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Short communication

Nationwide Survey of *Klebsiella Pneumoniae* Strains Producing CTX-M Extended-spectrum β-lactamases in Croatia

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ABSTRACT

Extended-spectrum β-lactamases (ESBL) producing bacteria have been increasingly reported in both hospital and community patients. Production of ESBLs is the major mechanism of resistance to oxymino-cephalosporins and aztreonam in Gram-negative bacteria[1,2]. Recently a new family of ESBLs with predominant activity against cefotaxime (CTX-M β-lactamases) has been reported. Over 80 CTX-M enzymes have been described so far, which can be grouped into five main subgroups according to amino acid sequence identity (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25)[3]. In some countries, CTX-M β-lactamases are the most prevalent types of ESBLs, for instance in Russia[4], Greece[5], Spain[6], Switzerland[7], Japan[8], Taiwan[9], China[10] and Argentina[11]. These enzymes have been identified in countries near Croatia such is Italy[12], Hungary[13] and Austria[14]. The aim of this study was to determine the prevalence and the types of CTX-M β lactamases produced by Klebsiella pneumoniae clinical isolates collected from October 2006 to January 2007 from both community- and hospital-based isolates were included (Figure 1.). 128 ESBL isolates were subjected to further analysis: screening with double disc diffusion test and confirmed by ESBL E test[15].

Key words: *Klebsiella pneumoniae, CTX-M, survey*

Introduction

Extended-spectrum β-lactamases (ESBL) producing bacteria have been increasingly reported in both hospital and community patients. Production of ESBLs is the major mechanism of resistance to oxymino-cephalosporins and aztreonam in Gram-negative bacteria[1,2]. Recently a new family of ESBLs with predominant activity against cefotaxime (CTX-M β-lactamases) has been reported. Over 80 CTX-M enzymes have been described so far, which can be grouped into five main subgroups according to amino acid sequence identity (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25)[3]. In some countries, CTX-M β-lactamases are the most prevalent types of ESBLs, for instance in Russia[4], Greece[5], Spain[6], Switzerland[7], Japan[8], Taiwan[9], China[10] and Argentina[11]. These enzymes have been identified in countries near Croatia such is Italy[12], Hungary[13] and Austria[14].

The aim of this study was to determine the prevalence and the types of CTX-M β lactamases produced by *Klebsiella pneumoniae* clinical isolates collected from October 2006 to January 2007 from both community- and hospital – based isolates were included (Figure 1.). 128 ESBL isolates were subjected to further analysis: screening with double disc diffusion test and confirmed by ESBL E test[15].

Methods and Results

Percentage of ESBL producing isolates was 56 and 44 from the hospitals and community, respectively. CTX-M was detected in 33 (25.8%) isolates. Seventy two strains originated from hospitals and 56 strains from community. Community isolates were obtained from outpatients deparment and according to medical records did not stay in hospitals within previous year. Duplicate isolates were excluded. Only single isolates (one isolate per patient) were analyzed.

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Fig. 1. Map of Croatia showing 12 hospitals and 13 Institutes of Public Health

CHC Zagreb-1, GH Sv.Duh Zagreb-2, CH Dubrava Zagreb-3, IPH Zagreb-4, IPH Varaždin-5, IPH Čakovec-6, GH Koprivnica-7, IPH Bjelovar-8, IPH Virovitica-9, GH Pakrac-10, GH N. Gradiška-11, GH Požega-12, IPH Sl.Brod-13, GH Našice-14, GH Vukovar-15, IPH Osijek-16, IPH Zabok-17, GH Zabok-18, IPH Pula-19, IPH Gospić-20, IPH Zadar-21, GH Split-22, IPH Split-23, IPH-Dubrovnik-24 and GH Ogulin-25.

CHC-Clinical Hospital Center, GH-General Hospital and IPH-Institute of Public Health

Circles indicate participating laboratory, whereas squares indicate laboratory in which a detected CTX-M positive strains.

CTX-M positive isolates were more prevalent in hospitals (57.6%) than in community (42.4%).

The gender ratio of hospital patients was [29.5%:70.5% (female: male)]. The CTX-M positive isolates from hospitals were found mostly in urine samples (47.4 %) but also from respiratory tract and wounds. A majority of patients were over 60 years old. Sixty patients were older than 60 years. Twenty one strain (16%) was obtained from respiratory tract and twelve (9%) from wound swabs. Urinary catheter was not significant risk factor for colonization with ESBL producing organism in our study. Previous antibiotic therapy was found to be significant risk factor for infection or colonization with ESBL producer. Hi square test was used to determine the statistical significance of the risk factors.

