Emergence of carbapenem-non-susceptible extended-spectrum β -lactamase-producing *Klebsiella pneumoniae* isolates at the university hospital of Tübingen, Germany

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The spread of Gram-negative bacteria with plasmid-borne extended-spectrum β -lactamases (ESBLs) has become a worldwide problem. This study analysed a total of 366 ESBL-producing *Enterobacteriaceae* strains isolated from non-selected patient specimens at the university hospital of Tübingen in the period January 2003 to December 2007. Although the overall ESBL rate was comparatively low (1.6 %), the percentages of ESBL-producing *Enterobacter* spp. and *Escherichia coli* increased from 0.8 and 0.5 %, respectively, in 2003 to 4.6 and 3.8 % in 2007. In particular, the emergence was observed of one carbapenem-resistant ESBL-producing *E. coli* isolate and five carbapenem-non-susceptible ESBL-positive *Klebsiella pneumoniae* isolates, in two of which carbapenem resistance development was documented *in vivo* under a meropenem-containing antibiotic regime. The possible underlying mechanism for this carbapenem resistance in three of the *K. pneumoniae* isolates was loss of the *Klebsiella* porin channel protein OmpK36 as shown by PCR analysis. The remaining two *K. pneumoniae* isolates exhibited increased expression of a tripartite AcrAB–TolC efflux pump as demonstrated by SDS-PAGE and mass spectrometry analysis of bacterial outer-membrane extracts, which, in addition to other unknown mechanisms, may contribute towards increasing the carbapenem MIC values further.

Carbapenem-non-susceptible ESBL isolates may pose a new problem in the future due to possible outbreak situations and limited antibiotic treatment options. Therefore, a systematic exploration of intestinal colonization with ESBL isolates should be reconsidered, at least for haemato-oncological departments from where four of the five carbapenem-non-susceptible ESBL isolates originated.

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INTRODUCTION

In contrast to the USA and the Western Pacific area, the prevalence of extended-spectrum β -lactamases (ESBLs) in Germany was comparably low in 2001 (8.2 % of *Klebsiella pneumoniae* and 0.8 % of *Escherichia coli* isolates; Sturenburg & Mack, 2003). However, the prevalence of ESBL-producing Gram-negative bacteria is increasing, both

in hospitals and in the community (Sturenburg & Mack, 2003; Pitout *et al.*, 2005).

ESBLs confer resistance to amino- and ureidopenicillins, oximino-cephalosporins and monobactams. As co-resistance to non- β -lactam antimicrobials is common among ESBL isolates, the treatment options are limited. Among the *E. coli* and *K. pneumoniae* isolates predominantly found to produce ESBLs in Europe, carbapenem-non-susceptible isolates are becoming more widespread (Martínez-Martínez *et al.*, 1999; Mena *et al.*, 2006). The underlying

Abbreviations: ESBL, extended-spectrum β -lactamase; FCCP, trifluoromethoxyphenylhydrazine; PA β N, phenylalanine arginine β -naphthylamide.

mechanism for carbapenem resistance of ESBL-positive *K. pneumoniae* isolates is loss of porin channels (OmpK35 and/or OmpK36) for antibiotic uptake (Martínez-Martínez *et al.*, 1999) combined with the expression of AmpC β -lactamases (Bradford *et al.*, 1997) or expression of carbapenemases (Woodford *et al.*, 2004).

The present study was conducted to evaluate the development of ESBL-producing isolates at our university hospital between 2003 and 2007. We also analysed the mechanisms of resistance and epidemiology of five carbapenem-non-susceptible *K. pneumoniae* isolates found during this study.

METHODS

Collection and phenotypic detection of ESBL-positive strains.

From January 2003 to December 2007, 366 non-duplicated ESBL-producing isolates were collected from non-selected patient samples in our tertiary-care hospital in the south-western part of Germany. The hospital has 1500 beds and treats approximately 68 000 inpatients and 220 000 outpatients per year. *Enterobacteriaceae* isolates were identified by using API 20 E or Vitek2 (bioMérieux). Susceptibility testing was performed by using Vitek2 or agar diffusion following the Clinical and Laboratory Standards Institute guidelines. For the detection of ESBLs, combination disc testing was performed with cefpodoxime (January 2003–August 2004 and February 2007–December 2007), ceftazidime and cefotaxime discs and their clavulanate-containing counterparts (January 2003–December 2007), and by ESBL combination disc testing with cefepime and its clavulanate-containing counterpart (AB Biodisk and Mast Diagnostica) (September 2004–December 2007). To confirm mer-

openem resistance, a microbroth dilution assay and meropenem Etest (AB Biodisk) were performed.

Phenotypic identification of ESBL producers that were resistant or intermediate to carbapenems was performed in a second, confirmatory microbroth dilution test detecting resistance to three third-generation cephalosporins (cefotaxime, ceftazidime and cefpodoxime) with and without clavulanic acid (CLSI, 2008). If the MIC of cefpodoxime was $\geq 8 \mu\text{g ml}^{-1}$ and the MIC of ceftazidime and/or cefotaxime was $\geq 2 \mu\text{g ml}^{-1}$, and, in addition, if the MIC ratio of an oximino-cephalosporin with and without inhibitor was ≥ 8 , the strain was inferred to be an ESBL producer (Sturenburg & Mack, 2003). The presence of metallo- β -lactamases was tested by using Etest MBL (AB Biodisk) (Walsh *et al.*, 2002).

