CLSI interpretative guidelines.[2] The presence of AmpC was further confirmed by analytical isoelectric focusing demonstrating a band at a pI of ~8.5–9.0, disappearing in the presence of oxacillin.

Fourteen out of the 83 AmpC hyper-producing isolates (Table 1) were resistant to ceftazidime and cefotaxime by current EUCAST breakpoints[3] and intermediate to cefepime (MIC above 1 mg/L; 1.5–8 mg/L). Sequence analysis of their chromosomal *ampC* genes revealed that eight of the isolates harboured an S287N mutation, while two harboured an H296P mutation, one harboured a V298L mutation and one harboured an L293P mutation. All these mutations, except the L293P mutation that is reported here for the first time, have already been reported in *E. coli* and were shown to be involved in the ESAC phenotype.[4,5]

No mutation was found in the chromosomal AmpC of the two remaining isolates. Instead, IS*Ecp1*-bla_{CMY-2} (plasmid-mediated *ampC* gene) was detected in those *E. coli*. In one of the isolates (MIC of cefepime=4 mg/L), the reduced susceptibility to cefepime could be ascribed to the presence of a bla_{OXA-30}

Table 1. Phenotypic and genotypic characterization of AmpC-overproducing *E. coli* isolates recovered at two Belgian hospitals

Isolate(s)	Number of isolates	Overproduced AmpC type	Phylogenetic group	MIC (mg/L)				
				CAZ	CTX	FEP	FOX	MEM
EC01[a]	1	plasmid bla_{CMY-2}	A	24	12	4	192	0.064
EC02	1	plasmid bla_{CMY-2}	D	32	32	1.5	>256	0.064
EC03	1	ESAC (S287N)	D	96	12	6	96	0.094
EC04 to EC10	7	ESAC (S287N)	A	24 to >256	3–12	2–8	16–192	0.023–0.064
EC11 and EC12	2	ESAC (H296P)	A	48–256	3–6	3–6	24–64	0.023–0.032
EC13	1	ESAC (V298L)	A	24	2	2	16	0.023
EC14	1	ESAC (L293P)	A	192	8	8	32	0.047
EC WT AmpC[b]	1	wild-type AmpC (L293)	A	3	1	0.047	24	0.027
E. coli TOP10 transformants								
L293P TOP10	1	ESAC (L293P)	NA	>256	24	16	24	0.047
WT TOP10	1	wild-type AmpC (L293)	NA	96	12	0.5	>256	0.032
C-TOP10	1	none	NA	0.75	0.25	0.38	4	0.0023

ESAC, extended-spectrum AmpC; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; FOX, cefoxitin; MEM, meropenem; NA, not analysed.
[a]The reduced susceptibility to cefepime could be ascribed to the presence of a bla_{OXA-30} β-lactamase detected in this isolate.
[b]EC WT AmpC is an *E. coli* clinical isolate hyper-producing a wild-type AmpC presenting exactly the same sequence as ESAC (L293P) except for the absence of a mutation at amino acid L293.

β-lactamase gene, which was identified by PCR/sequencing and isoelectric focusing (pI=7.3, not susceptible to clavulanate and oxacillin). A TEM-1-β-lactamase-encoding gene alone was evidenced in the last isolate (MIC of cefepime=1.5 mg/L).

In 69 out of the 83 isolates with cefepime MICs ≤1 mg/L, no single mutation assigned to the ESAC phenotype could be detected in the chromosomal AmpC.

Among the 12 isolates expressing ESAC β-lactamases, 11 strains (9 of which were recovered from urine or stool samples) belonged to phylogenetic group A, a group usually associated with the commensal non-virulent flora, and one isolate (from a stool sample) belonged to phylogroup D, a group usually associated with virulent and extra-intestinal strains.[6] Ten out of 12 of the ESAC-expressing isolates were closely related by PFGE (>80%) and rep-PCR (DiversiLab™ System, bioMérieux, France) analysis (>95%) while the ESAC-expressing isolate EC03, belonging to phylogroup D, harboured a different rep-PCR and PFGE profile sharing <70% and 60%, respectively, with the previous isolates. Isolate EC12, belonging to phylogroup A, was not related to the other ESAC-producing isolates.

The putative new ESAC-β-lactamase-encoding gene (accession number FJ439686) was cloned in *E. coli* TOP10 using pCR®-Blunt II-TOPO® plasmid as vector (Invitrogen®, Merelbeke, Belgium) (Table 1). AmpC from *E. coli* EC WT, expressing a wild-type AmpC with exactly the same sequence as L293P ESAC except for the absence of a mutation at amino acid L293, was used as a control. ESAC transformant L293P TOP10 was resistant to cefepime while transformant WT TOP10 remained susceptible to this cephalosporin. Since the AmpC sequences of L293P TOP10 and WT TOP10 only differed by the L293P amino acid substitution located inside the R2 loop region, already shown to be involved in the ESAC phenotype in Enterobacteriaceae,[1] this strongly suggested that this mutation was responsible for the resistance to cefepime.

