Antimicrobial susceptibility and molecular epidemiology of drug-resistant bacterial pathogens of healthcare-associated infections

Doctoral thesis

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Budapest
2012
INTRODUCTION

Healthcare-associated infections are an increasingly recognized problem. The emergence of healthcare-associated infections caused by multidrug-resistant microorganisms is of increasing concern as well.

The author initiated, conducted, or participated in the presented studies and examined the antimicrobial resistance and/or the molecular epidemiology of drug-resistant pathogens of healthcare-associated infections.

The objectives, the methods, the results, and the conclusions of the following studies performed on important drug-resistant healthcare-associated pathogens (such as penicillin-nonsusceptible *Streptococcus pneumoniae*, vancomycin-resistant *Enterococcus spp.*, multidrug-resistant *Enterobacter spp.* and *Acinetobacter spp.*) are outlined briefly in this thesis book.
OBJECTIVES

1. The aim of the study was the examination of antimicrobial susceptibility of clinical *S. pneumoniae* isolates collected in Hungary in 2000. A further aim of the study was to examine the correlations between the MICs of penicillin-cefotaxime, penicillin-levofloxacin, and cefotaxime-levofloxacin;

2. The aim of the study was to collect and characterize vancomycin resistant *Enterococcus faecium* (VREF) clinical isolates from Hungary and Serbia and to examine the genetic relationship between the collected isolates and the previously identified international high-risk Enterococcal Clonal Complex, the *Enterococcus faecium* clonal–complex, CC-17;

3. The aim of our study was to help a Hungarian hospital trying to stop an outbreak caused by multidrug-resistant *A. baumannii*;

4. The aim of the study was to investigate the molecular epidemiological background of the high occurrence of multidrug-resistant *E. cloacae* isolates in a Hungarian perinatal intensive care unit in a one-year-long period;

5. The aim of our study was the examination of the molecular epidemiology of confirmed ESBL isolates sent in a three-year-long period (2002-2004) for ESBL confirmation to the
National Center for Epidemiology in the national ESBL surveillance from 25 healthcare facilities throughout the country. The outbreak strain isolated from the first reported outbreak caused by ESBL *E. cloacae* in Hungary was further examined to characterize the resistance determinants of the ESBL plasmid;

6. molecular epidemiology, cyclohexane tolerance, and Phe-Arg-β-naphthylamide (PAβN) susceptibility of multidrug-resistant *E. cloacae* isolates with third-generation cephalosporin and high-level fluoroquinolone resistance collected in Hungarian health-care facilities in a nationwide survey are reported in the last presented study.
MATERIALS AND METHODS

1. Penicillin susceptibility of 327 *Streptococcus pneumoniae* strains isolated from clinical specimens between January and June 2000 was determined by 1 μg oxacillin discs. Two hundred and seven strains (63.3%) were proved to be penicillin-susceptible (PS). Minimal inhibitory concentrations for penicillin of the remaining 120 strains (36.7%) were measured by the E-test method. Further 19 strains belonged to PS category, while 71 strains (22.3%) exhibited intermediate penicillin susceptibility (IPS) and 30 strains (8.9%) proved to be penicillin-resistant (PR). IPS and PR strains were examined further by cefotaxime and levofloxacin E-test.

2. Identification at species level and detection of *van* genes were performed by PCR on all the isolates (n=65). VREF isolates (n=36) were initially typed by PFGE, followed by multiple locus variable-number tandem repeat analysis (MLVA) and MLST performed on the vanB positive *E. faecium* outbreak strain.

3. Molecular epidemiological examinations were performed on 18 strains collected from 13 infected and two symptomless patients, and from the environment sampled in the hospital hygienic screening, performed investigating the small outbreak occurred in the particular unit in 2003. As
only a few data were available about the discriminatory power of the molecular epidemiological techniques for Hungarian \textit{A. baumannii} strains, all the collected isolates have been examined not only with the gold standard technique (PFGE) but other frequently used techniques (AP-PCR with ERIC-2 primers and RAPD with DAF-4 primer) as well. The class-1 integron patterns were also examined, as the collected strains were multidrug-resistant, and the presence of some class-1 integrons had been found previously by others to be connected with a high epidemic potential.