PCR and sequence analysis of resistance genes. The genes for ESBLs of the carbapenem-intermediate and -resistant ESBL producers (*bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M}) were amplified by using multiplex PCR. The presence of relevant carbapenem-resistance genes (*bla*_{KPC}, *bla*_{OXA-23}, *bla*_{OXA-24}, *bla*_{OXA-48}, *bla*_{OXA-58}, *bla*_{VIM} and *bla*_{IMP}) was investigated by using the primers given in Table 1. PCR for detection of *bla*_{GIM}, *bla*_{SIM} and *bla*_{SPM} genes was carried out with primer pairs described previously (Mendes *et al.*, 2007). Intrinsic and acquired *ampC* genes in cefoxitin-resistant isolates were investigated by using a multiplex PCR that differentiated between the six gene families of plasmid-borne *ampC* and their chromosome-encoded progenitor genes (Pérez-Pérez & Hanson, 2002). Furthermore, the *ampC* promoter region of the cefoxitin-resistant, carbapenem-non-susceptible *E. coli* isolate was sequenced as described previously (Mulvey *et al.*, 2005). Sequencing of all amplicons was carried out by using an ABI PRISM BigDye 3.1 Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems).

Resistance gene transfer and plasmid analysis. Transfer of third-generation cephalosporin and carbapenem resistance was tested

Table 1. Primer sequences for detection of different β -lactamase genes

Primer name	Sense/antisense	Sequence (5'→3')	Target	Product length (bp)	Source
TEMu	Sense	ATGAGTATTCAACATTTCCG	All <i>bla</i> _{TEM} genes	851	Grimm <i>et al.</i> (2004)
TEMu	Antisense	TTAATCAGTGAGGCACCTAT			
SHVu	Sense	GCAAAACGCCGGGTATTTC	All <i>bla</i> _{SHV} genes	940	This study
SHVu	Antisense	GGTTAGCGTTGCCAGTGCT			
CTX-Mu	Sense	CGCTTTGCGATGTGCAG	All <i>bla</i> _{CTX-M} genes	551	Paterson <i>et al.</i> (2003)
CTX-Mu	Antisense	ACCGCGATATCGTTGGT			
CTX-M9	Sense	GCAGTACAGCGACAATACCG	<i>bla</i> _{CTX-M9} -related genes	356	This study
CTX-M9	Antisense	TATCATTGGTGGTGCCGTAG			
OXA-23u	Sense	AACCCCGAGTCAGATTGTTC	<i>bla</i> _{OXA-23} -related genes	642	This study
OXA-23u	Antisense	ATTTTCCATCTGGCTGCTC			
OXA-24u	Sense	GTTTCTCTCAGTGCATGTTTCATC	<i>bla</i> _{OXA-24} -related genes	740	This study
OXA-24u	Antisense	CGAATAGAACCAGACATTCCTTC			
OXA-48u	Sense	AAATCACAGGGCGTAGTTGTG	<i>bla</i> _{OXA-48} -related genes	555	This study
OXA-48u	Antisense	GACCCACCAGCCAATCTTAG			
OXA-58u	Sense	AAGTATTGGGGCTTGTGCTG	<i>bla</i> _{OXA-58} -related genes	456	This study
OXA-58u	Antisense	TCAACTTCCGTGCCTATTGT			
IMPu	Sense	CATGGTTGGTGGTCTTGT	All <i>bla</i> _{IMP} genes	447	This study
IMPu	Antisense	ATAATTGGCGGACTTTGGC			
VIMu	Sense	AGTGGTGAGTATCCGACAG	All <i>bla</i> _{VIM} genes	261	This study
VIMu	Antisense	ATGAAAGTGCGTGAGAC			
KPCu	Sense	CAGCTCATTCAAGGGCTTTC	All <i>bla</i> _{KPC} genes	533	This study
KPCu	Antisense	AGTCATTTGCCGTGCCATAC			

by broth mating assays using a sodium azide-resistant *E. coli* J53 recipient. Selection of transconjugants was performed on Mueller–Hinton agar plates containing sodium azide ($200 \mu\text{g ml}^{-1}$), ampicillin ($100 \mu\text{g ml}^{-1}$) and meropenem ($2 \mu\text{g ml}^{-1}$). The plasmid DNA of the donor and transconjugants was isolated with a Plasmid Mini kit (Qiagen) and analysed on a 0.4% agarose gel using *E. coli* V517 as a size marker (Macrina *et al.*, 1978).

For Southern blot hybridization, specific probes for *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M} were labelled with a PCR DIG Labelling mix (Roche Diagnostics) using DIG-DNA Ladder III (Roche) as a size marker.

Investigation of porins. To determine the presence or absence of the two major porins (outer-membrane proteins) of *K. pneumoniae*, OmpK35 and OmpK36, the gene fragments of *ompK35* and *ompK36* of the carbapenem-non-susceptible *K. pneumoniae* ESBL producers were amplified and sequenced by using primers described previously (Lee *et al.*, 2007).

