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

Doctoral thesis

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II. List of abbreviations

AFLP	amplified fragment length polymorphism
AMR	antimicrobial resistance
AP-PCR	arbitrarily-primed PCR
BAZ	Borsod-Abaúj-Zemplén
Bp	Budapest
BSI	bloodstream infection
CC	clonal complex
CDC	Centers for Disease Control
CFU	colony forming unit
CH	county hospital
CNS	Coagulase-negative staphylococci
CNSI	central nervous system infection
CLSI	Clinical Laboratory Standards Institute
CML	clinical microbiology laboratory
CREC	cephalosporine-resistant *E. cloacae*
CVC	central venous catheter
EARSS	European Antimicrobial Resistance Surveillance System
Env	environmental
ERIC	Enterobacterial repetitive intergenic consensus sequences
ESBL	extended-spectrum β-lactamase
EU	European Union
FQ	fluoroquinolone
HAI	healthcare-associated infections
HLAR	high level aminoglycoside resistance
ICU	intensive care unit
IEF	isoelectric focusing
MDR	multidrug-resistant, multidrug resistance

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List of abbreviations

MIC	minimal inhibitory concentration
MH	municipal hospital
MLST	multilocus sequence typing
MLVA	multiple locus variable-number tandem repeat analysis
MMA	Military Academy, Belgrad
MRSA	methicillin-resistant *S. aureus*
NBS	national bacteriological surveillance
NCCLS	National Center for Clinical Laboratory Standards
NCE	National Center for Epidemiology
NHI	National Health Institute
NPHMOS	National Public Health and Medical Officers' System
OEK	Országos Epidemiológiai Központ
og	obstetrics-gynecology
PAβN	Phenylalanine-Arginin-ß-naphtylamide
PCV	pneumococcus conjugate vaccine
PFGE	pulsed field gel electrophoresis
PICU	perinatal intensive care unit
PNSP	penicillin-nonsusceptible *S. pneumoniae*
PREA	plasmid restriction analysis
PRSP	penicillin-resistant *S. pneumoniae*
PYR	pyrrolidonyl-beta-naphtylamide
QRDR	quinolone-resistance determining region
RAPD	randomly amplified polymorphic DNA
RFLP	restricted fragment length polymorphism
ST	sequence type
SU	Semmelweis University

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List of abbreviations

TH	teaching hospital
Tn	transposon
UPGMA	unweighted pair group mathematical analysis
VAP	ventilator-associated pneumonia
VRE	vancomycin-resistant *Enterococcus spp*.
VREF	vancomycin-resistant *Enterococcus faecium*

III. Introduction

Healthcare-associated infections are of great concern as these are diseases with increasingly limited therapies. Hospitals represent unique ecological systems and provide the settings for nosocomial (hospital-acquired) infections. The principal components making up these systems are patients, medical-care personnel, equipment and devices employed in the treatment of patients with complicated medical illnesses, and the commensal microbiota of patients and the microbial population in the hospital environment. Modern acute-care hospitals are complex institutions consisting of a variety of specialized components: burn services, oncology wards, coronary care units, intensive care units, and transplantation units. Individual units may have particular nosocomial infection problems related to the type of patient being treated or the nature of their underlying illnesses, procedures employed in individual units, and the selection pressure exerted by antimicrobial usage patterns. We have now lived through more than sixty years of the antibiotic era. During this time we have seen the development of a remarkable array of antimicrobial agents – drugs that have clearly altered the course of medical history. On the other hand, the benefits from this class of drugs have been tarnished to varying degrees by the development and spread of antimicrobial resistance in many bacterial species. Antibiotic resistance surveys are published widely, citing percentage resistance rates, sometimes for vast transcontinental regions. Such data seem straightforward, but when one drills deeper, great complexity emerges. Rates for e. g., methicillin resistance among Staphylococcus aureus (*S. aureus)* from bacteriaemia vary from <1% to 50% among European countries, and vary greatly among both hospitals and hospital units [1, 2, 3, 4]. Methicillin-resistant *S. aureus* (MRSA) resistance rates are typically higher for tertiary-care hospitals and intensive care units than in general hospitals and wards. The likelihood of resistance also varies according to patient characteristics: those patients from nursing homes and with underlying disease, recent antibiotic treatment and hospitalization are more likely to harbor resistant pathogens. Percentage rates themselves also may be misleading; they may be high only because the denominator is small or inaccurate; i. e., resistance may be common but the pathogen rare. Measures of disease burden - cases per 1000 bed-days or per 10^5 individuals – overcome this deficiency but are harder to collect, influenced by case mix, and

associated with other problems: how to count part days or infections acquired elsewhere; most important, are all cases captured? National or international resistance statistics may illustrate trends and provide benchmarks, but for patient management, good local data are essential. Which units are most affected? Are the resistant infections locally acquired or imported with transferred patients? Are the resistant isolates clonally related, indicating cross-infection, or diverse, indicating repeated selection or reflecting antibiotic policy? Unless these aspects of infection are considered, interventions to reduce resistance may be misdirected. Managing antibiotic resistance also requires understanding the processes by which resistance mechanisms evolve and disseminate in populations of wild-type bacteria. As bacterial strains pose ever greater challenges to human health, including increased virulence and transmissibility, resistance to multiple antibiotics, expanding host spectra, and the possibility of genetic manipulation for bioterrorism, identifying bacteria at the strain level is increasingly important in modern microbiology. Bacterial strain typing, characterizing a number of strains in detail and ascertaining whether they are derived from a single parental organism is a way to identify bacteria at the strain level what is extremely important in hospital epidemiology and to uncover the genetic diversity and the genetic background of important phenotypic characteristics. That is why molecular epidemiology is essential trying to control healthcare-associated infections and to prevent the spread of drug-resistant pathogens not only in the hospitals but also in the communities over the hospital-walls. In the presented studies, the author examined the antimicrobial resistance and/or the molecular epidemiology of drug-resistant pathogens of healthcare associated infections.

The methods, the results, and the clinical importance of the antimicrobial susceptibility testing and the molecular epidemiological examinations performed in the presented studies on important drug-resistant healthcare-associated pathogens (drug-resistant *Streptococcus pneumoniae*, VRE, MDR *Enterobacter spp.* and *Acinetobacter baumannii*) are outlined in this work. MDR is defined in this work as usually as resistance to at least three classes of antimicrobial agents.

Respiratory tract infections are referred as the most frequent type of pneumococcal diseases. Beta-lactams and macrolides are two major groups of antibiotics used to treat respiratory tract infections, thus it is not surprising that severe treatment problems caused by the MDR strains (resistant to=/>3 antibiotic classes) have been reported from

different parts of the world. The emergence and spread of PRSP has been known to cause treatment failures all over the world since the early nineties [5, 6, 7]. The incidence and level of penicillin resistance in these bacteria was found to be varying greatly from one country to another [6, 8, 9], and Hungary was found to be one of the ten main foci of resistant organisms in the 1990s [8]. In many countries, resistance to other beta-lactams, macrolides, and cotrimoxazole was found more prevalent amongst PNSP isolates [6]. The newer fluoroquinolones with good antipneumococcal activity marketed first in the late 1990s may be considered for use in the treatment protocols of pneumonia caused by PRSP or MDR strains [10, 11]. Levofloxacin for example, is highly concentrated in lung tissue and macrophages and has long duration of effect after oral administration. Penicillin resistance in *S. pneumoniae* was found to be associated with resistance to broad spectrum cephalosporins, macrolides, and sulphonamides many times, but not to levofloxacin or vancomycin: little or no resistance was detected in Asia and Europe to levofloxacin and no resistance to vancomycin in the same period [11]. The laboratory examinations performed by the author and its colleagues at the turn of the century and reported here were performed following the actual recommendations given by the EARSS [12] and the results were interpreted following the instructions given by the NCCLS in the year of 2000 [13, 14].

Vancomycin was introduced as an antimicrobial agent in the late 1950s but it was not extensively used until the late 1970s when MRSA became prevalent. Since the first reports of VRE in 1988, these pathogens have emerged as an important cause of hospital acquired infections, particularly in North America: in 2002, 17.7% of *Enterococcus spp*. isolates derived from bloodstream infections and in 2003, 28.5% of enterococci isolated from ICU patients were resistant against vancomycin [15, 16]. Rates of VRE in nosocomial infections are typically lower in Europe where these figures varied from 0% in Switzerland to 21.2% in Ireland between 2002 and 2004 [17]. In the Eastern or Central European countries, the rates for vancomycin-nonsusceptible *Enterococcus faecium* (*E. faecium*) invasive isolates varied between 0% and 13.7% in 2005 as reported by the EARSS [18]. MLST on more than 400 VRE and vancomycin-susceptible *E. faecium* isolates, recovered from human and non-human sources and community and hospital reservoirs in 5 continents identified a clonal lineage designated clonal-complex-17 (CC-17), previously designated C1 lineage, that represents most

hospital outbreak and clinical VRE isolates. This clonal complex is also characterized by high level ampicillin-resistance and a novel putative pathogenicity island [19]. The spread of CC-17 in hospitals was also confirmed by studies conducted in other European countries like Germany and Italy [20-26]. The recently developed MLVA proved also useful in typing *E. faecium* isolates. This method groups CC-17 isolates into a corresponding distinct MLVA cluster designated MLVA-C1 [27]. A number of studies are available characterizing VRE clinical isolates by various molecular methods from Western or Southern European countries [17-25], however, such information was scarce with regard to VRE isolates from the Central-East European countries such as Hungary or Serbia.

Acinetobacter baumannii is an important pathogen of HAIs, mainly in the ICUs, where colonization and thereafter, infection of hospitalized patients with *A. baumannii* can be seen frequently. As an ubiquiter bacterium, *A. baumannii* is generally present in the hospital environment. Many studies have documented the rise of antibiotic resistance in clinical isolates of *Acinetobacter baumannii* (*A. baumannii*) on a global scale. A large study conducted in ICUs in the USA over 12 years indicated that, out of 74,394 Gram-negative bacilli collected, *A. baumannii* ranked fifth in frequency, at 6.2% [28]. Over this study period, mean rates of resistance increased in this species to nine out of 12 antibiotics tested. Rates of resistance steadily increased to ciprofloxacin, amikacin, piperacillin-tazobactam and ceftazidime from 1995 to 2004. To better understand the epidemiology and in particular the mode of spread of *A. baumannii*, a number of molecular typing systems have been developed, including PCR-based methods such as RAPD analysis [29], integrase gene PCR [30], infrequent-restriction-site PCR [31], ribotyping [32, 33], amplified fragment length polymorphism (AFLP) analysis [34], and PFGE [33, 35]. All of these methods rely on the generation of a distinct pattern or DNA “fingerprint” that is usually visualized by ethidium bromide staining or nucleic acid hybridization. So-called comparative typing systems, i.e., methods that depend on comparisons of DNA fragment patterns on gels, such as PFGE and RAPD analysis, are told to be well suited for local outbreak investigation. According to the results of a Hungarian nationwide survey at the turn of the century (unpublished results, NCE), *Acinetobacter spp*. were the second most prevalent among the potentially pathogenic bacteria isolated from the environment at the ICUs in Hungary. Although no significant

increase was seen in the number of *A. baumannii* isolates in the microbiology laboratories operated by the Hungarian National Public Health and Medical Officers' Cervices in the same period, summarized data in the annual reports sent from the Hungarian healthcare facilities to the NCE show a notable increase in the number of *A. baumannii* isolates between 2000 and 2002: the number of total isolates increased by 37% (from 906 to 1240), and moreover, the number of BSI/CNSI isolates increased by 83% (from 105 to 192) from 2000 to 2002 [36-38].

