

A Large Hospital Outbreak of *Klebsiella pneumoniae* (DHA-1 and SHV-11 Positive): Importance of Detection and Treatment of ampC β -Lactamases

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Abstract: Plasmid-borne ampC β -lactamases are emerging worldwide. There is no gold standard in detecting them so it is presumed their prevalence is underestimated. They may confer resistance to broad-spectrum cephalosporins. In this paper the first outbreak in Europe of a *Klebsiella pneumoniae* harbouring a plasmid-borne DHA-1 β -lactamase and a SHV-11 β -lactamase is reported. Following CLSI (Clinical Laboratories Standards Institute) guidelines the majority of isolates would have been reported susceptible to third generation cephalosporins in spite of increased MIC values. No failures were observed in patients treated with cefepime or meropenem. Infections were linked to stay on the intensive care unit and/or urological interventions. The outbreak was stopped after a meeting was organised between the department of infection control of the hospital and the complete ICU and urology staff in which the importance of strict hand hygiene to limit transmission was stressed.

Keywords: β -lactamases, AmpC, DHA-1, SHV-11, Cefepime treatment, *K. pneumoniae*.

INTRODUCTION

In the ever evolving world of bacterial resistance to antibiotics, one of the more frequent and powerful weapons of bacteria is the production of β -lactamases. In Gram-negatives this is the most important way of bacterial resistance against β -lactam antibiotics. These bacterial enzymes hydrolyze the β -lactam ring and inactivate the β -lactam antibiotic before it reaches the penicillin-binding protein (PBP) [1-3]. The β -lactamases are structurally very similar to PBP. Their active site contains either a serine residue or a metal ion which together with the conformation of that active site determines the substrate. Based on the substrate Ambler has made a classification of β -lactamases. In this classification with four classes, Ambler class C consists of cephalosporinases that hydrolyze cephalosporins more effectively than penicillins [4]. These plasmid-borne or chromosomal enzymes are often called ampC β -lactamases due to the specific activation of the ampC gene. Genes encoding ampC β -lactamases are much more frequently chromosomal than plasmid-mediated. *K. pneumoniae* is one of the few Gram-negative bacteria which do not possess a chromosomal ampC β -lactamase, but can acquire these enzymes *via* transfer of ampC-containing plasmids. In this article an outbreak of *K. pneumoniae* with a plasmid-borne ampC β -lactamase in a Belgian hospital is described. The importance of antibiotic resistance and treatment, and techniques of detection of acquired ampC enzymes is discussed.

Preliminary accounts of this work have been presented previously as a short report [5].

MATERIALS AND METHODS

Routine identification and antibiotic susceptibility testing were performed by **Phoenix[®] (BD)**. Clinical isolates identified as *K. pneumoniae* and flagged as "possible extended-spectrum β -lactamases (ESBL) positive" were investigated with our modified double-disk synergy test. Flagging is based on a combination of BD and home made rules.

Modified Double-Disk Synergy Test (DDST): Double disk testing according to Jarlier [6] with amoxicillin-clavulanic acid, ceftazidime, ceftriaxone, supplemented with aztreonam, cefepime, and cefotaxime was performed as ESBL confirmation assay. An extra cefoxitin disk was added as a screening measure for ampC β -lactamases [7].

AmpC Disk Test: presented by Black *et al.* [8], was performed in order to verify the presence of an ampC β -lactamase. EDTA makes the bacterial wall permeable to the ampC β -lactamase resulting in enzymatic desactivation of cefoxitin. Visually a flattening or indentation of the zone of the cefoxitin-susceptible *E. coli* is seen (Fig. 1).

18 clinical isolates were tested by PCR and pulse field gel electrophoresis.

DNA Macrorestriction and PFGE Analysis: DNA macrorestriction using the XbaI restriction enzyme (Genepath group 6 reagent kits, Bio-Rad, Hercules, USA) was performed according to the manufacturer's instructions. PFGE restriction patterns were obtained on a GenePath system (Bio-Rad, Hercules, USA). The PFGE patterns were analysed and clustered into dendrograms with the Fingerprinting II software (Bio-Rad, Hercules, USA). Interpretation was done using the criteria of Tenover *et al.* [9].