Analysis of outer-membrane protein profiles by SDS-PAGE and MS. The carbapenem-non-susceptible *K. pneumoniae* isolates and, as a control, several *E. coli* BL21(DE3) mutant strains (Prilipov *et al.*, 1998) were cultivated overnight in Luria broth at 37 °C, diluted (1:20) in fresh medium and incubated for a further 2 h. The outer membranes were isolated from cultures as described previously (Osborn *et al.*, 1972), washed three times with water, added to SDS-PAGE sample buffer and subjected to SDS-PAGE (11% polyacrylamide, 8 M urea) according to a method described previously with some modifications (Schägger & von Jagow, 1987).

The gels were stained overnight with Coomassie solution (0.25% Coomassie R250; 8%, v/v, acetic acid; 40%, v/v, methanol) at 4 °C, and then destained for 1–2 h with 10% (v/v) acetic acid, 40% (v/v) ethanol. Slices containing the proteins of interest were cut out and in-gel protein digestions were carried out overnight at 37 °C with trypsin (porcine, sequencing grade, modified; Promega).

Reversed-phase nano-liquid chromatography tandem MS was performed as described elsewhere (Liu *et al.*, 2006). In brief, the tryptic peptide mixture was separated using an Ultimate nanoflow LC system (Dionex/LC Packings) coupled to a QSTAR Pulsar I hybrid QqTOF mass spectrometer (Applied Biosystems/MDS Sciex), equipped with a nano-electrospray ion source (New Objective).

Proteins were identified by correlating the data from the MS/MS spectra with the NCBI.nr protein sequence database (version 20080206, taxonomy bacteria) using the MOWSE algorithm as implemented in the search engine MASCOT (Matrix Science; Perkins *et al.*, 1999). All experimental data obtained by gel electrophoresis and MS were stored in a LIMS database (Proteinscape 1.3; Bruker Daltonics). Database search results were reviewed as described elsewhere (Barjaktarovic *et al.*, 2007) with the following modification according to specific database characteristics: individual MASCOT ion scores >49 indicated identity or extensive homology.

Statistical analysis. Hybase software (Cymed AG) was used for statistical analysis of the microbiological findings.

RESULTS AND DISCUSSION

Development and epidemiology of ESBL isolates from 2003 to 2007

From January 2003 to December 2007, 366 non-duplicated ESBL-producing isolates were collected from non-selected patient samples. Fig. 1(a) shows that, among these, *E. coli*

Fig. 1. ESBL isolates 2003–2007 ($n=366$). (a) Total number of ESBL isolates per species. The corresponding organisms were *Citrobacter amalonaticus* ($n=1$), *Citrobacter braakii* ($n=2$), *Citrobacter farmeri* ($n=1$), *Citrobacter freundii* ($n=9$), *Citrobacter koseri* ($n=2$), *Enterobacter aerogenes* ($n=13$), *Enterobacter cloacae* ($n=43$), *Escherichia coli* ($n=200$), *Klebsiella ornithinolytica* ($n=4$), *Klebsiella oxytoca* ($n=3$), *Klebsiella pneumoniae* ($n=66$), *Klebsiella terrigena* ($n=1$), *Morganella morganii* ($n=4$), *Proteus mirabilis* ($n=10$), *Proteus penneri* ($n=1$), *Proteus vulgaris* ($n=1$), *Serratia fonticola* ($n=4$) and *Serratia marcescens* ($n=1$). (b) The percentage of ESBL isolates per species with respect to the overall number of isolates (ESBL-positive and -negative) of the corresponding species.

was the most frequent ($n=200$), followed by *Klebsiella* spp. ($n=74$), *Enterobacter* spp. ($n=56$) and other species (*Citrobacter* spp., $n=15$; *Proteus* spp., $n=12$; *Serratia* spp., $n=5$; *Morganella* spp., $n=4$). When the number of ESBL-positive isolates was compared with the overall number of isolates of the corresponding bacterial species (Fig. 1b), it became obvious that the rate of ESBL-positive *Enterobacter* spp. and *E. coli* isolates continuously increased from 0.8 and 0.5%, respectively, in 2003 to 4.6 and 3.8%, respectively, in 2007. Although an overall increase in ESBL-positive *E. coli* isolates was observed from 2003 to 2007, on average the *E. coli* ESBL rate was low (1.8%), which is comparable to the low rate of 0.8% ESBL-producing *E. coli*

isolates reported for 2001 in Germany (Sturenburg & Mack, 2003). The percentage of ESBL-positive *Klebsiella* strains in our hospital was much lower (between 1.3 and 2.9 %) compared with the Germany-wide rate of 8.2 % reported in 2001 (Sturenburg & Mack, 2003).

The results of studies determining whether ESBL production has an effect on clinical outcomes are divergent (Ramphal & Ambrose, 2006). Nevertheless, at least 62.8 % of the ESBL producers isolated in our hospital caused infections, as they were isolated from urine samples exhibiting bacterial monoculture and high bacterial counts ($>10^4$ ml⁻¹; 42.1 %), intraoperative swabs and punctates (12.8 %), blood (4.9 %), bronchoalveolar lavages (1.6 %) and other relevant specimens (1.4 %). The fact that most ESBL producers (42.1 %) were cultivated from urine is in accordance with a study performed at a Croatian university hospital reporting that the most abundant source of ESBL-producing strains were urinary-tract infections (Tonkic *et al.*, 2005). Of the ESBL-producing isolates, 16.4 % were derived from wounds. However, it is difficult to distinguish between infection and colonization of wounds, as the isolation of ESBL producers from deep wounds such as bedsores or ulcers does not necessarily indicate infection. By contrast, 20.8 % of the ESBL isolates were considered to have colonized patients without infection, as they were isolated from the upper respiratory tract (13.7 %), vaginal swabs (3.8 %), and stools and anal or rectal swabs (3.3 %). Compared with a recent study reporting a rate of 8.1 % faecal carriage of ESBL-producing *Enterobacteriaceae* in hospitalized patients (Castillo García *et al.*, 2007), the rate of faecal ESBL carriage at our clinic was low (3.3 %). Hence, we assume that, in our hospital, a considerable number of patients carrying faecal ESBL producers remain undetected, as screening for faecal carriage of ESBL producers has not been implemented in our hospital to date.