Enterobacter cloacae is a well known opportunistic pathogen. It has been repeatedly associated with sporadic or clustered cases of hospital acquired infection. MDR is a property that may account for the maintenance of bacterial clones in hospital environments under high antibiotic pressure. The national ESBL surveillance was initiated in 2001 by the NCE as there was little information on the presence of ESBL producing strains in Hungary, high incidence of CREC isolates in Hungarian hospitals had already been reported previously [39]. Appearance and spread of ESBLs can be attributed first of all to the excessive use of broad-spectrum cephalosporins. Since 1983, when ESBLs were described the first time [40] several derivatives of parental TEM and SHV enzymes have been characterized [41]. The worldwide distribution of them is of special interest. Epidemiological follow up for ESBL strains is extremely important to prevent and control infections caused by these strains as ESBL genes are harbored mainly in mobile genetic elements. The severe therapeutic problem caused by these strains is enhanced by the potential co-resistance to other antimicrobial agents explained by the frequent occurrence of ESBL genes on large conjugative plasmids carrying resistance determinants for aminoglycosides, tetracycline, sulphonamides and chloramphenicol as well [42]. Cyclohexane tolerance of Gram-negative bacteria is a well known indicator of the presence of cell-membrane-associated resistance mechanisms in MDR strains [43], but only one cyclohexane-tolerant *E. cloacae* isolate has been reported as yet [44]. In the last presented study, the author reports the nationwide spread of a MDR *E. cloacae* clone with cyclohexane tolerance.

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IV. Objectives

IV. I. Drug-resistant Streptococcus pneumoniae

The aim of the study performed by the author and its colleagues at the turn of the century and presented here first was the examination of antimicrobial susceptibility of clinical *Streptococcus pneumoniae* (*S. pneumoniae)* isolates collected in Hungary in 2000. Further aims of the study were: (i) to examine the incidence of resistance to penicillin, cefotaxime, and levofloxacin (the later has been administered since the autumn of 1999 in Hungary) in respiratory tract isolates of *S. pneumoniae* - isolated in Hungarian hospitals between 1st January and 30st June 2000 - following the recommendations given by the EARSS [12] and the NCCLS in the year of 2000 [13, 14]; (ii) to perform correlation analysis on the penicillin, cefotaxime and levofloxacin MIC values of the PNSP respiratory tract isolates of *S. pneumoniae* strains (n=96).

IV. II. Vancomycin-resistant Enterococcus spp.

The aim of the second presented study was to examine the molecular epidemiology of VRE clinical isolates collected in Hungary and Serbia. The author participated in the presented study performed in the NCE. This study was the first molecular study performed on VanB VRE isolates from Hungary and VanA *E. faecium* isolates from Hungary or Serbia. In this study, VRE isolates provided on a voluntary basis between august 2003 and October 2005 - by clinical microbiological laboratories in Hungary and by the Military Medical Academy (MMA) in Belgrade, Serbia - were characterized by molecular techniques. The study presented here highlighted outbreaks, among others the first VRE outbreak investigated by molecular methods in Hungary.

IV. III. MDR Acinetobacter baumannii

The aim of the third presented study was to help a Hungarian hospital trying to stop an outbreak caused by multidrug-resistant *Acinetobacter baumannii* (*A. baumannii*). In the lack of a reliable method for epidemiological typing of *A. baumannii* isolates, the source

of infections remained unknown many times in the ages before the molecular epidemiological techniques. The aim of the presented study was the molecular epidemiological examination of Hungarian MDR *A. baumannii* isolates. As with only a few exceptions there were no data about molecular epidemiological examinations performed on Hungarian *Acinetobacter spp*. isolates, a further aim of the presented study (in which the author participated) was to evaluate current molecular techniques (RAPD, AP-PCR, class-I integron PCR, PFGE), which has been used recently for epidemiological purposes worldwide.

IV. IV-VI. MDR Enterobacter cloacae

IV. IV.

The aim of the fourth presented study was to investigate the molecular epidemiological background of the high occurrence of MDR *Enterobacter cloacae* (*E. cloacae*) isolates in a Hungarian neonatal ICU in a one-year-long period. In a survey for extended-spectrum cephalosporin resistant *E. cloacae* strains isolated from Hungarian outbreaks, the antimicrobial susceptibility and molecular epidemiology of a collection of MDR *E. cloacae* isolates obtained from the samples of a Hungarian neonatal ICU in 1998 has been examined. The fourth study presented here was the first one examining the molecular epidemiology of CREC strain in Hungary.

IV. V.

The aim of the fifth study presented here was the examination of the molecular epidemiology of the confirmed ESBL isolates sent in the first three years of the national ESBL surveillance (2002-2004). In this period, eighty-five *Enterobacter spp.* strains were sent for ESBL confirmation from 25 laboratories throughout the country to the Department of Bacteriology in the NCE. The three isolates derived from the first reported cluster of cases caused by confirmed ESBL *E. cloacae* strains were further examined to characterize the resistance determinants of the ESBL plasmid.

IV. VI.

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As high incidence of MDR and coincidence of MDR with high-level ciprofloxacin resistance was recognized in 2001 among third-generation-cephalosporin-resistant *E. cloacae* isolates at the NCE, a nationwide survey was initiated in Hungary to collect MDR *E. cloacae* strains with high-level ciprofloxacin resistance and third-generation-cephalosporin resistance. In the last presented study, the author reports on the molecular epidemiology of the MDR *E. cloacae* isolates collected in this survey from health-care facilities in Hungary. A further aim of the study was to examine the cyclohexane tolerance and PAβN susceptibility of the collected strains, as these phenotypic features might be associated with MDR and might have been connected with the wide distribution of these strains in Hungary.

V. Materials and methods

V. I. Clinical settings and bacterial strains

Streptococcus pneumoniae

Between 1 January and 30 June 2000 a total of 3826 bacterial isolates from 12062 specimens from the in- and outpatients of Pál Heim Municipal Children's Hospital and several departments of University Hospitals, Semmelweis University (the Departments of Internal Medicine II., Oto-Rhino-Laryngology, Ophthalmology I., Pulmonology and perinatal intensive care units of the 1st and 2nd Departments of Obstetrics and Gynecology, Faculty of Medicine), Budapest, Hungary were examined for the presence of *S. pneumoniae*. Samples, except sputum specimens were taken in Mast transport medium with small swabs (Mast Diagnostica, Reinfeld, Germany) from the eyes and middle ears; the Biotest Transport System with normal sized swabs (Biotest, Dreieich, Germany) was used for other sites. Specimen collection and culture were done within 24 h on workdays and 48 h on weekends. Culture was carried out on 5% sheep blood agar and chocolate agar plates in 5% CO_2 at 35–37°C for 24–48 h. In our laboratory procedures, *S. pneumoniae* ATCC 49619 was used as a control strain. Strains were stored in a Mast Microbank System at –80°C.

Enterococcus spp.

The following isolates were examined in the presented study: (i) 21 vancomycin-resistant *E. faecium* (VREF) clinical, fecal, and environmental isolates sensitive for teicoplanin and one VREF strain resistant also to teicoplanin recovered from the outbreak detected at the Department of Hematology and Transplantation, NHI, Budapest, Hungary in 2004 (a brief description of the outbreak investigation can be read below); (ii) further 27, non *E. faecium Enterococcus spp*. isolates recovered from the departments of the same institute by the hospital hygienic screening of the patients the staff and the hospital environment performed in consequence of the outbreak described below; (iii) three fecal and two clinical VREF isolates obtained from the Ferenc Flór Hospital in Kistarcsa, Hungary; (iv) one further VREF clinical isolate provided by the I. Dep. of Internal Medicine, Semmelweis University; (v) in total, 9 VREF and 2

Enterococcus gallinarum (*E. gallinarum*) isolates with high-level vancomycin and teicoplanin resistance provided by the MMA (Belgrade, Serbia). Strains 7217 EARSS QA 2004 and 7218 EARSS QA 2004 provided by the EARSS as test strains for proficiency testing of *vanA* and *vanB* carriage in enterococci (EARSS QA 2004) were used as *vanA* and *vanB* positive control strains, respectively.

Acinetobacter baumannii

In a hospital-hygienic investigation performed in an ICU of a Hungarian secondary care hospital on the occasion of a small outbreak which affected two patients in 2003, respiratory tract of further two patients were found to be colonized. One of the infected patients died, the cause of death was indirectly the hospital-acquired pneumonia caused by a MDR *A. baumannii* strain. By the hospital-hygienic examination, three environmental samples of the 66 hygienic samples taken were found to be positive for *A. baumannii*. Altogether 15 human and 3 environmental isolates were drawn into our study: 11 samples of 11 patients who had been cared and infected in the particular ICU in the previous year (2002) were examined retrospectively; two isolates derived from the two patients affected by the small outbreak in 2003; the remaining five isolates (two from symptomless patients, three from the hospital environment) were obtained by the hospital-hygienic investigation performed in 2003, on the occasion of the small outbreak weltered the same year. Through the good offices of Kevin Towner, *A. baumannii* RUH 2037 [45] was used as control strain in the molecular epidemiological examinations. *Escherichia coli* (*E. coli)* ATCC 25922 was used as control strain in antimicrobial susceptibility testing assays.

Enterobacter spp.

Regarding the high clinical incidence, CREC isolates (n=142) derived from clinical samples of 94 patients of the neonatal ICU of a secondary and tertiary care, university affiliated department of obstetrics and gynecology were collected by the staff of the clinical microbiological laboratory in 1998. This collection of isolates included a) isolates from samples taken for general microbial monitoring with surface culture of external ear for infection screening purposes (2%), b) isolates from endotracheal aspirate surveillance cultures of mechanically ventilated patients (68%), c) isolates

from samples taken for clinical reasons when infection was suspected (30%). 28% of CREC positive patients were considered infected, the further 72% of patients were considered only colonized by CREC strains by the clinicians. The following drugs were used in the unit: combination of ampicillin and netilmicin regularly as empiric therapy, cefotaxime or ceftazidim frequently but only intentionally, chloramphenicol only by vital indication or externally, tetracycline only externally. Derivatives of trimethoprim, sulphonamide or quinolons were not used in the unit. For CREC infected patients imipenem was administered. The collection of CREC isolates were examined in NCE in 2004-2005. In our laboratory tests (NCE, 2004-2005) *E. coli* ATCC 25922 (for antimicrobial susceptibility retesting), *K. pneumoniae* ATCC 700603 (for ESBL screening) *E. cloacae* ATCC 13047 (for ERIC-PCR and PFGE) and *E. coli* V517 (for plasmid profile analysis) have been used as control strains.

Examining the strains sent in the national ESBL surveillance between 2002 and 2004 to the NCE, all the *Enterobacter spp.* strains sent for ESBL confirmation were identified to species level using the API-20E system (bioMérieux, Marcy l'Etoile, France) and stored in N2 bouillon with 10% glycerin at –80 °C in the NCE. *E. coli* ATCC 25922, *E. cloacae* ATCC 13047 and *K. pneumoniae* ATCC 700603 were used as control strains for antimicrobial susceptibility testing, *E. coli* J5-3 F^- R^- rif^R was used as recipient for conjugation experiments. *E. coli* V517 was used as a standard for plasmid profile analysis. The strains (*E. cloacae* 101/02, 102/02 and 112/02) from the first reported outbreak caused by ESBL *E. cloacae* in Hungary were isolated in 2002 from two nasal specimens of six days interval of the first (Eb101/02 and Eb112/02) and a blood culture of a second premature infant (Eb102/02), at a neonatal ICU in the county of Csongrád.

In the last study presented in this work, Hungarian microbiological laboratories were called (November, 2001) to send MDR *E. cloacae* isolates with the personal and institutional data. In this study, for the senders, in this study, MDR was defined as resistance to third-generation-cephalosporines, ciprofloxacin, and a third, non-ß-lactam agent for the senders. Eighty-six isolates were sent from 11 Hungarian counties. A further 27 isolates collected by the NCE in previous years were also drawn into the study. The laboratory control strains were *E. coli* ATCC 25922 (biochemical identification and antimicrobial susceptibility testing), *K. pneumoniae* ATCC 700603

(ESBL screening), *E. cloacae* ATCC 13047 (biochemical identification, antimicrobial susceptibility testing, ERIC-PCR, PFGE, cyclohexane, and PAβN tests).