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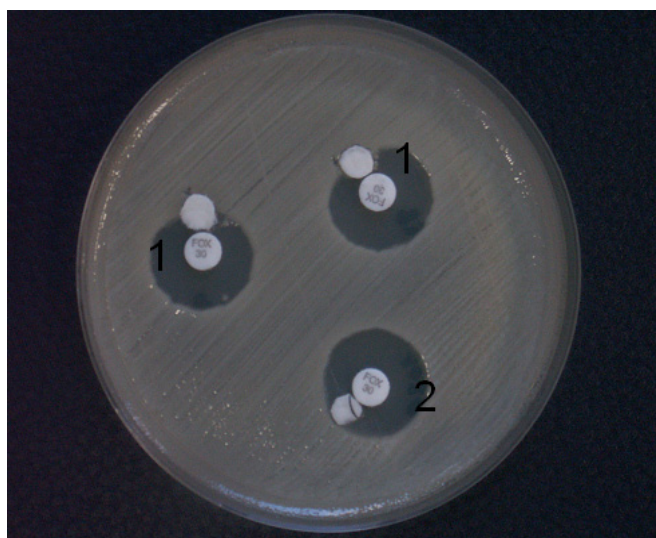


Fig. (1). AmpC disk test: indentation towards cefoxitin points to the presence of an AmpC β -lactamase. The test strain is applied on a paper disk containing TRIS-EDTA and is placed near a cefoxitin (FOX) disk. (1: isolate nr 67224, 2: negative control).

Polymerase Chain Reaction (PCR)

DNA Preparation. Bacterial solutions were boiled for 10 min. PCR experiments were performed with these crude lysates.

Amplification of β -Lactamase (BLA) Genes: Strains suspected of ESBL production (based on DDST) were examined for the presence of *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M} by PCR using sequence specific primers. The gene encoding the 16s rRNA was used as an amplification control. PCR products were separated on a 1.5% agarose gel stained with ethidium bromide. Isolates positive for *bla*_{TEM} were additionally examined for the presence of *bla*_{TEM-24} with sequence specific primers (Table 1).

Sequencing of BLA Genes: PCR products positive with the consensus primers that were previously described, but negative for *bla*_{TEM-24}, were used for direct sequencing. Dye terminator cycle sequencing was performed and analysis was performed on a CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA). The obtained sequences were compared with sequences in Genbank (National Centre for Biotechnology

Information) and with sequences for TEM, SHV and CTX-M extended spectrum β -lactamases ([10], Available from: URL: <http://www.ncbi.nih.gov/BLAST>).

The Presence of DHA-1 was confirmed by G. A. Jacoby (Lahey Clinic, Burlington, USA). A PCR product was seen for DHA-1 primers but not for ACT-1, CMY, FOX, or MIR-1 β -lactamases.

Epidemiology Data on ampC Positive *K. pneumoniae*

From the 1st August 2006 on, the department of Infection Control and Hospital epidemiology was informed, by daily mail, on each sample harbouring an ampC positive *K. pneumoniae*. The lab results and medical charts for the respective patients were carefully reviewed and all data were collected in a database. This database contained information on patient demographics, medical history, medical interventions, microbiology and antimicrobial treatment. Descriptive statistical analysis was performed using SPSS 15.0. The Kolmogorov-Smirnov test was used to assess normality. The difference in distribution of sample site location for both ampC positive and ampC negative *K. pneumoniae* was tested by means of a two-tailed χ^2 test. The results were considered statistically significant at a p-value of <0.05.

Meetings with all involved partners (medical wards, department of microbiology, department of infection control and hospital epidemiology) were held at regular basis to discuss the collected data and work out preventive measures.

RESULTS

Phenotype

The 42 strains (isolated from August 2006 till July 2007) of *K. pneumoniae* shared the same phenotype (Fig. 2). All were cefoxitin-resistant ($\emptyset = 6$ mm). Amoxicillin-clavulanic acid (AMC) induced resistance for all β -lactams except cefepime. Scattered colonies were seen in the inhibition zones of ceftazidime, ceftriaxone, aztreonam, cefotaxime, and AMC, but again not for cefepime. The cefepime inhibition zone showed a small enhancement (phantom zone) near AMC.