4. All the isolates (n=142) have been examined by antimicrobial susceptibility testing and ERIC-PCR. Representative isolates (n=54 were further tested by PFGE. The seven ESBL positive isolates (derived from six patients) have been further tested by class-1-integron PCR and plasmid electrophoresis.

5. ESBL confirmation was performed on all the isolates with double-disc test, ESBL E-test, and PCR based tests. Molecular epidemiological examinations were performed on the confirmed ESBL isolates with ERIC-2 primer. Isolates of the first outbreak were examined by plasmid electrophoresis, PFGE, PCR-sequencing, IEF, and, were drawn in conjugation experiments. Transconjugants were characterized by the same techniques and PREA. MIC
testing and specific PCR assays to detect transmissible resistance determinants were performed on the transconjugants and the parental strains simultaneously.

6. A total of 113 multidrug-resistant *E. cloacae* isolates (recovered in 1997-2005) were subjected to disc diffusion tests, ERIC-PCR, and *XbaI* PFGE. Representatives of the ERIC-types were further tested (n=67) with cyclohexane and PAβN using ciprofloxacin as substrate. Chloramphenicol and tetracycline MICs of 39 isolates were also tested.
RESULTS

1. Out of 68 IPS strains (the further three strains were died out during the study) 45, 21 and 2 were cefotaxime-susceptible, -intermediate and -resistant, respectively. Out of the 28 surviving PR strains 2, 8 and 18 proved to be cefotaxime-sensitive, -intermediate, and -resistant, respectively. Out of 28 PR and 68 IPS strains isolated from respiratory tract samples 85 proved to be susceptible, 7 (7.2%) intermediate and 4 (4.1%) resistant to levofloxacin. All these four strains were isolated from inpatients of that particular clinic where levofloxacin has been administered since the autumn of 1999. There was seen strong positive correlation between the MICs for penicillin and cefotaxime and week positive correlation between the MICs for levofloxacin and β-lactams by rank order correlation analysis.

2. Examining the macrorestriction profiles of the VanB VRE isolates recovered from blood, urine, and fecal cultures at a Budapest hospital between August 2003 and December 2004, ≥ 87.5 % similarity was found calculating the Dice coefficient suggesting the monoclonal origin of isolates (type A). Type B strains were isolated from a small outbreak in Kistarcsa, Hungary; the type C strain was a sporadic isolate from a second hospital in Budapest. Examining the VanA VRE isolates from a Belgrade hospital in Serbia, an outbreak involving two clones affecting six departments was
identified by PFGE (types D and E). The vanA gene cluster of the type E clone was found transferable by in vitro conjugation experiments. The two vanA positive *E. gallinarum* blood culture isolates identified at the Belgrade hospital was also found epidemiologically related by PFGE. Molecular typing of the VanB *E. faecium* outbreak strain by MLVA and MLST revealed that the outbreak strain belongs to the CC-17 and the corresponding MLVA cluster 1.

3. While three 100% corresponding types could be discriminated with the methods used in our study among the PCR-based typing techniques, one of these PCR types was found two different PFGE types by the year of isolation.

4. The ESBL isolates have been found indistinguishable in each of these laboratory tests, one genetic clone has been revealed in the background of ESBL cases by PFGE. The ESBL positive isolates have been proven to harbour a ~62 Md plasmid and two class-1 integrons (0.9 kb, 1.875 kb). PCR-sequencing has been performed on the ESBL outbreak strain and has revealed a *bla*<sub>SHV</sub> gene that encodes for an SHV-2a. All but one of the further representative isolates of the collection belonged to a second PFGE clone.