V. II. Identification and antimicrobial susceptibility testing

Streptococcus pneumoniae

S. pneumoniae isolates were identified as described by Ruoff [46]. Repeat isolates from individual patients were excluded. The presence of some capsular antigens was tested by Slidex Pneumo latex agglutination reagent (bioMérieux, Lyon, France). Susceptibility testing to appropriate antimicrobials was carried out by the disc diffusion method [13] with Oxoid discs (Oxoid, Basingstoke, UK). Penicillin resistance in *S. pneumoniae* isolates was screened using 1 μg oxacillin discs and strains showing ≥20 mm inhibition zones were assessed as susceptible. Strains exhibiting smaller inhibition zones around the 1 μg oxacillin discs were provisionally identified as penicillin non-susceptible [13] and further examined by low concentration penicillin E-test (AB Biodisk, Solna, Sweden). The results were interpreted as described previously [14]. Respiratory strains with penicillin MICs of 0.125 mg/L or higher were further tested for cefotaxime and levofloxacin susceptibility according to the protocol of the EARSS [12]. The E-test results were interpreted as described by the NCCLS [14].

Enterococcus spp.

Phenotypic screening for vancomycin-resistant *Enterococcus spp*. was carried out by Bile Esculin Azide (BEA, Oxoid) plates [47] and 6 mg/L vancomycin containing Brain Heart Infusion (BHI, Oxoid) plates as recommended by the CLSI [48], coupled with the application of the PYR test (Remel, Lenexa, USA) and motility tests. MICs were determined by the E-test method (AB Biodisk). Identification at species level and detection of *van* genes were carried out by PCR [49] and sequencing of PCR products. These results were also confirmed by the GenoType® Enterococcus Kit (Hain Lifescience, Nehren, Germany). The nucleotide sequence of the coding region of *vanB* of a blood culture isolate from the NHI, Budapest was deposited in GenBank with the accession number AY958220.

Acinetobacter baumannii

Susceptibility testing of *A. baumannii* isolates for 14 antibacterial agents such as ceftazidime (Caz), cefepime (Fep), imipenem (Ipm), meropenem (Mem) norfloxacin (Nor), ciprofloxacin (Cip), streptomycin (Str), gentamicin (Cn), tobramycin (Tob), kanamycin (K), netilmicin (Net), amikacin (Ak), chloramphenicol (C), tetracycline (Te) was performed by disk-diffusion test (disks: Oxoid) according to the instructions by the NCCLS [50], using Mueller Hinton agar plates (Oxoid).

Enterobacter spp.

Antimicrobial susceptibility of the CREC isolates - ESBL screening, using the modified synergy test [51], and further antimicrobial susceptibility retesting (as recommended for the standard disc diffusion tests [44]) for Ipm, nalidixic acid (Nal), Cip, Cn, Tob, Net, Ak, Str, C, Te, trimethoprim (Tri) and trimethoprim-sulfamethoxazole (Sxt) – collected in 1998 was tested with Oxoid discs.

All the 85 isolates sent for ESBL confirmation in the national ESBL surveillance were reexamined for ESBL production. ESBL confirmation was performed using the double-disk test (ESBL SET, MAST Diagnostics, Merseyside, UK) performed and interpreted following the instructions by the manufacturer; and the synergy test as described previously [51] respecting the modifications determined for *E. cloacae* [52]. Enhancement of the inhibition zone toward the amoxicillin-clavulanic acid (AMC) disk indicating a synergy between clavulanate and any one of the tested cephalosporins was interpreted as presumptive evidence of ESBL production. The synergistic activity of clavulanate (Cla) with both of Caz and cefotaxime (Ctx) was confirmed with ESBL E-test strips (AB Biodisk, Solna Sweden) containing Caz (0,5-32 mg/L)/Caz plus Cla (Caz, 0,064-4 mg/L; Cla, 4 mg/L) and Ctx (0,25-16 mg/L)/ Ctx plus Cla (Ctx, 0,016-1 mg/L; Cla, 4 mg/L). Production of ESBL was assumed when the MIC for any of combinations containing Cla was 8 fold lower than the MIC for the particular cephalosporine. Detailed susceptibility testing of the outbreak isolates for Ctx, Caz, ceftriaxon (Cro), cefpodoxime, Fep, AMC, Ipm, Nal, Cip, Str, Cn, Tob, Net, Ak, Sxt, Tet, and C was performed by disk-diffusion test (disks: Oxoid) and interpreted according to the guidelines by the NCCLS [69]. E-test (AB Biodisk) was performed for Caz, Ctx, Cro, Fep, AMC, Cip, Cn, Tob, Net, AK, Te, Sxt on Mueller-Hinton agar plates (Oxoid) following the instructions by the manufacturer and interpreted according to the NCCLS [53]. In 2010, antimicrobial susceptibility of 17 isolates - 9 sporadic and

all the outbreak isolates (n=8) – were retested for Ipm, ertapenem, Cn, Ak, Cip, Sxt, Te, tigecycline by disc diffusion test as described above, the results were interpreted following the actual guideline by the CLSI [54].

In the last study, biochemical identification of all the MDR *E. cloacae* isolates was performed using the API 20E identification system (bioMerieux, Marcy L'Etoile, France) according to the manufacturer's instructions. ESBL screening [51] and further disc diffusion tests [55] for Ipm, Cip, Cn, Tob, Net, Ak, Str, C, Te, nitrofurantoin (Ni), and Sxt were carried out with Oxoid discs. The MICs for Cip, C, and Te (Sigma-Aldrich Chemie, Steinheim, Germany) were determined as recommended by the CLSI [55].

V. III. Phage typing

Phage typing was performed on 118 cephalosporine-resistant *E. cloacae* isolates with a Hungarian set of 22 phages. Considering that this set of phages has been used since 1993, reproducibility was checked in preliminary studies, briefly: phage lysis was defined as more than 50 plaques and phage types were established for lysis patterns differing by two or more phages to achieve a higher than 90% (91%) reproducibility of results. In this study arrangement for phage lysis patterns was made in descending order and the types denoted. Subtypes were established for lysis patterns differed by one phage lysis from the typical patterns defined as types. Subtypes were signed in alphabetic order (with a and b) for patterns with one phage lysis more, and with d (degraded) for patterns with one phage lysis less. Regarding the presence of numerous similar patterns, cluster analysis was performed by the unweighted pair group method with arithmetic avarages (UPGMA) with Statistica for Windows version 4.5 (StatSoft Inc., StatSoft Hungary Ltd., Hungary), phage lysis patterns were transformed to a 22 digit number in a binary system: lysis was indicated with a 1, its absence with an 0.

V. IV. Analytical isoelectric focusing (IEF)

IEF was performed using PhastSystem (Pharmacia PhastSystem, Uppsala, Sweden) with ready polyacrylamide gel supplied by the manufacturer (Phastgel IEF 3-9,

Amersham Biosciences Ltd., Budapest, Hungary) on the chloroform lysates [56] of all the three *E. cloacae* isolates obtained in the first reported outbreak caused by ESBL *E. cloacae* in Hungary, the transconjugants (TCEC119 and TCEC120), and the supernatant of a crude sonic extract of *K. pneumoniae* ATCC 700603 as described previously [57]. Briefly, for the rapid enzyme activity test, 150 µl of nitrocefin solution (0.05 mg/ml) was added to 50 µl of bacterial sonic extract in a microdilution plate and if there was a change in color from yellow to orange or red in less than 20 to 30 s, 1 to 3 µl of sonic extract was applied to the gel. Less than an hour was needed to complete the IEF run. The gel was stained with 0.5mM nitrocefin (Oxoid) solution.

V. V. Organic solvent tolerance test

Organic solvent tolerance of the representative MDR *E. cloacae* isolates (n=67) selected on the basis of the PFGE results and the clinical epidemiological data was examined with cyclohexane (Sigma-Aldrich) by repeated testing as described by White *et al* [58]. Briefly: (i) strains were let grow to late logarithmic phase, (ii) cultures were diluted to a concentration of approximately 10^5 CFU/ml, (iii) a 500-µl aliquot of each bacterial suspension was plated on LB agar (Difco, Detroit Mich., USA) and allowed to dry, (iv) cyclohexane was overlaid to a depth of 2 to 3 mm, (v) the plates were sealed and incubated overnight at 30 ˚C. Efficiency of plating was checked with a parallel series of inoculated plates incubated without cyclohexane under the same conditions. Reproducibility was determined by repeated testing: two independent suspensions of each strain were obtained from single-colony cultures prepared from the stock cultures at different times. Growth was recorded as confluent growth (+), visible growth (<= 100 colonies; +/-), or no growth (-) [58].

V. VI. Efflux-pump inhibitor test with Phe-Arg-ß-naphtylamide (PAßN)

Efflux-pump inhibitor test was performed with ciprofloxacin and PAßN (Sigma-Aldrich) as described by Lomovskaya *et al* [59]. Briefly, interactions between ciprofloxacin and PAßN were assessed by a checkerboard titration assay: ciprofloxacin was tested at 11 concentrations (0.5 to 512 µg/ml), while PAßN was tested at 7 concentrations (0.06 to 40 µg/ml) in one plate (with 4 bacterial strains). With regard to

the results of this assay, the efflux-pump inhibitor test was performed using PAßN in a 20 μg/ml concentration on the same MDR isolates and the same control strain as the organic solvent tolerance test.

V. VII. Molecular typing methods

V. VII. I. PFGE

Enterococcus spp.

Genetic relatedness of the *Enterococcus spp*. isolates was initially determined by PFGE. Briefly, bacteria from overnight L-agar cultures were harvested and washed twice with cell suspension buffer (100 mM Tris-HCl [pH 8] and 100 mM EDTA), and the suspensions were diluted with cell suspension buffer to a final optical density at 610 nm (1-cm light path) of 3.7 to 4.0 (ca. 2.5×10^9 CFU/ml). Aliquots (0.2 ml each) of the suspensions were lysed, an equal volume of 1.2% molten SeaKem Gold agarose (Bio-Rad Laboratories, Hercules, USA) containing 1% sodium dodecyl sulfate was added, and the mixtures were poured into 2-cm by 1-cm by 1.5-mm reusable plug molds (Bio-Rad Laboratories) and allowed to solidify at 4° C for 10 min. The proteolysis, washing, *Sma*I restriction analysis, electrophoresis (performed using the CHEF DR II apparatus (Bio-Rad Laboratories), staining and destaining steps were performed as described by Turabelidze *et al* in 2000 [60] (see the section of "Modified PFGE protocol"). Representative VRE isolates were selected for further characterization by MLVA and MLST based on the origin and PFGE profile.

Acinetobacter baumannii

DNA agarose blocks from *A. baumannii* isolates for PFGE were prepared following the standard procedure by ARPAC [61] and first described by Bannerman *et al* [62]. Briefly, a two mm slice of each block was digested with 20 U *Apa*I (New England Biolabs, Hitchin, UK) overnight, according to the instructions given by the ARPAC [61]. PFGE was performed following the standard protocol [61]. Briefly, fragments were separated in a Chef-DR II apparatus (Bio-Rad Laboratories, California, USA) in 1.5 % agarose gel (Pulsed Field Certified Agarose, Bio-Rad Laboratories), 0.5 x TBE

buffer at 200 V, 14 °C for 20 hours with pulse times linearly ramped from 5 s to 13 s. Lambda ladder (Bio-Rad Laboratories) as an external reference standard was run in every sixth track of each gel.

Enterobacter cloacae

DNA agarose blocks from *E. cloacae* isolates were prepared following a standard procedure [63]. A two mm slice of each block has been digested following the instructions by the manufacturers with 50 U *Xba*I (New England BioLabs) [64] overnight. Fragments have been separated in a Chef-DR II apparatus (Bio-Rad Laboratories) in 1% agarose gel (Pulsed Field Certified Agarose, Bio-Rad Laboratories), 0.5x TBE buffer at 200 V, 14 °C for 26-29 hours with pulse times linearly ramped from 3 to 60s. Lambda concatemer (Bio-Rad Laboratories) has been used as molecular weight standard. Dendrogram for macrorestriction profiles has been prepared calculating Dice coefficient, applying UPGMA at 1% band position tolerance, 1% optimization, and 2% band size correction to the end of gel. Lambda concatemer has been used as external reference systems in this procedure. Evaluation of PFGE results was made in accordance with recent publications [64, 65].