This study represents the first report of ESAC-expressing *E. coli* outside of France. As reported by Mammeri *et al.*,[5] our study confirms the occurrence of ESAC isolates among AmpC-overproducing *E. coli* isolates in Belgian hospitals (12 out of 83, i.e. 14.5%).

In contrast to previous reports, two different molecular typing methods showed a consistent clonal relatedness between the vast majority of the ESAC isolates, most of which belong to phylogroup A. It is possible that the selection of specific commensal endemic clones of chromosomal AmpC hyper-producers might be the reflection of similar antibiotic policies that obviously favour the clinical usage of β-lactam/β-lactamase inhibitor associations and of expanded-spectrum cephalosporins. In any case, this observation underlines the need to further investigate the epidemiology of AmpC-producing *E. coli* on a wider scale at a national level, as well as to evaluate the clinical relevance of this mechanism of resistance.

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Transparency declarations

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Reduced susceptibility of multidrug-resistant *Acinetobacter baumannii* to tigecycline in combination with 1-(1-naphthylmethyl)-piperazine is not a pH-dependent phenomenon

D. W. Wareham[1,2]*, N. C. Gordon[2], J. B. Casals[3] and D. C. Bean[1]

[1]*Centre for Infectious Disease, Institute of Cell and Molecular Science, Barts and The London, Queen Mary's School of Medicine and Dentistry, London, UK;* [2]*Division of Infection, Barts and The London NHS Trust, London, UK;* [3]*Rosco Diagnostica, Taastrup, Denmark*

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*Corresponding author. Centre for Infectious Disease, Institute of Cell and Molecular Science, 4 Newark Street, Whitechapel, London E1 2AT, UK. E-mail: d.w.wareham@qmul.ac.uk

Sir,

Acinetobacter baumannii has emerged as an important cause of nosocomial infection in the immunocompromised and critically ill. The organism frequently exhibits multidrug resistance with only polymyxin derivatives and the glycylcycline antibiotic tigecycline retaining significant activity *in vitro*. Resistance to these agents has been reported[1] and in the case of tigecycline is thought to be mediated by the AdeABC resistance-nodulation-division efflux pump. Recently, we described a curious phenomenon whereby strains of *A. baumannii* belonging to the multidrug-resistant OXA-23 clone 1 appeared to decrease in susceptibility to tigecycline in the presence of the efflux pump inhibitor 1-(1-naphthylmethyl)-piperazine (NMP). The converse was observed when NMP was combined with doxycycline, tetracycline or minocycline.[2] In an attempt to explain this finding, we considered whether low pH could contribute to this observation, as the activity of many tetracyclines is known to be enhanced by pH[3] and the preparation of NMP requires acidification of the solvent (0.2 M HCl, pH 2). We were unable to detect any significant changes in the pH of the medium when NMP was added to Iso-Sensitest agar (Oxoid, Basingstoke, UK), presumably due to the low volume (640 μL of 10000 mg/L stock solution per 100 mL of agar) and the buffering capacity of the medium. We therefore proceeded to study the effect of directly supplementing antimicrobial discs with NMP and the NMP solvent. In disc diffusion tests, blunting of the zones of inhibition was observed when discs containing 64 μg of NMP were placed 10 mm from 15 μg tigecycline discs (Figure 1a). When this was repeated using minocycline, the converse occurred with enhancement of the zone size adjacent to the NMP-containing disc (Figure 1b). Addition of solvent alone to discs placed 10 mm from tigecycline discs had no effect on zone sizes (Figure 1c).

It is well documented that *in vitro* testing of the susceptibility of *A. baumannii* to tigecycline is highly dependent on the method used and the level of manganese supplementation.[4] However, the addition of acidic substances in disc diffusion tests had no effect on tigecycline susceptibility and certainly did not account for the paradoxical decrease in susceptibility observed with NMP versus other tetracyclines.

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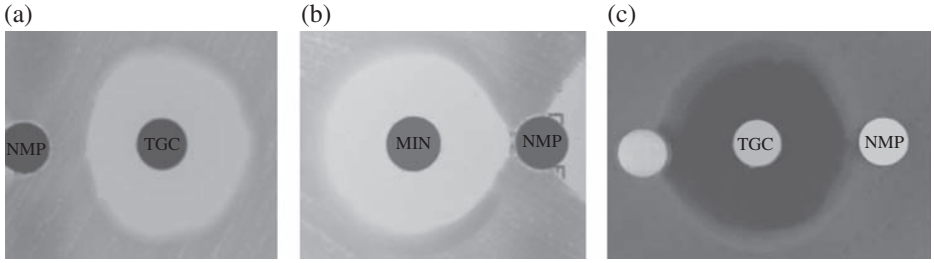

Figure 1. Disc diffusion tests with tigecycline (TGC), minocycline (MIN) and NMP versus *A. baumannii* OXA-23 clone 1. Blunting (a) of the tigecycline and expansion (b) of the minocycline zone size when placed adjacent to a disc containing 64 μg of NMP. No effect (c) on tigecycline zone size when placed adjacent to a disc containing the NMP dissolving solution alone.