Most of the ESBL producers were isolated from patients from intensive care units (17.8 %), internal medicine departments (16.9 %) and surgery departments (11.2 %). A considerable number of ESBL-producing strains (23.8 %) were isolated from patients admitted to one of the four external hospitals that are associated with our university hospital (all a maximum of 20 km from the university hospital). Although there is no definite consensus on the hospital care of patients carrying or being infected with ESBL-producing bacteria, the management of ESBL-positive patients at our hospital follows uniform hospital-wide guidelines based on generally accepted recommendations such as those from the Robert Koch Institute on a national level and the Centers for Disease Control and Prevention on an international level to prevent the transmission of bacteria.

In spite of strict hygiene management, we observed an increase in ESBL-positive isolates from 2003 to 2007, in particular *Enterobacter* spp. and *E. coli*, at our hospital. As there was no suggestion of a possible outbreak caused by ESBL-positive organisms, it cannot be excluded that

community-acquired ESBL producers were increasingly imported to our hospital. Several studies reporting the influx of ESBL-producing organisms into the hospital suggest that ESBL-producing *Enterobacteriaceae* should be considered not only as nosocomial pathogens but also as community-acquired pathogens (Ben-Ami *et al.*, 2006; Oteo *et al.*, 2006; Pitout *et al.*, 2005). Recently, it has been reported that even household contacts of community ESBL patients lead to high rates of intestinal colonization of the contact persons (16.7 %) (Valverde *et al.*, 2008). Unfortunately, in our hospital, the rate of patients who carried ESBL-producing bacteria at admission could not be evaluated, as patients were not routinely screened for ESBLs when they were admitted to the hospital.

Antimicrobial susceptibility profiles of the ESBL producers

The occurrence of ESBLs in members of the *Enterobacteriaceae* results in resistance to many different β -lactam antibiotics. The prevalent location of ESBL genes and further resistance genes on conjugative plasmids leads to co-resistance to non- β -lactam antibiotics and the transmission of resistance among enterobacterial species. Antimicrobial testing of the ESBL producers isolated in our hospital revealed that 83.9 % (47/56) of *Enterobacter*, 73.3 % (55/75) of *Klebsiella*, 72.7 % (8/11) of *Proteus* and 60.0 % (9/15) of *Citrobacter* species were susceptible to levofloxacin, and 80.0 % (4/5) of *Serratia*, 63.6 % (7/11) of *Proteus* and 53.3 % (8/15) of *Citrobacter* species were susceptible to cotrimoxazole (Fig. 2), providing antibiotic treatment options other than carbapenems for patients infected with these bacteria. Detailed analysis of the 102 ESBL-producing *E. coli* isolated from urine revealed that co-resistance to non- β -lactam antibiotics was frequent among these isolates [64.7 % (66/102) to cotrimoxazole and 76.4 % (78/102) to levofloxacin] when compared with the overall 27.6 % (1103/3990) cotrimoxazole-resistant and 14.4 % (576/3990) levofloxacin-resistant non-ESBL *E. coli* isolates from urine over the same time frame. Strikingly, all four ESBL-producing *Morganella morganii* isolates were resistant to cotrimoxazole, whereas the overall resistance rate to cotrimoxazole among non-ESBL *M. morganii* isolates over the same time frame was only 18.3 %. Susceptibility to the 7- α -substituted β -lactam cefoxitin was 100 % (7/7) among the *Proteus*, 90.9 % (40/44) among the *Klebsiella*, 82.6 % (138/167) among the *E. coli* and 75.0 % (3/4) among the *Serratia* ESBL producers, whilst susceptibility to cefotetan was 100 % (1/1) among the *Proteus*, 98.6 % (71/72) among the *E. coli* and 93.3 % (28/30) among the *Klebsiella* ESBL-positive isolates tested for these antibiotics. Unfortunately, although cefotetan has better susceptibility rates, it is not currently available in the domestic German trade.

Carbapenem-non-susceptible ESBL isolates

Carbapenems are usually used for antibiotic treatment of infections with ESBL producers. Although most of the

Fig. 2. Susceptibility testing of ESBL isolates. Percentage of ESBL producers per species susceptible to the indicated antibiotics. All of the ESBL-positive isolates were tested against levofloxacin and cotrimoxazole. Only selected isolates were tested against cefoxitin and cefotetan (cefoxitin: *Citrobacter*, $n=11$; *Enterobacter*, $n=47$; *E. coli*, $n=167$; *Klebsiella*, $n=44$; *Morganella*, $n=2$; *Proteus*, $n=7$; *Serratia*, $n=4$; cefotetan: *Citrobacter*, $n=4$; *Enterobacter*, $n=8$; *E. coli*, $n=72$; *Klebsiella*, $n=30$; *Proteus*, $n=1$; *Serratia*, $n=3$). *, Not applicable (no *Morganella* isolate was tested).