In the last study, DNA blocks for PFGE [59] were digested following the instructions by the manufacturers with *Xba*I (New England BioLabs), *Not*I (New England BioLabs) [66], and *Spe*I (New England BioLabs) [67], respectively. PFGE was performed as described above, dendrograms for macrorestriction profiles were prepared calculating the Dice and the Pearson's coefficients.

V. VII. II. *MLVA and MLST*

Enterococcus faecium

MLVA and MLST were performed on the vancomycin-resistant *Enterococcus faecium* isolates as described by Top [27], and Homan [68], respectively.

V. VII. III. *PCR based molecular epidemiological examinations*

Acinetobacter baumannii

Chromosomal DNA for PCR reactions was extracted from overnight colonies of *A. baumannii* isolates grown on nutrient agar plates. For the RAPD analysis, two to three colonies were suspended in 100 µl PCR-quality water, vortexed for 10 s, and centrifuged at 10,000 *g* for 2 min. The supernatant (up to 20 µl) was used as a template in PCRs.

RAPD fingerprints were generated with the DAF-4 primer as described by Grundmann *et al* [29].

AP-PCR fingerprints were generated with the ERIC-2 primers as described by Steinbrueckner *et al* [69].

The class-1 integron PCR was performed as described by Ploy *et al* [70]. Amplification of the class 1 integron gene cassettes was carried out in 50-µl volumes with primers 5'CS and 3'CS, as described previously by Le´vesque et al [71]. Amplification products were separated through agarose 1.75% w/v gels (Sigma Type II: Medium EEO, Sigma-Aldrich) in 1x TAE buffer (Merck KgaA, Darmstadt, Germany), at 120 V/200 mA, with bromophenol blue as the tracking dye. An external reference standard was run in every sixth track of each gel (in DAF-4 gels: 100-bp DNA ladder, Amersham Pharmacia Biotech, Little Chalfont, USA; in ERIC-2 and class-1 integron gels: pGEM DNA Markers, Promega, Madison, USA). Sequencing of the class-1 integron cassette PCR products amplified from *A. baumannii* isolates by the 5' - 3'CS primers were cleaned using Qiaquick purification columns (QIAGEN, Crawley, UK) according to the manufacturer's instructions and sequenced by using the ABI PRISM dRhodamine terminator protocol as recommended by the manufacturer (Perkin-Elmer Applied Biosystems, Les Ulis, France). Products were analyzed with an ABI PRISM 373 automated DNA sequencing apparatus (Perkin-Elmer Applied Biosystems).The nucleotide sequence analysis procedure was obtained online over the Internet at the National Center for Biotechnology Information website [72].

Enterobacter spp.

Chromosomal DNA for PCR reactions from *Enterobacter spp.* isolates were extracted from overnight cultures (N2 agar plate) with a commercial kit (Wizard Genomic DNA Purification Kit; Promega, Madison, USA), based on alkaline lysis.

ERIC-PCR was performed with 100 pmol of ERIC2 and ERIC1 primers (Sigma-Aldrich), respectively, on the CREC and the MDR isolates as described previously

[73]. PCR reactions for ERIC sequences were performed by standard techniques [74, 75]. Amplification products (20 μl of samples) have been separated by horizontal electrophoresis in 2% agarose gels (Sigma Type II: Medium EEO, Sigma-Aldrich) in 1x TAE buffer (Merck), at 110 V for 4 h. Molecular weight standards were included in each PCR gel (ERIC-PCR gels: 100-1000 bp DNA marker, Sigma-Aldrich; class-1 integron PCR gels: 300-2645 bp, pGEM DNA Markers, Promega). Results by the ERIC-PCR were evaluated in accordance with recent publications [76, 77].

Molecular typing of all the isolates collected in the national ESBL surveillance between 2002 and 2004 and proved to be *Enterobacter sp*. and ESBL positive with the confirmatory tests was performed by ERIC-PCR as described above, but only with ERIC-2 primers. The isolates obtained from the first reported outbreak (101/02, 102/02, 112/02) were further tested with PFGE, and plasmid electrophoresis.

Class-1 integron PCR was executed with 5’-3’ CS primers (Sigma-Aldrich) as described by Levesque *et al* [71].

V. VII. IV. Plasmid profile analysis

Plasmid DNA was extracted by the standard procedure published by Kado and Liu [78]. Plasmids were separated in a vertical system on 0.7 % agarose gels (Sigma type II: Medium EEO, Sigma-Aldrich) in 1x TAE buffer at 120 V for 4-5 hours. *E. coli* V517 was used as molecular weight standard in each gel. The gels were stained, detected, and the molecular weight of the plasmids were calculated by the gene detection/analysis system and software described below (see section “Analysis of DNA gels”).

V. VIII. Specific PCR assays

The *esp* gene encoding the *E. faecium* variant of the enterococcal surface protein (Esp) and the *erm*B erythromycin resistance determinant were detected by PCR [79, 80]. ESBL genes were detected from the total DNA prepared by a standard technique [81] with primers described previously for SHV, TEM, and CTX-M genes [82-85].

PCR-s for tetracycline resistance determinants were performed as described by Aminov *et al* [86, 87], PCR-s for aminoglycoside resistance determinants were performed as described by van de Klundert *et al* [88].

PCR reactions for the quinolone resistance-determining regions (QRDRs) of *gyrA*, and *parC* genes [44], and ESBL (*bla*$_{SHV}$ and *bla*$_{CTX-M}$) genes [89, 90] were performed by standard techniques.

PCR-RFLP for *bla*$_{SHV}$ genes with 25 pmol of each of SHV5' and SHV3' primers (Sigma-Aldrich) and *Nhe*I (New England BioLabs) was carried out as described Nuesch-Inderbinden *et al* [91].

PCR-sequencing was performed as follows: amplified PCR product were purified with Prep-A-Gene DNA Purification Kit (Bio-Rad Laboratories) and analyzed in Genetic Analyser310 ABI Prism (Applied Biosystems, California, USA) with ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and AmpliTaq DNA polymerase (PerkinElmer, Massachusetts, USA).

DNA sequences were analyzed by using BLASTN (www.ncbi.nlm.nih.gov/BLAST/) [72].

The nucleotide sequence of the *esp* PCR product of a blood culture isolate from the NHI, Budapest was deposited in GenBank with the accession number AY960761.

V. IX. Analysis of DNA gels

All the DNA gels were visualized under UV light by staining with ethidium bromide 5 mg/L for 30 min, recorded and stored as files with GelDoc 2000 (Bio-Rad Laboratories). All the DNA gels were subjected to visual examination, computer assisted analysis were performed with Quantity-one and Fingerprinting II, version 3.0 softwares (Bio-Rad Laboratories). Molecular weight standards were used as an external reference system in each gel.

Results by the molecular typing methods (RAPD, AP-PCR, PFGE) were evaluated in accordance with recent publications [64, 65, 76, 77].

V. X. Mating out assays

Conjugation experiments of the VRE study were performed on MH agar plates with a representative clinical isolate from Serbia as donor, and the Rif^{R} *E. faecalis* JH2-2 and *E. faecium* 64/3 strains as recipients [92, 93]. The initial donor/recipient ratio was 0.3. Mating plates were incubated at 30 °C or 37 °C for 14 hours. Transconjugants were

selected on BHI agar plates containing 50 mg/L and 6 mg/L rifampicin and vancomycin, respectively.

Conjugation experiments examining the first reported outbreak caused by ESBL *E. cloacae* in Hungary were performed with the first *E. cloacae* isolate of both patients (101/02 and 102/02). A 0.5 McFarland suspension of both of donor isolates and the recipient strain *E. coli* J5-3 F $^-$ R $^-$ rif^R were prepared in Nutrient broth (Oxoid), respectively. The broth for the donor isolates were supplemented with 4 mg/L cefotaxime (Sigma Aldrich). Suspensions were cultured up to 3.0 McFarland by dynamic incubation at 37 ºC. 4 ml of the recipient, 1 ml of the donor strain broth cultures were centrifuged for 3 minutes with 3000 rpm, respectively. The sediments were washed with nutrient broth, mixed (one of clinical isolates with the recipient, respectively) and incubated at 37 ºC for one hour. One ml sterile nutrient broth was added to each and the suspensions were incubated at 37 ºC overnight. 50 µl of these overnight cultures were plated on to selective Mueller-Hinton agar plates (4mg/L cefotaxime, 300 mg/L rifampicin). Identification of transconjugants (TCEC119, TCEC120) was performed by API 20E, ESBL screening by double disc and synergy tests, and ESBL E-test. PCR amplifications of bla_{SHV} and bla_{TEM} genes were performed on both of the transconjugants, and the recipient strain (*E. coli* J5-3 F $^-$ R $^-$ rif^R).

The following laboratory procedures were performed to examine the transconjugants derived from the first reported ESBL outbreak: plasmid isolation and gel electrophoresis was performed on the recipient strain, and the transconjugants as described above; plasmid restriction enzyme analysis (PREA) was performed on the plasmid DNA of TCEC119 and TCEC120 obtained using QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany) using *Eco*RI (New England Biolabs), fragments were detected by gel electrophoresis in 0.7% agarose at 110V for 2 h; DNA sequencing of the amplified SHV gene of TCEC120 was performed as described above.

V. XI. Statistical analyses

To examine the correlations between the penicillin, the cefotaxime, and the levofloxacin MICs of the PNSP respiratory tract isolates of *S. pneumoniae* isolates collected in the first presented study, we attempted to apply a linear regression analysis as described by Brueggemann *et al* [94], but the distribution of MICs did not correspond to a normal

distribution. Instead, therefore, the Spearman rank correlation coefficients were calculated from the MIC data using the software Statistica for Windows, v. 4.5.
Statistical comparisons to calculate one-tailed P values on tetracycline, chloramphenicol, and ciprofloxacin MICs of the MDR *E. cloacae* isolates representing all but one year of the study period were performed using the Mann-Whitney test [95].

VI. Results

VI. I. Drug-resistant Streptococcus pneumoniae

Prevalence of Streptococcus pneumoniae

Between 1st January and 30st June 2000 a total of 3826 clinically relevant isolates were recovered and 327 (8,5 %) of them proved to be *S. pneumoniae*; 75% of these strains ware isolated from the upper respiratory tract (URT), 15% from the lower respiratory tract (LRT), 4 %, 2 %, 1 %, 3 % from eye, wound, bloodstream and urogenital tract infections, respectively. Figure I shows the distribution of isolates by the site of infection.

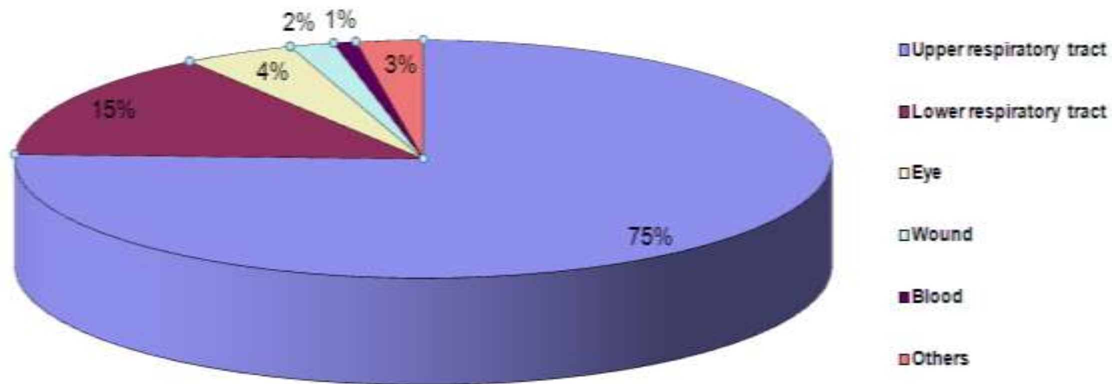

Figure I. Distribution of isolates by the site of infection

Out of the 327 *S. pneumoniae* strains 221 (68%) and 106 (32%) were isolated from children aged ≤12 years and adults, respectively. In- and outpatients were the sources of 72%, and 28% of the isolates, respectively.