The MIC values for the third and fourth generation cephalosporins respectively ranged from 4 to >16 μ g/ml for ceftazidime, ≤ 2 to 4 μ g/ml for ceftriaxone, and ≤ 1 to 16 μ g/ml for cefepime. MIC values for aztreonam ranged from

Table 1. PCR Primers

Gene	Primer Sequence (5'-3')	Amplicon Length (bp)	Annealing Temperature (°C)
<i>bla</i> _{TEM}	ATGAGTATTMAACATTTCCG CCAAWGCTTAATCAGTGAGG	858	50
<i>bla</i> _{TEM'}	ATGGATCCTCAACATTTCCG CCAAWGCTTAATCAGTGAGG	858	50
<i>bla</i> _{SHV}	ATGCGTTWDTTCGCCTGTG AGCGTTGCCAGTGCTCGATC	859	58
<i>bla</i> _{CTX-M}	SCVATGTGCAGYACCAGTAA ACCAGAAYVAGCGGBGC	570	58
<i>bla</i> _{TEM-24}	GGGCAAGAGCAACTCGGT AGACCCACGCTTACCGGT	470	58

≤ 2 to 8 $\mu\text{g/ml}$ (Table 2).

Based on the phenotype it was very likely that these strains expressed an inducible plasmid-mediated ampC β -lactamase, suggesting the DHA-type [11, 12]. In these strains the ampC disk test was used which was positive pointing to the presence of an ampC β -lactamase (Fig. 1).

PCR and PFGE

PCR using consensus primers (Table 1) and sequencing of the BLA gene showed the presence of the *bla*_{SHV-11} gene, which is not classified as an ESBL-producing gene. Another polymerase chain reaction in order to detect ampC β -lactamases, revealed a DHA-1-like product. Besides that a *qnrB* gene which confers resistance to quinolones was found (GA Jacoby, personal communication).

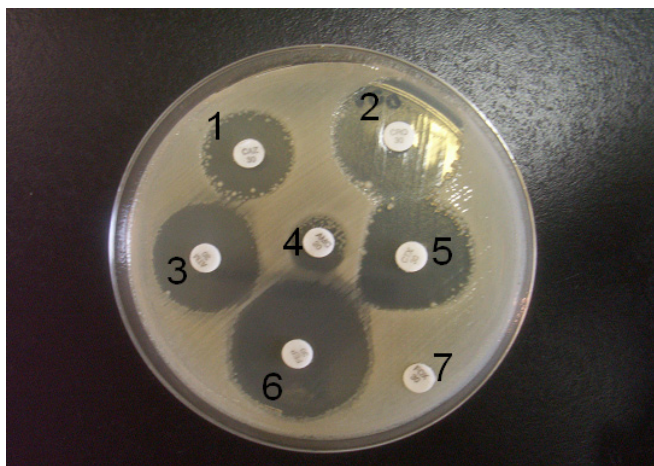


Fig. (2). Phenotype of one of the isolates on our modified double-disk synergy test (1: ceftazidime, 2: ceftriaxone, 3: aztreonam, 4: amoxicillin-clavulanic acid, 5: cefotaxime, 6: cefepime, and 7: cefoxitin).

Pulse field gel electrophoresis gave 3 types. All strains were 'closely' or 'possibly related' to the outbreak according to the Tenover criteria [9]. This suggests a single clonal spread.

Epidemiology

A retrospective analysis was performed on all clinical isolates that were identified in our hospital from the beginning of 2004 until the 1st August 2006. Based on identification and antimicrobial susceptibility data available in the Phoenix database, this analysis revealed that in 2004, only 8 of the 264 *K. pneumoniae* isolates had a similar antibiotic profile as the ampC β -lactamase positive *K. pneumoniae*. A similar antibiotic profile was identified in 9/287 *K. pneumoniae* isolates in 2005 and in 10/130 in the first semester of 2006. Unfortunately, none of these strains

was available for genotypic/phenotypic analysis.

From August 2006 until July 2007, the incidence of *K. pneumoniae* with the above described phenotype was prospectively monitored. During this period, a total of 42 patients were identified harbouring an identical strain and patient characteristics are given in Supplemental Data 1. The mean age of the patients was 72.7 years (std 9.7) and the male to female ratio was 1.8. More than 30% of the patients were staying at the ICU at the time of isolation, followed by a ward of internal medicine (21%) and a surgical ward (19%). AmpC positive *K. pneumoniae* was isolated in 6 outpatients (14%). The median number of days of hospitalisation until isolation of ampC β -lactamase positive *K. pneumoniae* was 9.5 days (IQR 22 days). The majority of first isolates came from urine samples (27/42 – 64%) followed by pus samples (7/42 - 17%) and sputum samples (5/42 - 12%). This distribution of sample sites corresponds to the distribution within our hospital of ampC negative *K. pneumoniae*.