ESBL-positive isolates in our hospital were susceptible to meropenem, we found that five *K. pneumoniae* isolates and one *E. coli* isolate, which together represented 1.6% of the total ESBL-producing strains, were of intermediate susceptibility (two *K. pneumoniae*, one *E. coli* isolate) or were resistant (three *K. pneumoniae* isolates) to meropenem, confirmed by a microbroth dilution assay and by Etest. The meropenem and imipenem MIC values of the carbapenem-non-susceptible *K. pneumoniae* isolates and their carbapenem-susceptible counterparts, as well as the resistance profile to other antibiotics and some information concerning patients (A–E) that the isolates were derived from, are given in Table 2.

Resistance to cefoxitin was observed in all isolates, with increased MIC values for imipenem and meropenem, whereas the carbapenem-susceptible counterparts (isolate 187 from patient A and isolate 46 from patient B) were susceptible to cefoxitin. Moreover, all carbapenem-non-susceptible *Klebsiella* isolates exhibited intermediate susceptibility or resistance to colistin.

Strikingly, isolates 202 (patient A) and 201 (patient C) had the same antibiotic susceptibility profile and were isolated at an interval of 1 day, indicating a possible relatedness among these two strains. This assumption was confirmed by PCR analysis (Table 3) showing that both isolates carried the ESBL gene *bla*_{CTX-M-15} and the classical *bla*_{SHV-1} gene and exhibited the same amino acid substitutions in the *ompK36* gene, indicating that these two isolates were

identical. However, there was no indication of the possible route of transmission from patient A (child) to patient C (adult), as these patients did not share the same ward and were not treated in the same operation area or other 'transfer areas' such as computed tomography at the same time.

Whereas most of the carbapenem-non-susceptible *K. pneumoniae* isolates with increased carbapenem MIC values only colonized patients (A–C, E), one of the *K. pneumoniae* isolates (patient D) caused an infection. This patient was transferred by emergency to Germany by family members and admitted to our hospital after she had undergone several revision operations in Turkey because of a hip prosthesis infection due to ESBL-positive *K. pneumoniae*. Therefore, this isolate was probably imported to our hospital from Turkey.

We have documented that two ESBL-positive *K. pneumoniae* isolates (patients A and B) developed carbapenem resistance *in vivo* after treatment with meropenem for 60 days (patient A) and 3 days after discontinuation of the meropenem-containing regime for 13 days (patient B) (Table 2). Also patients D and E had received meropenem prior to the isolation of the carbapenem-non-susceptible ESBL *Klebsiella*, whereas patient C did not receive carbapenems as he had been admitted to our hospital 23 days before the detection of ESBL. Thus, in four of the five patients, antibiotic treatment with meropenem preceded the isolation of carbapenem-non-susceptible *K. pneumoniae* isolates. Moreover, two of these patients developed carbapenem resistance *in vivo*, indicating that antibiotic treatment of ESBL producers with carbapenems is not sufficient and, additionally, that carbapenems may select for resistance. It is being increasingly reported that carbapenem resistance in *K. pneumoniae* develops in strains producing plasmid-encoded AmpC β -lactamase or other broad-spectrum β -lactamases such as ESBLs due to the selection of mutants with reduced permeability to these drugs or, alternatively, carbapenemases (Jacoby *et al.*, 2004; Kaczmarek *et al.*, 2006; Mena *et al.*, 2006; Queenan & Bush, 2007; Yigit *et al.*, 2001).

Resistance gene location and transfer

In order to analyse whether ESBLs were present in the carbapenem-non-susceptible *K. pneumoniae* isolates, PCR analyses of the strains using universal primers for detection and sequencing of *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M} genes were performed and *bla* genes were identified in all isolates. Five isolates harboured a single ESBL gene (*bla*_{TEM-29}, $n=1$; *bla*_{SHV-2}, $n=4$). The remaining isolates contained the ESBL-type CTX-M-15 and a SHV-1 and/or TEM-1 β -lactamase (Table 3). The gene *bla*_{CTX-M-15} was found to be located on a plasmid of ~40 kb in three clonally related *K. pneumoniae* isolates. In isolate 263, *bla*_{TEM-1} and *bla*_{CTX-M-15} were found on a 40 kb plasmid, whereas isolate 467 contained an 80 kb plasmid encoding *bla*_{SHV-12} as well as *bla*_{CTX-M-15} (Table 3). Via conjugation in two isolates, the

Table 2. Susceptibility patterns and sources of the carbapenem-non-susceptible ESBL-producing *K. pneumoniae* isolates and their susceptible counterparts

Overall, five carbapenem-intermediate or -resistant *K. pneumoniae* isolates (bold) were detected. Carbapenem-susceptible, ESBL-producing *K. pneumoniae* isolates were also cultivated from patients A and B. Isolate numbers are consecutive numbers from our collection of ESBL-producing organisms. MPM, meropenem; IP, imipenem; AMK, amikacin; CIP, ciprofloxacin; COL, colistin; FOS, fosfomycin; FOX, ceftiofur; GEN, gentamicin; LEV, levofloxacin; OTE, oxytetracycline; SXT, sulfamethoxazole/trimethoprim; TOB, tobramycin.