Antimicrobial susceptibility (disc diffusion test) and incidence of MDR strains

Table I shows the incidence of resistance to penicillin, macrolides, cotrimoxazole, tetracyclines and chloramphenicol in *S. pneumoniae* strains tested (n=327) comparing these results with those of PRSP isolated in the same period. The incidence of MDR was 6%. In the rest of our study we focused on the PNSP strains.

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Table I. Comparison of antimicrobial susceptibility of *S. pneumoniae* strains tested between 1 Jan and 30 Jun 2000 and PRSP strains isolated during the same period

Antimicrobials	number of strains	susceptibility	susceptibility of PRSP strains
Penicillin	n=327	64%	-
Erythromycin	n=321	54%	11%
Cotrimoxazole	n=227	48%	32%
Tetracycline	n=261	71%	89%
Chloramphenicol	n=294	96%	100%

Results of penicillin MIC testing of S. pneumoniae strains non-susceptible with 1 μg oxacillin disc

One hundred and twenty strains (36.7 %) were predicted as PNSP by the 1 μg oxacillin test. By further examinations performed on these strains by the E-test method, additional 19 strains were found as penicillin susceptible with MICs between 0.008 mg/L and 0.094 mg/L. Figure II shows the distribution of these 120 strains by their penicillin MIC values measured by the E-test method. Following the interpretative MIC standards given by the NCCLS [14], the overall incidence of penicillin susceptibility, and resistance was 69.1%, and 9.2%, respectively (21.7% of the strains were qualified intermediate susceptible).The incidence of penicillin resistance was 12% (n=221) in children, and 3% (n=106) in adults (Figure III). 73% of PRSP strains were isolated from children younger than six years. Susceptibility of PRSP isolates for erythromycin, sulphamethoxasole-trimethoprim, and tetracycline varied between wide ranges (see Table I), glycopeptides, however proved to be 100% effective *in vitro*. Almost all of the 101 *S. pneumoniae* strains qualified PNSP by the E-test method were obtained from respiratory tract specimens (28 PRSP and 68 penicillin-intermediate-sisceptible); three penicillin-intermediate-susceptible strains were cultured from eye infections; two PRSP strains (with penicillin MICs of 4mg/L and 3 mg/L) were isolated from a bloodstream and a urogenital tract infection. The PNSP respiratory tract isolates were examined further following our surveillance protocol (Figure IV) created on the basis of the

recommendations given by the EARSS. In the rest of our study we have focused on the PNSP respiratory tract isolates.

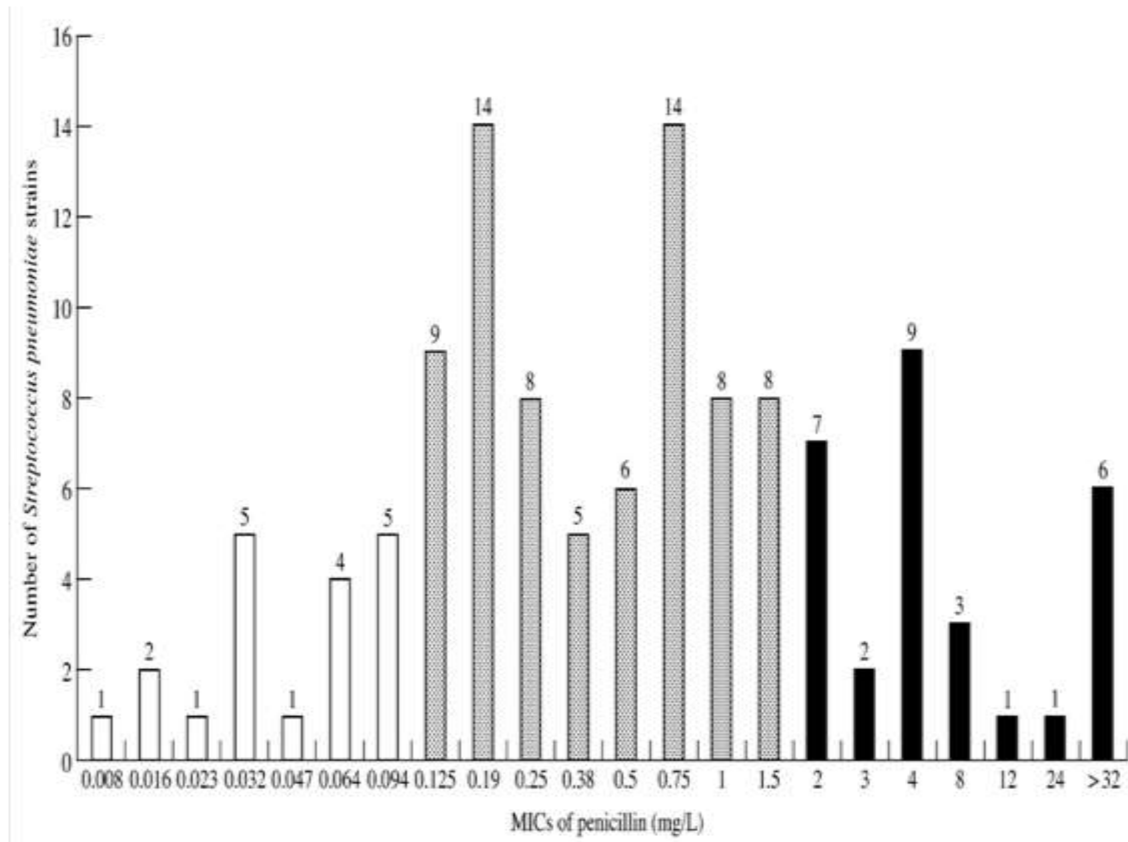

Figure II. Distribution of PNSP strains by their penicillin MICs by E-test method

MICs of penicillin for 120 *S. pneumoniae* strains proved to be penicillin non-susceptible with 1 μg oxacillin disc. White columns: susceptible; gray columns: intermediate; black columns: resistant.

Cefotaxime and levofloxacin susceptibility of penicillin non-susceptible respiratory tract isolates

The 96 PNSP respiratory tract isolates were examined further by the E-test method for their quantitative susceptibility to cefotaxime and levofloxacin. Table II/a shows the MIC range, MIC50 and MIC90 of the strains studied, while Table II/b summarizes the results of cefotaxime and levofloxacin susceptibility testing using the interpretative standards given by the NCCLS [14]. Figure V shows the distribution of PNSP, respiratory tract isolates by their cefotaxime (a) and levofloxacin (b) MICs.

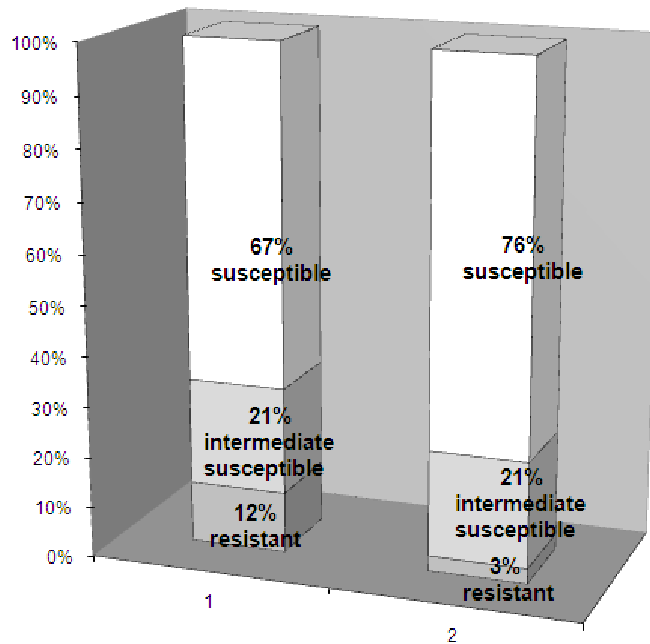

Figure III. Penicillin susceptibility of *S. pneumoniae* strains (n=327) distributed by age (1: children; 2: adults)

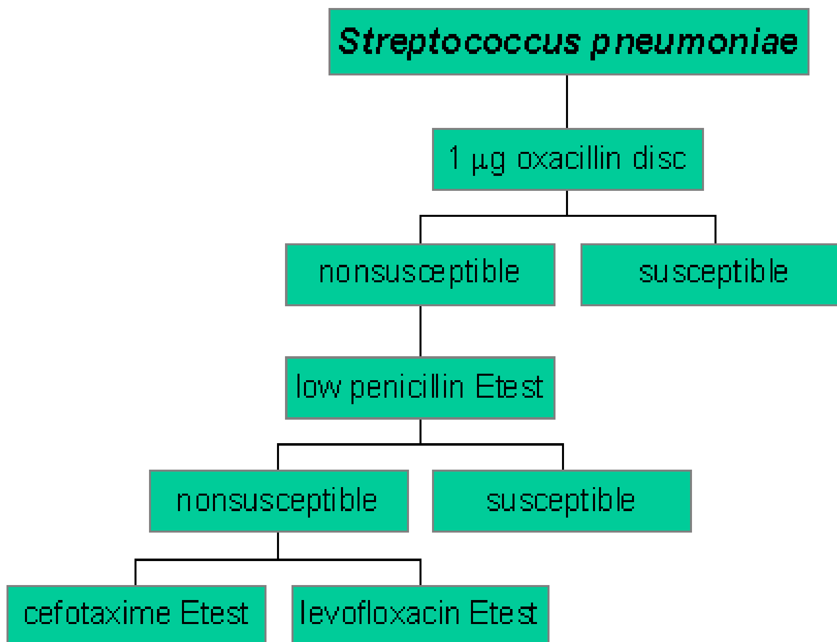

Figure IV. Protocol for the quantitative antimicrobial susceptibility testing of respiratory tract isolates of *S. pneumoniae*

Our surveillance protocol was created on the basis of the recommendations given by the EARSS in the year of 2000.

Table II. a Antimicrobial activity (mg/L) of penicillin, cefotaxime and levofloxacin on respiratory tract PNSP isolates

	penicillin			cefotaxime			levofloxacin		
PRSP	MIC	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range
URT[1] (n=19)	3	24	2-32	1.5	4	0.25-24	1	1.5	0.38-2
LRT[2] (n=9)	>32	>32	2-32	>32	>32	1-32	3	4	1-4

	penicillin			cefotaxime			levofloxacin		
PISSP[3]	MIC	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range	MIC_{50}	MIC_{90}	MIC range
URT (n=58)	0.38	1.5	0.1-1.5	0.19	0.75	0.032-0.75	0.75	1	0.25-1.5
LRT (n=10)	0.75	1	0.1-1.5	0.38	0.5	0.1-0.75	3	32	0.75-32

[1] Upper respiratory tract isolates

[2] Lower respiratory tract isolates

[3] Penicillin intermediate susceptible *S. pneumoniae* strains

Cefotaxime	S	I	R
Penicillin I (n=68)	67%	30%	3%
Penicillin R (n=28)	3%	33%	64%

Levofloxacin	S	I	R
Penicillin I (n=68)	91%	4%	5%
Penicillin R (n=28)	82%	18%	0%

Table II. b Cefotaxime and levofloxacin susceptibility of PNSP strains isolated from the respiratory tract

Carbapenem and vancomycin MICs of PRSP strains with extremely high penicillin and/or cefotaxime resistance

The PRSP strains with extremely high penicillin and /or cefotaxime resistance (MICs ≥8 mg/L) were examined with carbapenem and vancomycin E-test as well (see Table III). These examinations were needed to be performed because of the severe treatment problems caused by these strains. More than half of them had high level resistance to carbapenems as well. None of them were found to be resistant to levofloxacin but four out of them were proved to be intermediate susceptible to levofloxacin. All the 12 strains with extremely high level penicillin and/or cefotaxime resistance were isolated from children under 6 years of age. Although the E-test method was reported to be one of the most appropriate from the commercially available ones to examine the MICs of penicillin for *S. pneumoniae* isolates [96], but strains with extremely high level penicillin or cefotaxime resistance (>8 mg/L) are isolated rarely worldwide, so the penicillin and cefotaxime MIC values measured in our study with the E-test method for the 12 strains with extremely high penicillin or cefotaxime resistance were compared with those of measured by the conventional agar-dilution method [14]. Table IV shows, that MICs determined by using the E-test method did not compare within one two-fold dilution interval with those determined by conventional agar dilution method in five of 24 cases (grey cells), in four of which the MIC values reached or exceeded the highest MIC value measurable by the particular E-test strip (grading goes to 32 mg/L on the

penicillin, and the cefotaxime E-tests, and 128 mg/L on the high-level penicillin E-test strips). But, it has to be noted, that the strains were frozen in 2000, held frozen at – 80 °C in dextrose bouillon containing 20% glycerin and recultured before the second seria of examinations performed in 2001.