Most patients have been hospitalized in urology units or intensive care units. All of CTX-M-15 producers were isolated from urine from elderly men and woman (> 60 years). The gender ratio of community patients was [63.3%:36.7% (female: male)]. The CTX-M positive community isolates originated mostly from urine (93.8%) which is in concordance with previous reports[16].

After determination of minimal inhibitory concentration (MIC), a phenotype consistent with production of CTX-M-type β-lactamase was defined by a cefotaxime MIC ≥ 8-fold higher than ceftazidime MIC, with the MICs of both agents reduced ≥ 8-fold in the presence of 4mg/L clavulanic acid. Strains producing CTX-M of group 1 β-lactamase demonstrated high-level resistance to ceftazidime with uniform susceptibility to carbapenems[17,18]. Elevated MICs of ceftazidime in our strains is consistent with the production of CTX-M-15 β-lactamase.The rates of resistance to non-β-lactam agents, including tetracycline, amikacin, ciprofloxacin and cotrimoxazole for CTX-M producing *K.pneumoniae* from hospitals were 85.7%, 29.4%, 25.3% and 72.1%, from community 87.3%, 25.0%, 27.1% and 75.2% respectively. There was no resistance observed to carbapenems. Based on the conjugation assay as described previously[19], seven *bla* genes could be transferred to transconjugants with the frequency of transfer (10^{-4}–10^{-6}) to recipient strain *E.coli*, suggesting that they were plasmid mediated. *E. coli* A15R- resistant to rifampicin was used as recipient in conjugation experiments.

Resistance to chloramphenicol, tetracycline and cotrimoxazole were cotransferred alongside with CTX resistance from five, two and two isolates, respectively.

Only two isolates cotransfer tetracycline and co-trimoxazole resistance to *E.coli* recipient. In other strains, these resistance determinants were not located on transferable plasmids.

Isolates with a CTX-M phenotype were screened for bla_{CTX-M} alleles by multiplex PCR with primers MA-1 (5'-SCS-ATG-TGC-AGY-ACC-AGT-AA-3') and MA-2 (5'-CGC-CRA-TAT-GRT-TGG-TGG-TG-3'). PCR was performed under following conditions: 94° for 3 min, the 35 cycles consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s each, followed by a final extension at 72°C for 5 min. All 33 isolates were confirmed to produce group 1 CTX-M enzyme. More hospital isolates were positive [19 out of 33 (57.6%)] compared to community [14 out of 33 (42.4%)]. Twenty eight isolates gave a 400 bp PCR product apparently linking an IS26 element with bla_{CTX-M} which previously reported in the UK[18]. The bla_{CTX-M} genes from representative strains were sequenced using forward (MA-1) and reverse (MA-2) primers and primers for group – 1 CTX-M beta-lactamases in order to amplify the whole open – reading frame of bla_{CTX-M} gene.

Sequencing of PCR products of representative strains belonging to major clusters revealed the presence of CTX-M-15-β-lactamases.

Isolation of chromosomal DNA was performed as described by Kaufman et al.[20]. Bacterial genomic DNA was compared by using PFGE of *Xba* I- digested fragments as described previously[21]. The PFGE patterns were compared following the criteria of Tenover[21] for bacterial strain typing and analyzed by computer software GelComparII (Applied Maths, Belgium). An optimization of 0.50% and position tolerance of 3.00% was applied during the comparison of PFGE fingerprinting patterns. Three of the 33 isolates could not be typed using PFGE, due to either difficulties

with lyses of the bacterial cell wall or the digestion of the DNA. Typing of the CTX-M producing strains by PFGE revealed 14 similarity clusters (I to XIV) three of which, I, III and V, were classified as major similarity clusters, which have shown specific geographical distribution (Figure 2). Similarity cluster I harboured 5 of the 33 (15.2%) isolates, similarity cluster III harbored 8 of the 33 (24.2%) isolates; two isolates produced identical *Xba*I PFGE patterns, whereas similarity cluster V harboured 3 of the 33 (9.1%) isolates and they all originated from urine. Cluster VII, X and XII harboured two isolates each. Most isolates from both community and hospitals belonged to same large clusters. The remaining isolates showed unique, unrelated PFGE profiles and were unlikely to be considered as outbreaks strains.