Patient	Hospital ward	Isolate no.	MIC (MPM/IP; $\mu\text{g ml}^{-1}$)	Specimen	Date of specimen receipt	Antibiotic profile		
						Susceptible	Intermediate	Resistant
A	Paediatric haematology ICU	202	16/8	Stool	23.01.06	GEN	OTE, AMK	TOB, SXT, LEV, CIP, FOS, COL, FOX
B	Paediatric oncology	187	$\leq 0.063/0.25$	Stool	5.12.05	GEN, COL, FOX	OTE, AMK	TOB, SXT, LEV, CIP, FOS
		51	8/4	Urine	2.02.04	GEN, TOB, LEV, CIP, AMK		SXT, FOX, OTE, FOS, COL
		49	4/4	Vaginal swab	28.01.04	GEN, TOB, LEV, CIP, AMK		SXT, FOX, OTE, FOS, COL
		52	4/2	Vaginal swab	4.02.04	GEN, TOB, LEV, CIP, AMK		SXT, FOX, OTE, FOS, COL
		46	$<0.063/0.5$	Stool	7.01.04	GEN, TOB, LEV, CIP FOS, COL, FOX, AMK		SXT, OTE
C	Haemato-oncology ICU	201	16/8	Stool	24.01.06	GEN	OTE, AMK	TOB, SXT, LEV, CIP, FOS, COL, FOX
D	Orthopaedics	263	32/64	Wound	14.08.06	AMK	COL	GEN, TOB, OTE, SXT, LEV, CIP, FOS, FOX
E	Paediatric haematology ICU	467	8/4	Stool	29.10.07	AMK	COL	GEN, TOB, OTE, SXT, LEV, CIP, FOS, FOX

Table 3. Resistance and changes in porin genes of the carbapenem-non-susceptible *K. pneumoniae* and *E. coli* isolates and their susceptible counterparts

The presence of ESBL resistance genes of all five carbapenem-non-susceptible *K. pneumoniae* isolates and one *E. coli* ESBL isolate was investigated by conventional PCR. Transfer experiments were performed with *E. coli* J53. Changes in porin genes (*ompK35* and *ompK36*) in the *K. pneumoniae* strains were determined by sequence analyses. ESBL types are shown in italics, and carbapenem-non-susceptible isolates in bold. ND, Not determined; I-TGA/I-TAA, insertion resulting in the stop codon TGA or TAA, respectively; M-TGA, mutation resulting in the stop codon TGA.

Isolate no.	Species	Patient	<i>bla</i> _{TEM}	<i>bla</i> _{SHV}	<i>bla</i> _{CTX-M}	Transferred genes	Plasmids	<i>ompK35</i>	<i>ompK36</i>
202	<i>K. pneumoniae</i>	A	–	1	15	–	40 kb CTX-M+	+	+*
187			–	1	15	–	40 kb CTX-M+	+	+*
51	<i>K. pneumoniae</i>	B	–	2	–	–	6 kb SHV–	– (M-TGA)	– (I-TGA)
49			–	2	–	–	6 kb SHV–	– (M-TGA)	– (I-TGA)
52			–	2	–	–	6 kb SHV–	– (M-TGA)	– (I-TGA)
46			–	2	–	–	6 kb SHV–	– (M-TGA)	+
201	<i>K. pneumoniae</i>	C	–	1	15	–	40 kb CTX-M+	+	+*
263	<i>K. pneumoniae</i>	D	1	1	15	<i>bla</i> _{TEM-1} , <i>bla</i> _{CTX-M15}	40 kb (and >100 kb)	+	– (I-TAA)
467	<i>K. pneumoniae</i>	E	–	12	15	–	80 kb SHV/CTX-M+	+	–
194	<i>E. coli</i>	–	29	–	–	<i>bla</i> _{TEM-29}	90 kb TEM+	ND	ND

*Various amino acid substitutions.

resistance genes (isolate 194, *bla*_{TEM-29}, and isolate 263, *bla*_{TEM-1} + *bla*_{CTX-M-15}) could be transferred successfully in *E. coli* J53. The plasmid profiles of isolate 263 and its transconjugant showed a 40 kb plasmid and another weak plasmid band (>100 kb), but Southern blot hybridization was not successful. The *bla*_{SHV-2} gene of four clonally related *K. pneumoniae* isolates was not transferred and could not be identified on a plasmid by Southern blot hybridization.

As *K. pneumoniae* normally lacks a chromosomally encoded class C β -lactamase, acquisition of a plasmid-encoded *bla*_{AmpC} combined with porin loss or carbapenemases can confer resistance to carbapenems (Bradford *et al.*, 1997). However, PCR analysis of all five carbapenem-non-susceptible *K. pneumoniae* isolates for plasmid-borne *ampC* genes and relevant carbapenem-resistance genes (*bla*_{KPC}, *bla*_{OXA-23}, *bla*_{OXA-24}, *bla*_{OXA-48}, *bla*_{OXA-58}, *bla*_{VIM}, *bla*_{IMP}, *bla*_{GIM}, *bla*_{SIM} and *bla*_{SPM}) was negative, indicating the absence of AmpC β -lactamases or carbapenemases. Accordingly, analysis of a possible production of metallo- β -lactamases of all carbapenem-non-susceptible *K. pneumoniae* isolates by Etest MBL yielded negative results. Additionally, a modified Hodge test, a phenotypic test with a 100% sensitivity in detecting KPC and which is also positive when other carbapenemases are present (Anderson *et al.*, 2007), was negative for all five carbapenem-non-susceptible *K. pneumoniae* isolates, indicating the absence of KPC and other carbapenemases.