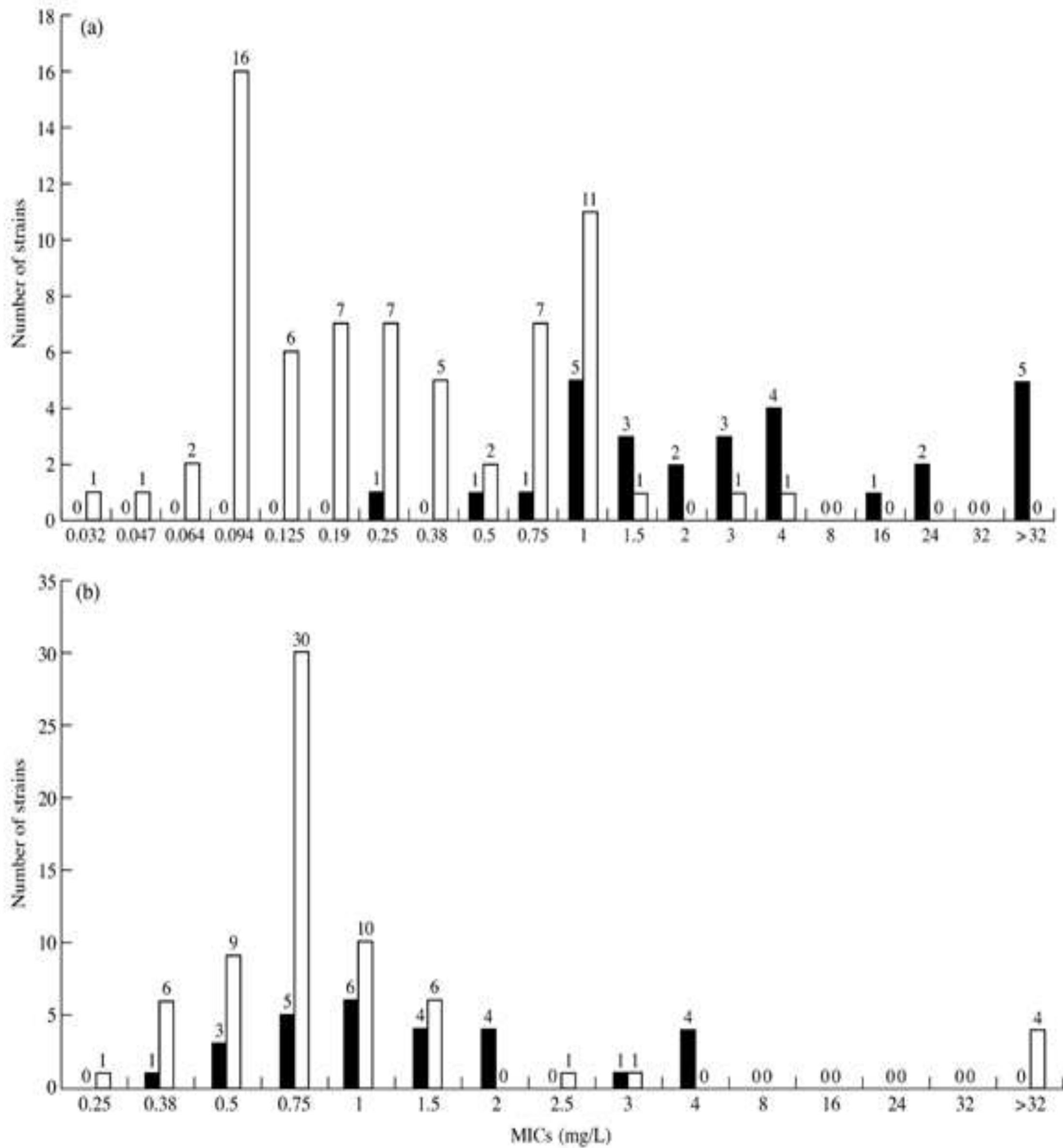

Figure V. Distribution of penicillin intermediate-susceptible and resistant strains *of S. pneumoniae* isolated from the respiratory tract according to their cefotaxime (a) and levofloxacin (b) MICs ($n = 96$).

Key: penicillin-resistant strains, ▪; penicillin intermediate-susceptible strains, □.

Table III. Penicillin, cefotaxime, carbapenem and vancomycin MICs of *S. pneumoniae* strains with extremely high penicillin and/or cefotaxime MIC

Sample	MIC (mg/L)					
	Penicillin	Cefotaxime	Imipenem	Meropenem	Vancomycin	Levofloxacin
Nasal swab	3	16	0.125	0.125	0.75	2
Tracheal aspirate	>32	24	1	0.38	0.5	2
Nasal swab	>32	24	6	6	0.75	1.5
Nasal swab	24	2	0.19	0.38	0.75	0.5
Nasal swab	24	3	0.75	0.5	0.75	0.75
Nasal swab	8	3	0.25	0.25	0.38	0.5
Nasal swab	>32	1.5	2	1.5	0.75	0.5
BAL	>32	>32	4	4	1	3
Tracheal tube	>32	>32	6	1.5	0.75	2
Tracheal tube	>32	>32	16	8	1	4
Tracheal tube	>32	>32	6	4	1	4
Tracheal tube	>32	>32	12	8	1	4

S. pneumoniae strains with low or high level resistance to levofloxacin

Four out of 96 PNSP respiratory tract isolates had high level resistance to levofloxacin (MIC ≥32 mg/L). All of them were isolated from sputa of inpatients hospitalized in the Department of Pulmonology. The strains with high level levofloxacin resistance were intermediate susceptible to penicillin and remained susceptible to cefotaxime and carbapenems.

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Table IV. Results of comparative testing of penicillin and cefotaxime MICs with E-test and agar dilution on 12 extremely resistant Hungarian *S. pneumoniae* isolates

Clinical data	Penicillin MICs				Cefotaxime MICs	
	E-test	high-penicillin E-test		agar dilution	E-test	agar dilution
	2000*	2000*	2001**	2001**	2000*	2001**
nasal swab	3	4	6	4	**16**	4
tracheal aspirate	>32	64	96	64	24	16
nasal swab	>32	48	64	64	24	32
nasal swab	24	24	12	16	2	2
nasal swab	8	8	6	8	3	2
nasal swab	24	24	16	16	3	2
nasal swab	**>32**	32	16-24 h	16	1.5	1
BAL	>32	48	96	64	**>32**	16
tracheal aspirate	>32	32	16-24 h	32	**>32**	16
tracheal aspirate	>32	48	48	32	>32	32
tracheal aspirate	>32	48	48	64	**>32**	16
tracheal aspirate	>32	48	32	64	>32	32

BAL: bronchoalveolar lavage; h: heteroresistance

* Examinations were made using one bacterial suspension of each isolate prepared at once in the year of 2000.

** Examinations were made from one bacterial suspension of each isolate prepared at once in the year of 2001. Isolates were held at -80°C in 20% glycerol between the two series of examinations (performed in 2000, and 2001, respectively).

Further seven out of the 96 PNSP respiratory tract isolates were found intermediate susceptible to levofloxacin. Four of these strains were resistant to penicillin, cefotaxime and macrolides, too (see Table III). Altogether, 11 levofloxacin non-susceptible strains were found in our study among the PNSP respiratory tract isolates. Eight of them

derived from inpatients treated at the Department of Pulmonology, where levofloxacin had been administered since the autumn of the year of 1999. Table V shows that 8 of the 11 isolates derived from the patients of the Department of Pulmonology in the study period were non-susceptible for levofloxacin.

Table V. Penicillin, cefotaxime, and levofloxacin MICs (mg/L) of *S. pneumoniae* isolates derived from patients treated at the Department of Pulmonology in the study period

	MIC (mg/L)	
Penicillin	Cefotaxime	Levofloxacin
0,38	0,25	0,75
0,5	0,38	0,75
4	4	0,75
2	1,5	2
0,5	0,38	2,5
0,75	0,25	3
3	1	4
0,5	0,094	>32
0,75	0,25	>32
0,75	0,38	>32
1	0,5	>32

MDR S. pneumoniae strains

Out of the 28 PRSP strains, 25 showed resistance to erythromycin, among these, 18 were resistant to a third drug (cotrimoxazole), and one of them displayed resistance to a fourth antimicrobial agent (tetracycline) as well. Out of the MDR strains (n=18) four were found non-susceptible for levofloxacin too.

Correlation analysis of penicillin, cefotaxime and levofloxacin MICs of penicillin non-susceptible respiratory tract isolates

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Strong positive correlation was found between penicillin and cefotaxime MICs (within the range of Spearman r coefficient ≥0.7). Weak positive correlation (within the range of Spearman coefficient $0.3 \leq r \leq 0.5$) was found between penicillin and levofloxacin MICs and also between cefotaxime and levofloxacin MICs.

The lines laid with the Statistica for Windows software on the values got by the Spearman rank correlation analysis (Figures VI.a, VI.b, and VI.c) demonstrate the positive correlation between any two of the three data sets (penicillin MICs vs cefotaxime MICs; penicillin MICs vs levofloxacin MICs; cefotaxime vs levofloxacin MICs).

Figure VI. a The line laid with the Statistica for Windows software on the penicillin MICs vs cefotaxime MICs got by the Spearman rank correlation analysis demonstrates the strong positive correlation.

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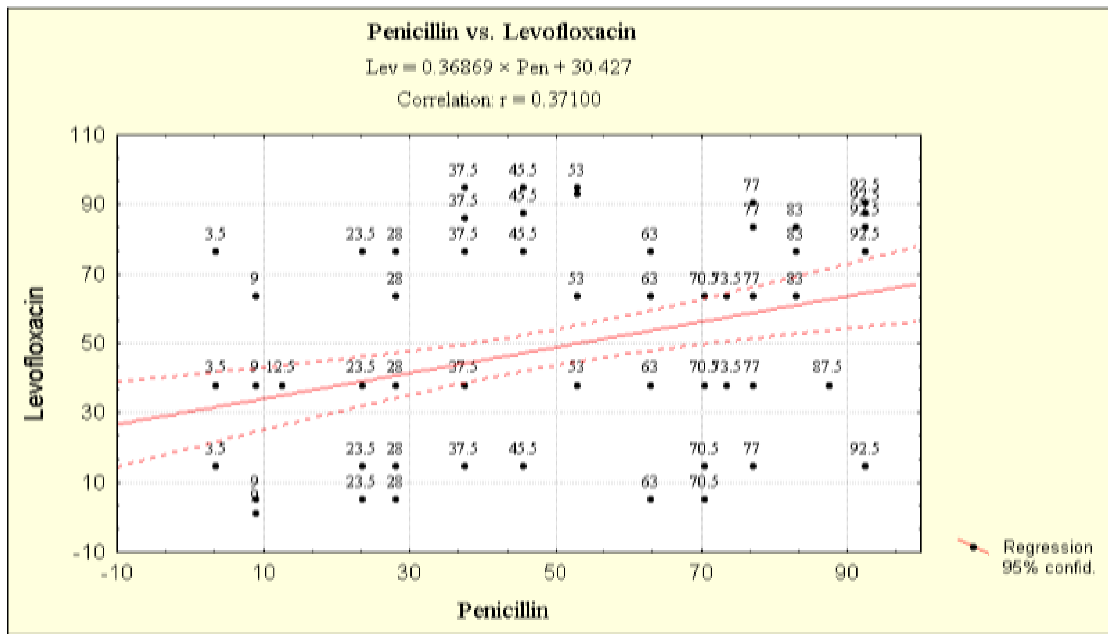

Figure VI. b The line laid with the Statistica for Windows software on the penicillin MICs vs levofloxacin MICs got by the Spearman rank correlation analysis demonstrates the week positive correlation.