Strains 13, 106, 26, 83 are single isolates which showed distinct PFGE patterns and were not related to other strains.

Although several studies addressed the issue of emergence of CTX-M – producing *Klebsiella pneumoniae* world-

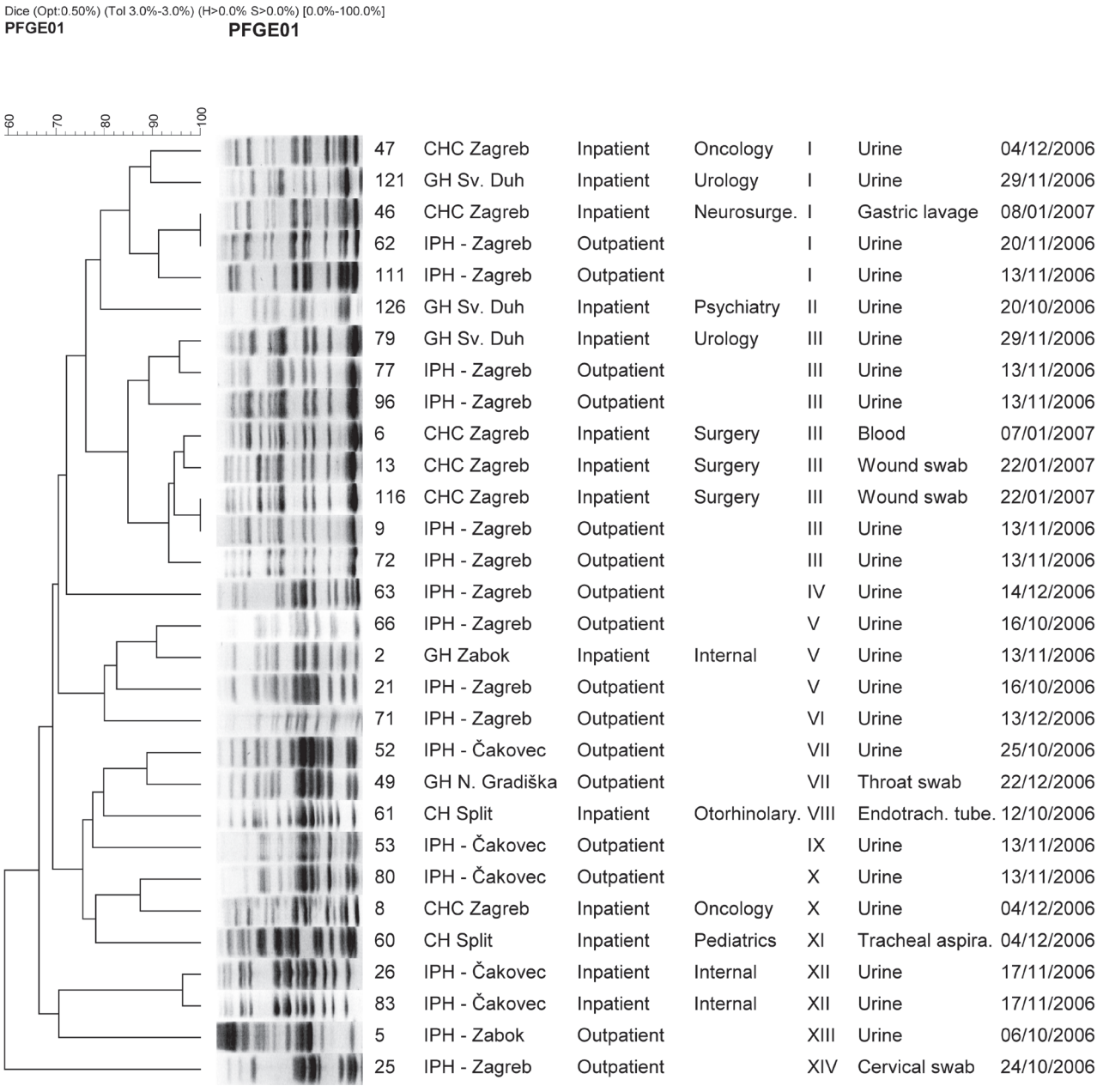

Fig. 2. Dendrogram of the 33 CTX-M producing Klebsiella pneumoniae.