5. Out of the 85 *Enterobacter sp.* isolates 41 (48 %) were confirmed as ESBL. ESBL strains were seen in an increasing number year by year. ESBL *E. cloacae* isolates were more frequent (n=36) than ESBL *E. aerogenes* strains
(n=5). Four small outbreaks affecting two or three patients were highlighted by ERIC-PCR; all of them were caused by *E. cloacae* strains. The further 23 cases were found to be sporadic as belonged to unique ERIC patterns. Presence and expression of SHV-2a ESBL gene was proved by the concordant results by MIC testing, IEF, and DNA-sequencing in the *E. cloacae* strains of both patients involved in the first recorded small outbreak caused by ESBL *E. cloacae* strains in Hungary. The conjugative ESBL plasmid harbored an aminoglycoside and a tetracycline resistance determinant as well.

6. 44%, 19%, 17%, and 15% of isolates derived from urinary tract, bloodstream, respiratory tract, and wound infections, respectively. Four ERIC-types (A, B, C, and D) were distinguished, but 109 isolates were found to belong to a single, epidemic ERIC-type: A. PFGE results suggested the monoclonal origin of the epidemic-type-isolates. Forty-two patients were involved in four outbreaks caused by the epidemic-type-strains. Eighty-one cases were found nosocomial. At least fourfold reduction in ciprofloxacin MICs was found in the presence of PAβN in 79% of representative isolates (representing types A, C, and D); at least eightfold reduction in ciprofloxacin MICs in the presence of PAβN (PAβN+) was found in 37% of representative isolates (types A, and C). Eighty-five percents of representative isolates were found to be
cyclohexane-tolerant (types A, C, and D).
CONCLUSIONS

1. Penicillin susceptibility of respiratory tract isolates is suggested to be examined with E-test. Weak positive correlation between β-lactam and levofloxacin MIC values of *S. pneumoniae* isolates is reported first here. Emergence of extremely high penicillin and cefotaxime resistance and high level levofloxacin resistance in Hungarian clinical isolates of *Streptococcus pneumoniae* is also reported first here.

2. This is the first molecular study on VanB VRE isolates from Hungary, and VanA *E. faecium* isolates from Hungary and Serbia. Our results provide further evidence for the special clinical importance of CC-17 in hospital outbreaks and extend the documented occurrence of the pandemic MLST type to a novel region in Europe.

3. Comparing the results by the ERIC-PCR, the DAF-PCR, and the PFGE examinations, PFGE was found to be the best for hospital epidemiological examinations discriminating the outbreak strain from those isolated from the environment and the patients cared at the particular unit previously, in 2002. Our results showed that the strains isolated from patients in 2002 were able to persist in the hospital environment for months, as were found in the environment in the hospital hygienic screening as well. On
the other hand, the strains isolated from the patients affected by the small outbreak in 2003 and were found in further two patients by the hospital hygienic screening were not found in the environment. By the examination of class-1 integron patterns, the isolates from 2002 were also found to be different from those from 2003. Comparing the nucleotide sequences found in the class-1 integron of the outbreak strain to those found in an international database, emergence of an epidemic type described previously in another country might be assumed in Hungary. The lower discriminatory power of the PCR-based techniques found in our study might be explained by the emergence of an epidemic type in the particular unit.

4. Considering the results by the molecular epidemiological examinations, there was a large outbreak, affecting 94 premature newborn babies, and, with respect to their clinical relatedness and our laboratory findings there was a small outbreak caused by the ESBL clone. This is the first description of SHV-2a positive *E. cloacae* strains isolated in Hungary.

5. More than 2/3 of cases were caused by sporadic strains. Only small outbreaks were found. No epidemic clone (causing at least two cases at least two times) was found, the outbreak clones were found to be ephemeral. The ESBL plasmid isolated from the first outbreak strain harbored
bla SHV-2a, aac-III/2, and tetA genes. As tetA is widely distributed in many species of the Enterobacteriaceae family, but unusual in E. cloacae, the presence of tetA in E. cloacae on a large conjugative plasmid harboring more resistance determinants and so, conferring multi-drug resistance suggests the interspecies spread of such plasmids in vivo between E. cloacae and other species of the Enterobacteriaceae family.

6. This is the first report of wide distribution of cyclohexane-tolerant or PAβN+ E. cloacae strains. These features, indicators of adaptive mechanisms that help bacteria to survive in the hospital wards might have contributed to the nationwide spread of type-A-strains.
Parts of these studies were published:


List of further publications
by the author