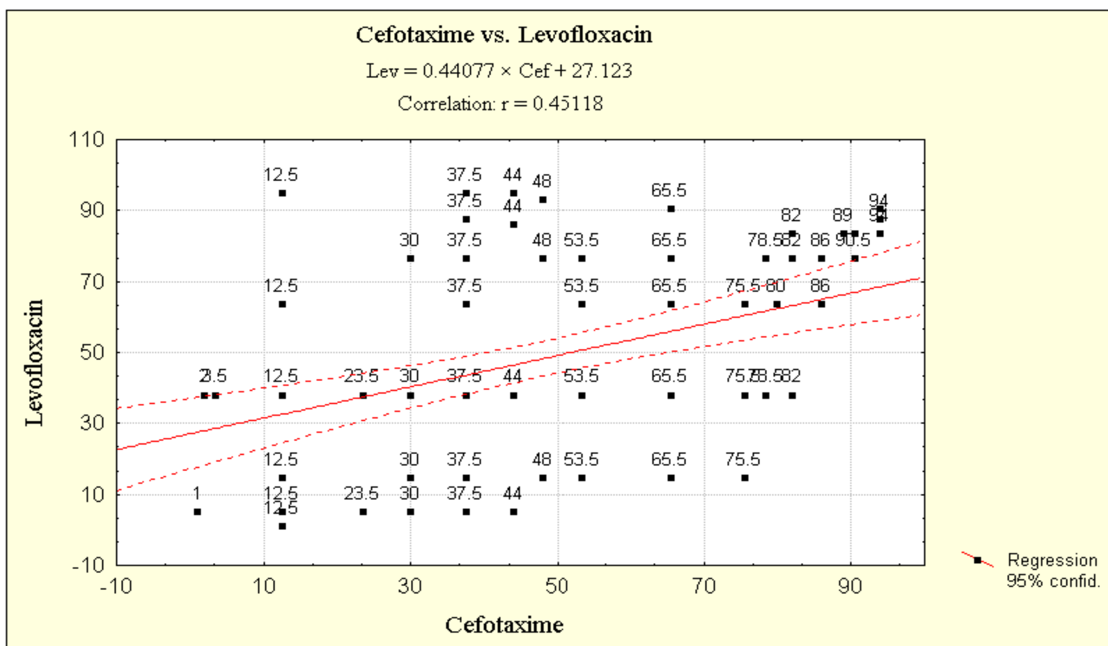

Figure VI. c The line laid with the Statistica for Windows software on the cefotaxime vs levofloxacin MICs got by the Spearman rank correlation analysis demonstrates the week positive correlation.

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VI. II. Vancomycin-resistant Enterococcus spp.

A cluster of VRE infections at the NHI, Budapest, Hungary

The first healthcare-associated vancomycin-resistant *E. faecium* (VREF) outbreak in Hungary occurred between April and September 2004 at a hematology and stem cell transplantation unit of a hospital. Fourteen cases of infection and seven cases of intestinal colonization were detected. During the outbreak, *E. faecium* was identified in blood samples (9 patients), urine (12 patients) and wound secretions (two patients). The vancomycin-resistant isolates had vancomycin MICs of 48-128 mg/L and were teicoplanin susceptible (MICs 1-2 mg/L) (the so-called VanB phenotype). During the epidemiological investigation at the hematology unit in September, *E. faecium* isolates were also identified in three environmental samples (a surgical bowl, a sheet from a ward, and a wash basin from the bedpan-washing room). As part of the investigation, stool samples from forty patients and the staff were tested. While none of the staff were colonized by *vanB* harboring VREF, eight of the screened patients (20%) were found carriers (colonization in seven cases and one symptomatic case). Two patients with symptomatic illness had undergone stem cell transplantation. Twelve of the 14 infected patients had malignant hematological disease. Five colonized patients also had hematologic malignancies.

A single *vanA* positive *E. faecium* was also recovered from the fecal sample of an administrator not in direct contact with the patients. An unexpectedly high rate (26.3%) of *vanC* enterococcal (*Enterococcus casseliflavus* n=5, or *Enterococcus gallinarum* n=13) carriage was observed among the staff and six patients not affected by the outbreak were also found positive for *vanC* harboring *E. gallinarum*.

Analysis of 13 selected *vanB* positive VREF isolates from blood, urine, fecal and environmental samples revealed 5 different PFGE patterns with ≥ 87% similarity by the Dice coefficient and belonged to a single PFGE type (PFGE type A) by eyes (not more than six band difference) suggesting the monoclonal origin of these isolates. In contrast, the single *vanA* positive VREF isolate and a vancomycin susceptible *E. faecium* from the same department displayed unrelated macrorestriction profiles (Figure VII). Sequencing the full length coding region of *vanB* from a blood culture isolate of the

index patient highlighted two amino acid substitutions compared to the originally described *vanB2* subtype [97].

The VREF outbreak clone (PFGE type A) displayed high-level ampicillin resistance (MIC ≥64 mg/L). Further antibiotic susceptibility testing showed vancomycin MICs ranging from 8 to 256 mg/L, with all but two of the isolates having an MIC ≥32 mg/L. All the outbreak isolates were sensitive to teicoplanin, linezolid, and quinupristin-dalfopristin. The gentamicin MICs varied between 128 mg/L and >1024 mg/L. Table VI shows the antimicrobial susceptibility patterns of the outbreak isolates representing the phenotypic diversity found in an outbreak setting in our study.

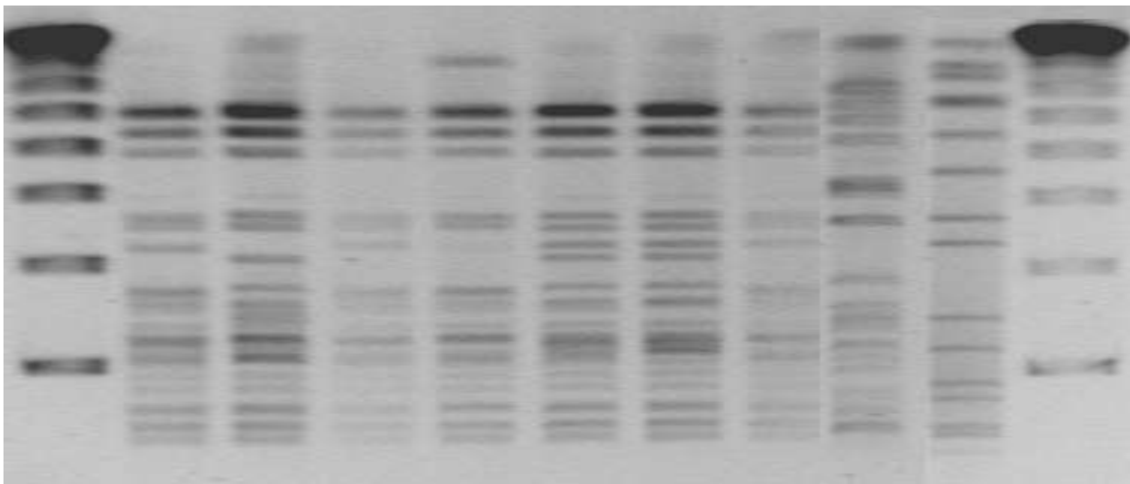

Figure VII. PFGE patterns of nine isolates derived from the NHI in 2004.

Lanes 1, 11: Lambda concatemer (molecular weight standard); Lanes 2, 3, 4, 5, 6, 7, 8: outbreak isolates selected on the basis of the phenotypic results (see Table VI) represented three of the five different PFGE patterns belonging to the outbreak PFGE type; Lane 9, 10: unrelated PFGE patterns of the *vanA* positive and a vancomycin susceptible *E. faecium* strains isolated at the NHI in the outbreak period.

Characterization of VanA VRE clinical and fecal isolates from further two Hungarian hospitals

In 2005, two vanA harboring *E. faecium* strains were isolated from a blood and a wound sample at the municipal hospital in Kistarcsa. These samples originated from the Departments of Intensive Care and Surgery, respectively, and proved to be closely related to each other by PFGE (PFGE type B). The two rectal swab VREF isolates of further two patients cared at the Department of Surgery of the same hospital were found

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also vanA positive, but clonally unrelated by the PFGE examinations. A further vanA VREF isolate cultured from a urine sample at the First Department of Internal Medicine (SU, Budapest) in October, 2005 was also found not related to PFGE types A or B, thus the macrorestriction profile of it was designated PFGE type C.

All the VanA VREF clinical and fecal isolates were highly resistant to ampicillin, vancomycin, teicoplanin, and gentamicin but were sensitive to linezolid and quinupristin/dalfopristin.

Table VI. Antimicrobial susceptibility patterns (P1-P7) displayed by the outbreak strain As MICs (mg/L) were measured by the E-test method, the table shows the MIC values according to the grades of the scale can be seen on the E-test strips.

AM agent	P1	P2	P3	P4	P5	P6	P7
ampicillin	128	128	192	256	128	192	192
vancomycin	128	48	48	64	48	256	64
teicoplanin	2	1	1	2	2	2	1.5
rifampicin	32	8	64	16	16	16	16
ciprofloxacin	32	32	32	32	32	32	32
linezolid	0.5	1	1	1	2	2	1
quinupristin/dalfopristin	0.5	1	1	1	1	2	1
erythromycin	4	256	256	256	4	6	256
gentamicin (HLAR)	512	256	256	256	256	256	256
streptomycin (HLAR)	256	192	>1024	>1024	256	256	>1024

Characterization of isolates from a cluster of VanA VRE infections at the Military Academy (MMA) in Belgrade, Serbia

The nine vanA positive VREF isolates from seven patients of six different departments at the MMA examined in our study were cultured from three blood samples, two abdominal drains, urine, a wound, and a peritoneal fluid clinical samples, and a fecal sample between August 2004 and May 2005. According to the PFGE patterns, two of these isolates displayed 91% similarity to each other, designated as PFGE type D, while the further seven isolates belonged to a distinct PFGE type (PFGE type E) with a

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similarity of ≥83% to each other. The antibiogram of these isolates was identical to that of the VanA VREF isolates from Hungary.

Mating out assay with a representative VanA isolate from the MMA

Mating out assay was performed with a blood culture isolate belonging to PFGE type E as the donor and two standard *Enterococcus spp*. strains as recipients. Both donor-recipient combinations yielded transconjugant colonies harboring the *vanA* gene cluster: the transconjugants were resistant to rifampicin, vancomycin, teicoplanin, and erythromycin, but remained HLAR sensitive oppose the donor strain. The presence of *vanA* gene in the transconjugants was confirmed by PCR experiments. As the transconjugants displayed erythromycin resistance, PCR reactions were performed with the *ermB* specific primers. The particular resistance determinant was found to be present in the transconjugants just like in the donor strains.