14 patients had subsequent positive samples for ampC positive *K. pneumoniae* - for the majority of these patients, *K. pneumoniae* was isolated from the same source as the first positive isolate. Two patients were diagnosed with a urinary colonization of ampC positive *K. pneumoniae*. AmpC positive *K. pneumoniae* isolation was related to ICU stay: 30% (13/42) of patients were staying at the ICU at the time of isolation and 57% (24/42) of patients had stayed at the ICU department in the 30 days prior to the isolation of ampC positive *K. pneumoniae*.

Environmental samples were taken at the ICU. In none of these samples ampC positive *K. pneumoniae* was detected.

Treatment

Of the patients treated for a clinically relevant (fever, elevated C-reactive protein, leukocytosis) ampC positive *K. pneumoniae* (n = 21), 43% received meropenem (n = 9) and 29% were treated with cefepime (n = 6). Treated infections were the following: urinary tract infections (n = 13), pneumonia (n = 2), sepsis (n = 2), wound infections (n = 2), peritonitis (n = 2). Of the admitted patients (n = 36), 8 patients died (22%), of which 2 deaths could be attributed to the infection with *K. pneumoniae*. The latter two patients received respectively either no therapy or ciprofloxacin (to which the ampC *K. pneumoniae* was resistant). All patients who did receive meropenem or cefepime had a good outcome with in most patients a proven eradication (Table 3).

DISCUSSION

Many gram-negative bacteria possess an ampC gene on their chromosome. The only known exceptions are

Table 2. Spread and Median of the MIC's of 45 Isolates of *K. pneumoniae* (ampC) for Amoxicillin-Clavulanic Acid (AMC), Aztreonam (ATM), Ceftazidime (CAZ), Ceftriaxone (CRO), Cefepime (FEP), Ciprofloxacin (CIP), and Piperacillin-Tazobactam (TZP)

n = 45	AMC	ATM	CAZ	CRO	FEP	TZP	CIP
MIC spread ($\mu\text{g/ml}$)	16 ->16	≤ 1 ->16	4 ->16	≤ 2 - 4	≤ 1 - 16	8 ->64	>2
MIC median ($\mu\text{g/ml}$)	>16	2	8	≤ 2	2	64	>2

Table 3. Outcome of Antibiotic Therapy of 20 Treated Patients

n = 20	Good Outcome (Eradication)	Unclear Outcome (Lost to Follow-Up, Early Antibiotic Switch, no Eradication)	Negative Outcome (Death)
Meropenem	5 (71%)	2 (29%)	0
Cefepime	3 (60%)	2 (40%)	0
Temocillin	1 (50%)	1 (50%)	0
Ciprofloxacin	0	1 (50%)	1 (50%)
Amikacin	0	2 (100%)	0
Piperacillin-tazobactam	0	1 (100%)	0
Phosphomycin	0	1 (100%)	0

Klebsiella spp, *Salmonella spp*, and *Proteus mirabilis* [13, 14]. In clinically important gram-negatives that produce an ampC this β -lactamase is usually repressed. AmpC expression results from a system including ampD (a cytosolic amidase), ampG (a channel), ampR (an activator/repressor), and intermediates in peptidoglycan recycling [15]. Actions that influence these factors such as β -lactam antibiotics or a mutation, can induce ampC expression.

The DHA-1 ampC β -lactamase belongs to the *Morganella morganii* group. It was first discovered in 1992 in *Salmonella enteritidis* and is named by its site of discovery: the Dharan hospital in Saudi Arabia [16]. The first detection of DHA-1 in *K. pneumoniae* was in 1998 in France [17].

Until now two other DHA enzymes are discovered, DHA-2 and DHA-3, in France and Taiwan respectively and remarkably both in *K. pneumoniae* [18, 19].

Strains of *K. pneumoniae* containing SHV-11 together with an AmpC β -lactamase are very rare and have been described mainly in Taiwan [20, 21]. The literature reveals that the prevalence of plasmid-mediated ampC β -lactamases in *K. pneumoniae* is rising worldwide and strains producing these enzymes have been reported from every continent, but particularly from East-Asia [22-26]. Since they aren't searched for routinely it is assumed their prevalence is underestimated.