Dendrogram of the 33 CTX-M producing Klebsiella pneumoniae.The seven columns on the right represent isolate code, centre, inpatient/outpatient, clinical ward, PFGE type, clinical specimen and date of collection.

wide, multicenter epidemiological survey has not been carried out in Croatia until now. It has only been reported that resistance to β-lactam antibiotics is increasing among clinical isolates from some hospitals, expressing the need for further investigation. In the previous report from our country, we have shown presence of group 1 CTX-M producing isolates in *E.coli* [22].

Discussion

The present study is first national survey from twelve Croatian hospitals and from the community and offers an insight in to the current prevalence and molecular types of CTX-M- producing *K.pneumoniae* in both the hospital and community setting. The previous studies have shown CTX-M-3 β-lactamase to be the most prevalent in Croatia[21]. Since $bla_{CTX\text{-}M\text{-}15}$ coding region differs from $bla_{CTX\text{-}M\text{-}3}$ by only a single amino acid substitution (Asp240 → Gly) it can be hypothesized that $bla_{CTX\text{-}M\text{-}15}$ evolved from $bla_{CTX\text{-}M\text{-}3}$ by point mutation in the coding region of the gene.

A number of risk factors have been identified as linked to the acquisition of community-acquired infection involving CTX-M-positive isolates. These are previous hospitalization or antibiotic therapy within the previous 3 month, old age (>60 years), male gender and urinary catheterization[4,23,24]. We have found two of above-mentioned risk factors in our community patients: old age (>60 years) and male gender, but did not find prior hospital contact, antibiotic therapy nor presence of urinary and vascular catheter in this group of patients.

Our data suggest that CTX-M producers are widespread in at least three regions of Croatia, possibly as a consequence of the dissemination of major clones between hospitals and community, between regions, and the horizontal transfer of plasmids or mobile elements. The presence of insertion sequences most likely facilitated the spread of $bla_{CTX\text{-}M}$ genes and enhanced the expression of the genes. The fact that isolates from different hospitals were clonally related points out that there was epidemic spread of related clones between hospitals in the same region. Our data suggest that these clones can spread from the community to the hospital and other way around. Their incidence may be rising with potential risk of transfer to other bacterial species, like *E.coli,* especially in community. This demonstrates the need to monitor both hospitalized and community patients for further emergence of transferable resistance to extended-spectrum cephalosporins.

In conclusion, this study highlights the need to establish an antimicrobial resistance surveillance network for *K. pneumoniae* and to further monitor the trends and new resistance mechanisms in the hospitals and the community. The factors responsible for the selection and dissemination of the plasmids encoding CTX-M type enzymes and clonal dissemination of strains have to be identified, controlled and prevented to avoid outbreaks.

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NACIONALNO ISTRAŽIVANJE SOJEVA BAKTERIJE *KLEBSIELLA PNEUMONIAE* KOJI PROIZVODE CTX-M PROŠIRENI-SPEKTAR B-LAKTAMAZA U HRVATSKOJ

S A Ž E T A K

Prošireni spektar beta-laktamaza (ESBL) kojr proizvode bakterije se povišeno pojavljuje kod bolničkih pacijenta I pacijenata u zajednici. Proizvodnja ESBL-a je glavni mehanizam otpornosti na oxymino-cefalosporine i aztreoname kod gram-negativnih bakterija[1,2]. Nedavno je zabilježena nova obitelj ESBL-a s dominantnim djelovanjem protiv cefotaksima (CTX-M beta-laktamaze). Više od 80 CTX-M enzima je do sada opisano, koji se mogu grupirati u pet glavnih podskupina prema istovjetnosti aminokiselinskog slijeda (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 i CTX-M-25)[3]. U nekim zemljama, CTX-M beta-laktamaze su najzastupljenije vrste ESBL-a, primjerice u Rusiji[4], Grčkoj[5], Španjolskoj[6], Švicarskoj[7], Japanu[8], Tajvanu[9], Kini[10] i Argentini[11]. Ovi enzimi su identificirani u zemljama u blizini Hrvatske poput Italije[12], Madžarske[13] i Austrije[14]. Cilj ovog istraživanja bio je utvrditi učestalost i vrste CTX-M beta laktamaza koje proizvode klinički izolati bakterjie *Klebsiella pneumoniae* prikupljeni od listopada 2006. do siječnja 2007. godine od oba u zajednici i bolnica-based izolata su bili uključeni (slika 1.). 128 ESBL izolati bili podvrgnuti daljnjoj analizi: screening s dvostrukim disk difuzije testa i potvrđuje ESBL E test[15].