The carbapenem-intermediate susceptible *E. coli* isolate (Table 3) with MIC values of 8 $\mu\text{g ml}^{-1}$ for meropenem and 2 $\mu\text{g ml}^{-1}$ for imipenem was isolated from urine in the paediatric haematology intensive care unit and displayed the ESBL phenotype of cefpodoxime MIC of >8 $\mu\text{g ml}^{-1}$,

ceftazidime MIC of >32 $\mu\text{g ml}^{-1}$, inhibited by clavulanic acid: MIC ratio ≥ 8 , and ESBL genotype TEM-29. Actually, there are only a few reports worldwide describing this ESBL variant, which is characterized by a noticeable resistance pattern conferring resistance to ceftazidime but not to cefotaxime. Analysis of the chromosomal *ampC* promoter sequence of this isolate revealed mutations at position –42 of the *ampC* promoter sequence associated with a mutation at position –18 whereby two new alternative promoter boxes were generated. It is known that these changes result in high-level expression of chromosomal *E. coli ampC* and mediate cefoxitin resistance in *E. coli* (Caroff *et al.*, 2000), suggesting that the cefoxitin resistance was due to the mutations of the chromosomal *ampC* promoter sequence. However, this suggestion could not be confirmed by agar disc diffusion testing for cefoxitin on Mueller–Hinton agar plates containing the AmpC inhibitor cloxacillin, as no increase in the cefoxitin inhibition zone diameter was observed when compared with testing on agar plates without the inhibitor. By contrast, meropenem MIC values were reduced in the presence of cloxacillin (250 $\mu\text{g ml}^{-1}$) by two twofold dilutions (tested by Etest), indicating that the high-level expression of chromosomal *E. coli ampC* might contribute to carbapenem resistance, as it has previously been described in combination with porin loss (Livermore & Woodford, 2006).

Investigation of porins

Loss of the two major porins, the outer-membrane proteins OmpK35 and/or OmpK36, has been associated with carbapenem resistance in *K. pneumoniae* (Kaczmarek *et al.*, 2006; Martínez-Martínez *et al.*, 1999). Moreover, the development of resistance to carbapenems *in vivo* in ESBL-

positive *K. pneumoniae* isolates due to the selection of mutants with a lack of porin expression and reduced permeability to these drugs has been reported previously (Lee *et al.*, 2007; Mena *et al.*, 2006). In particular, the C-terminal F residue of OmpK36 is necessary for outer-membrane expression (Struyve *et al.*, 1991).

Sequence analysis of the coding region of the *ompK35* gene in four clonally related *K. pneumoniae* isolates (isolates 46, 49, 51 and 52, all from patient B) revealed a change in position 135 from codon TGG to stop codon TGA (Table 3). Furthermore, three of these isolates that showed an increased MIC for carbapenems (isolates 49, 51 and 52) had an insertion of 8 bp (GCGCGGCG) in the *ompK36* sequence resulting in a frameshift and generation of a stop codon (TGA) and a lack of the last 155 aa. These changes, combined with expression of the SHV-2 β -lactamase, might confer resistance to carbapenems and produce elevated MIC values to carbapenems, respectively, on these isolates as described previously (Crowley *et al.*, 2002). By contrast, in the carbapenem-susceptible isolate 46, this frameshift was not found (Table 3), supporting a role for OmpK36 expression in carbapenem susceptibility.

In the *ompK36* gene of isolate 263 (patient D), a single base-pair insertion was detected that led to a preliminary stop codon, TAA, resulting in a porin truncated by 196 aa. Hence inactivation of OmpK36 in isolate 263 was caused by interruption of the coding sequence by a single base-pair insertion similar to a previous report describing the interruption of the coding sequence by an insertion sequence (Hernández-Allés *et al.*, 1999). In isolate 467 (patient E), *ompK35* but not *ompK36* was amplified by PCR. The failure of *ompK36* amplification in isolate 467 suggests either a deletion of part of the complete coding sequence or its interruption by an insertion sequence that was too large to be amplified by conventional PCR. The presence of CTX-M ESBL in isolates 263 (patient D) and 467 (patient E), along with inactivation of OmpK36, might contribute to the carbapenem resistance in these two isolates. Moreover, ceftiofloxacin resistance in ESBL-positive *K. pneumoniae* has been attributed to porin loss, as the resistance persists when the plasmid is eliminated, whilst the plasmid itself does not express ceftiofloxacin resistance when transferred to a new host (Martínez-Martínez *et al.*, 1996, 1999). Hence, ceftiofloxacin resistance in isolates 51, 263 and 467 could be attributed to this mechanism.

The carbapenem-resistant *K. pneumoniae* isolates 202 (patient A) and 201 (patient C) exhibited the same amino acid substitutions in the *ompK36* gene. However, these changes did not seem to be causative of the resistance to carbapenems, as this mutation was also found in a susceptible counterpart, isolate 187 (patient A). Hence we looked for other possible changes in the bacterial cell wall of isolates 202 and 201 that might contribute to the carbapenem resistance of these isolates.