Nowadays there is no gold standard to detect the presence of plasmid-mediated ampC β -lactamases. The laboratories searching for these ampCs use a variety of tests. Often a cephamycin such as cefoxitin is used as a screening method since cephamycin-resistance is typical to ampC β -lactamases. It is highly sensitive but not specific. As mentioned above cefoxitin-resistance points to a few possible resistance mechanisms: porin loss; ampC β -lactamase production or carbapenemase production (metallo- β -lactamase). Some exceptions are known. ACC-1 for instance, an ampC β -lactamase originating from *Hafnia alvei*, doesn't confer resistance to cefoxitin [27]. As strains with ampC β -lactamases are resistant to β -lactam/ β -lactamase inhibitor combinations this can differentiate them from ESBLs as long as there is no concomitant production of

ampC β -lactamase and an ESBL. This can be confirmed by using a cloxacillin-based plate in which ampC's are inhibited.

The presence of a plasmid-mediated ampC β -lactamase can be confirmed with methods as the three-dimensional test, the ampC disk test, inhibitor-based tests mostly using boronic acid, or a multiplex PCR [8, 28-30]. Of these confirmatory tests multiplex PCR is the only test that can differentiate plasmid-borne ampC β -lactamases from chromosomally ampCs. In a nice paper Doi and Paterson review the detection of ampC β -lactamases, suggesting an interesting flow chart [31].

The migration of chromosomal ampC genes into plasmids may pose a serious threat to the health care. Infections with *K. pneumoniae* strains harbouring an acquired AmpC β -lactamase are associated with a higher mortality and a high risk of therapeutic failure of third-generation cephalosporins, although these strains may appear susceptible *in vitro* [32]. It has to be stressed that strains bearing acquired ampC β -lactamases meet CLSI criteria for ESBL screening, but present with a negative ESBL confirmation test. Since these strains aren't resistant to broad-spectrum cephalosporins using CLSI breakpoints, they are usually reported as being susceptible which might hamper treatment outcome [31].

There's some controversy about the use of fourth generation cephalosporins to treat infections with ampC β -lactamases. Most authors state these antibiotics can be used unless there is concomitant production of ESBLs [26, 33, 34]. This matches an evaluation we did at our laboratory. Scattered colonies within the ceftazidime inhibition zone proved to be highly resistant with MIC values for ceftazidime above 32 μ g/ml, but remained susceptible for cefepime (data not shown). In the limited series of patients treated with cefepime in the mentioned outbreak no therapeutic failure was noticed. On the other hand in some strains MIC values for cefepime were already elevated and an *in vitro* inoculum effect and *in vivo* ampC mutants resistant to cefepime have already been observed by others [8, 35, 36]. Therefore it may be advisable to choose therapy on the basis of MIC values (Table 2).

Finding an ampC may also be clinically important because a carbapenem resistance can develop by further mutations, in particular when there is porin loss [37, 38].

There seems to be a remarkable association between DHA-1 enzymes and the plasmid-mediated resistance to quinolones by *qnrB* genes. It has been mentioned in these isolates and it has been seen before [39].

One third of all patients in our outbreak had a medical history of an urological intervention (urethral catheter excluded) in the 6 months prior to the isolation of ampC positive *K. pneumoniae*. Because of the association found between ampC positive *K. pneumoniae* isolates and a stay at the ICU and/or urological interventions, a meeting was organised between the department of infection control of the hospital and the complete ICU and urology staff. The outbreak of ampC positive *K. pneumoniae* was discussed and the importance of strict hand hygiene to limit transmission was stressed. After this meeting no more positive cases were observed.

CONCLUSION

The first outbreak in Europe with a *K. pneumoniae* harbouring a plasmid-borne DHA-1 ampC β -lactamase and SHV-11 is described. Such plasmid-borne ampCs are emerging in gram-negatives worldwide.

In the above mentioned outbreak the infections with *K. pneumoniae* were related to stay in the intensive care unit and/or history of urological conditions. The data were too few to draw meaningful conclusions with respect to antibiotic therapy. Selecting antibiotic therapy should be done carefully when dealing with *K. pneumoniae* harbouring acquired ampC β -lactamases. It may be advisable to guide therapy on the basis of MIC values when using cephalosporins. Susceptibility to carbapenems is universal. Large randomized studies are needed to clarify which antibiotics are of choice. Preventive measures are of great help in stopping these nosocomial infections.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Web site along with the published article.

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