Identification of efflux pumps by SDS-PAGE and MS

In order to analyse possible changes in the bacterial cell membrane of the carbapenem-non-susceptible *K. pneumoniae* isolates, outer-membrane extracts of these isolates were separated by SDS-PAGE and stained with Coomassie solution. Bands of proteins expressed at increased levels in the carbapenem-non-susceptible isolates or, alternatively, only in the carbapenem-susceptible counterparts were excised, digested and analysed by MS. As shown in Fig. 3, both isolate 202 (patient A, lane 1) and isolate 201 (patient C, lane 7) expressed all of the components of a tripartite AcrAB-TolC efflux pump (Bornet *et al.*, 2003; Fernandez-Recio *et al.*, 2004; Hu *et al.*, 2007), namely the outer-membrane channel TolC, the trimeric inner-membrane proton antiporter AcrB and the periplasmic membrane fusion protein AcrA, indicating that an efflux mechanism might contribute to the resistance to carbapenems in these two isolates. Accordingly, by MS analysis, these three proteins were not detectable in isolate 187 (patient A, lane 2), the carbapenem-susceptible counterpart to isolate 202. Moreover, isolates 202 and 201 both exhibited a reduction in meropenem MICs (tested by Etest) by one twofold dilution in the presence of the efflux pump inhibitors phenylalanine arginine β -naphthylamide (PA β N, 70 μ M) and carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP, 100 μ M), respectively, which is closely related to the more common efflux pump inhibitor carbonyl cyanide *m*-chlorophenylhydrazone. Although the mechanisms responsible for increasing the *K. pneumoniae* MIC to a rather high level remain unclear, it is possible that the efflux pump system might have contributed to further increasing the meropenem MICs in these two isolates. By contrast, when Etests were performed with isolates 51, 263 and 467, no synergistic inhibitory effect between meropenem and the efflux pump inhibitors was observed, suggesting that efflux pumps do not play a role in the carbapenem resistance of these isolates. This was in accordance with the lack of efflux pumps in these isolates demonstrated by MS.

There is a report describing efflux pumps as the causative mechanism for multi-resistant *K. pneumoniae* isolates from Turkey (Hasdemir *et al.*, 2004). Expression of this resistance-nodulation-cell division (RND) efflux system might also contribute to the concomitant resistance to ceftiofloxacin of these two isolates as reported for RND-family efflux pumps in clinical isolates of *Bacteroides fragilis* (Pumbwe *et al.*, 2006), as we observed a noticeable increase in the ceftiofloxacin inhibition zone diameter by 8 mm each in the presence of the efflux pump inhibitors PA β N and FCCP. By contrast, a synergistic inhibitory effect of ceftiofloxacin and the inhibitors was not observed in isolates 51, 263 and 467, suggesting that mechanisms other than efflux pumps are responsible for ceftiofloxacin resistance in these isolates.

Unfortunately, we were not able to differentially detect OmpK35, OmpK36 and OmpA of the *K. pneumoniae*

Fig. 3. Coomassie-stained SDS-polyacrylamide gel of outer-membrane proteins. The outer-membrane proteins of the nine meropenem-resistant or -intermediate ESBL-producing *K. pneumoniae* isolates and their meropenem-susceptible counterparts isolated from patients A–E (see Table 2) were separated by SDS-PAGE and 31 single bands were analysed by MS. As a control, *E. coli* BL21(DE3) wild-type (wt) (lane 10) and several deletion mutants (Prilipov *et al.*, 1998) of this strain [omp1=BL21(DE3) Δ lamB; omp4=BL21(DE3) Δ lamB ompF::Tn5 Δ ompA; omp7=BL21(DE3) Δ lamB ompF::Tn5 Δ ompC; lanes 11–13, respectively] were used. The corresponding outer-membrane proteins of these strains are indicated.

isolates from the SDS-PAGE analysis as these outer-membrane proteins co-migrated. The *E. coli* OmpC (homologous to OmpK35), OmpF (homologous to OmpK36) and OmpA also co-migrated. Co-migration of these proteins was confirmed by immunoblot analysis with specific antisera against OmpC/F and OmpA (data not shown).

In summary, we have reported here the emergence of five sporadic carbapenem-non-susceptible, ESBL-positive *K. pneumoniae* isolates, two of which developed carbapenem resistance *in vivo* under a meropenem-containing regime, and one *E. coli* isolate. The mechanism of carbapenem resistance in three of the *K. pneumoniae* isolates was loss of porins essential for uptake of antibiotics. However, the mechanisms responsible for carbapenem resistance of the other two *K. pneumoniae* isolates remain unclear. One could speculate that post-translational changes in the OmpK36 protein leading to loss of porin or loss of function were causative in increasing the meropenem MICs to a rather high level, and it is possible that the overexpression of an efflux pump system in these two isolates might have contributed towards further increasing the meropenem MICs. Neither an outbreak nor transmission to other patients was observed. However, it cannot be excluded that the development of carbapenem resistance is largely underestimated as ESBLs are not currently systematically explored for intestinal colonization. Therefore, from our study and recent studies reporting the emergence of ESBL-positive isolates resistant to carbapenems (Lee

et al., 2007; Liu *et al.*, 2008; Mena *et al.*, 2006), we conclude that medical professionals should be aware of these isolates, should continue strict hygiene procedures and, additionally, should implement an ESBL screening system, in particular for faecal carriage on haemato-oncological wards with increased use of carbapenems, in order to prevent possible outbreaks caused by these multi-resistant organisms.

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