Identification and characterization of two vanA positive Enterococcus gallinarum clinical isolates provided by the MMA

Two *E. gallinarum* isolates were recovered from the blood samples of two patients cared at the Department of Neurosurgery and Surgical Intensive Therapy of the MMA in August, 2004, and in May, 2005. Both isolates displayed high-level resistance to vancomycin, teicoplanin (MICs ≥256 mg/L), and gentamicin (MICs >500 mg/L). The isolates were susceptible for ampicillin, rifampicin, linezolid, quinupristin-dalfopristin and ciprofloxacin. Subsequent PCR experiments demonstrated that both isolates carried the vanA gene. The 93% similarity (Dice) found by the macrorestriction pattern analysis (PFGE) highlighted the close, clonal relationship of the two isolates. The first isolate derived from a 64 year old patient with cerebral contusion, intraventricular hemorrhage and coma by cerebral trauma. The patient received multiple antimicrobial chemotherapy including imipenem, teicoplanin, amikacin, and metronidazole. The patient died 5 weeks after the isolation of the VRE strain. The second patient was a 49 years old patient with intracranial hemorrhage and received only amikacin before the isolation of the VRE strain.

MLVA and MLST on representative VREF isolates from Hungary and Serbia

By MLVA, all the tested isolates (a selection of isolates representing all the PFGE types signed by letters) were found to belong to a single type, MLVA type 1, or represented single or double locus variants of type 1, like MLVA type 159, and two newly described variants, MLVA types 230, and 231.

Typing by MLST revealed that the representative VanB positive *E. faecium* isolates belong to CC-17, namely to ST 17 or its single (ST178) or double locus variants (ST18 and ST80).

Detection of esp gene in the representative isolates by PCR

The *esp* variant of the enterococcal surface protein was found to be present by PCR in all the representative VREF clinical isolates, but was absent from the transconjugants.

VI. III. MDR Acinetobacter baumannii

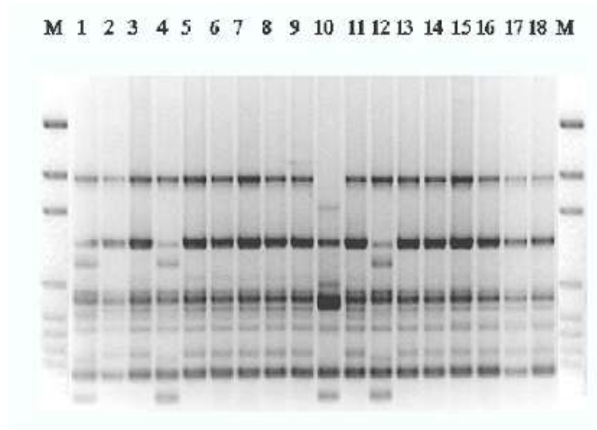
Molecular typing techniques

By the AP-PCR and the RAPD technique, three, 100% corresponding types could be distinguished (Figure VIII); fourteen of the 18 isolates belonged to the prevalent, the outbreak type (type B). This type comprised of the four isolates from 2003, and teen isolates from 2002. On the other hand, the type B isolates obtained in 2002 and 2003 belonged to two different PFGE types by the year of isolation (Figure IX). Three of the further four isolates belonged to a second type (type A), and the only remaining isolate to a third type (type C) by all the molecular epidemiological techniques used in our study.

Class-1 integron PCR, sequencing of class-1 integrons

Three representative amplicons (a 520 bp, a 1100 bp, and a 2500 bp amplicons obtained from one DNA sample) were sequenced. The amplicons of the fourth size (~700 bp) by this technique (Figure X) had been found non-specific products of this PCR reaction in previous studies [60]. No coding region could be identified by the computer assisted analysis of sequence data in either the 520 bp or the 1100 bp PCR product; two resistance genes (*aadA1* and *aacA1*) encoding for aminoglycoside-modifying enzymes

were found in the ~2500 bp amplicon. Namely, we got some results in effect only with the PFGE type D strains by this technique.



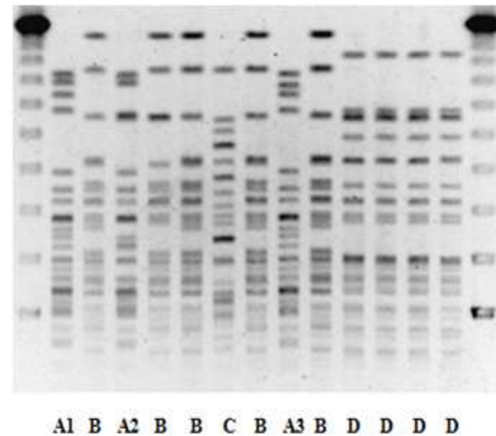


Figure VIII.

Figure IX.

Figure VIII. Patterns of the *A. baumannii* isolates obtained with the ERIC-2 primer
Lanes 1, 20: molecular weight standards; Lanes 2, 5, 13: type A; Lanes 3, 4, 6-10, 12, 14-19: type B; Lane 11: type C. M: molecular weight standard; 1-18: ID number of isolates

Figure IX. PFGE patterns of *A. baumannii* strains isolated in the present study
PFGE types (A, B, C, D) were established for patterns differed in at least six bands, subtypes (A1, A2, A3) were established for patterns differed in less than six bands [64].

Antimicrobial susceptibility testing

Non of the clinical strains involved in our study was found susceptible to the third generation cephalosporin, and only one isolate was found to be susceptible for cefepime. Some further isolates were found intermediate susceptible for ceftazidime or cefepime (Table VII). Decreased susceptibility for meropenem occurred in all the four groups, decreased susceptibility for imipenem occurred in three (A, C, D) of the four groups distinguished by PFGE.

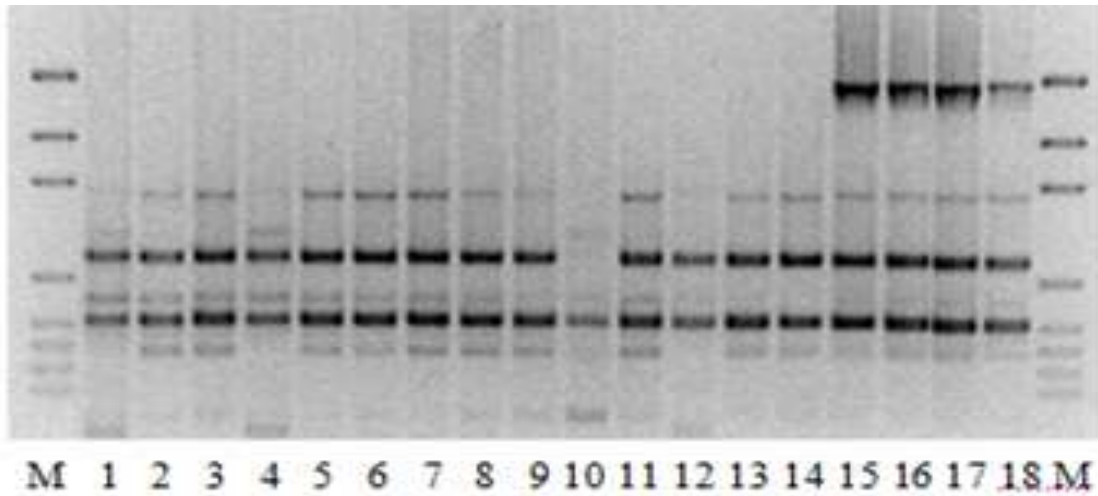


Figure X. Class-1 integron patterns of the *A. baumanni* strains isolated in this study Lanes 2-18: integron patterns of isolates obtained in the present study; Lanes 1, 19 (M): 2645, 1605, 1198, 676, 517, 460, 396, and 350 bp-s control bands (pGEM DNA Markers, Promega, Madison WI)

The amplicons of about 520 or 700 bp-s are non-specific products of this PCR assay, and correspond to the empty class-1 gene cassettes (strong bands); no gene could be identified in a product of about 1100 bp-s either; coding sequences could be identified only in the amplicon of about 2500 bp-s (see the text).

One strain was found to be resistant, and one strain intermediate susceptible for both of the carbapenems. Among the type A strains, fluoroquinolone resistance emerged in the study period: the third isolate was found yet to be resistant for the fluoroquinolones, but were all the outbreak type isolates, and the only sporadic type isolate as well. All the isolates were found to be resistant for amikacin. There was only one isolate from each PCR type which was susceptible for gentamicin. Type A and type C strains were susceptible for netilmicin, while type B strains were uniformly resistant to this agent. On the other hand, there were only two tobramycin-resistant isolates among the isolates collected. There was no isolate susceptible for streptomycin or kanamicin. The results of the PCR based typing techniques, the PFGE, and those of the antimicrobial susceptibility testing are summarized in Table VII.

Table VII. Results of PCR based typing, PFGE, and antimicrobial susceptibility testing

Year	ID	sample	PCR/PFGE	AMR pattern
2002	01	tracheal aspirate	A/A1	AkStrCTe
	04	tracheal aspirate	A/A2	TobGnAkCazStrTe
	12	tracheal aspirate	A/A3	AkNorCipImpMemFepCazStrKCTe
	10	blood culture	C/C	AkNorCipCazStrC
	02	blood culture	B/B	NetGnAkNorCipFepCazStrKCTe
	03	nasal swab	B/B	NetGnAkNorCipFepCazStrKCTe
	05	liquor	B/B	NetGnAkNorCipCazStrKCTe
	06	environmental	B/B	NetGnAkNorCipFepCazStrKCTe
	07	environmental	B/B	NetGnAkNorCipFepCazStrKCTe
	08	environmental	B/B	NetGnAkNorCipFepCazStrKCTe
	09	liquor	B/B	NetGnAkNorCipFepCazStrKCTe
	11	tracheal aspirate	B/B	NetAkNorCipFepCazStrKCTe
	13	urine	B/B	NetAkNorCipCazStrKCTe
	14	nasal swab	B/B	AkNorCipFepCazStr
2003	15	tracheal aspirate	B/D	GnAkGnNorCipCazStrKCTe
	16	nasal swab	B/D	NetTobGnAkNorCipFepCazStrKCTe
	17	nasal swab	B/D	TobGnAkNorCipFepCazStrKCTe
	18	nasal swab	B/D	TobGnAkNorCipFepCazStrKCTe

Year: year of isolation; ID: identity numbers of isolates examined in the present study; PCR/PFGE: PCR/PFGE types and subtypes; AMR: antimicrobial resistance patterns of the isolates; Net: Netilmicin, Tob: Tobramycin, Cn: Gentamicin, Ak: Amikacin, Nor: Norfloxacin, Cip: Ciprofloxacin, Mem: Meropenem, Imp: Imipenem, Fep: Cefepime, Caz: Ceftazidime, Cip: Ciprofloxacin, S: Streptomycin, K: Kanamicin, C: Chloramphenicol, Te: Tetracycline

VI. IV. MDR Enterobacter spp.

VI.IV.I. *Emergence of SHV-2a producing Enterobacter cloacae in Hungary*

Antimicrobial susceptibility testing, ESBL positive patients and bacterial strains

All the isolates (n=142) have been proven non susceptible for broad-spectrum cephalosporins, but only seven isolates have shown the typical disc diffusion pattern of ESBL productive strains [51], further isolates have shown the typical resistance pattern of derepressed mutants [51]. All the isolates have been found resistant to gentamicin, netilmicin, tobramycin, but susceptible for amikacin. No carbapenem resistance has been detected. Results with further antimicrobial drugs have provided the establishment of seven groups: signed with fonts in alphabetical order (Table VIII). Figure XI shows an assortment of isolates by antibiogram and patient distributed by the 52 weeks of 1998. ESBL positive isolates were isolated within an eight weeks period from 04. 28. to 05. 26, 1998 and have made up a separated group: "f". The ESBL isolates were derived from six patients: all of them needed mechanical ventilation, the last one of them had clinical signs of infection (pneumonia), and previous cases were considered cases for respiratory tract colonization by the clinicians. The five ESBL cases registered in April (Figure XI) have been found one cluster of unambiguously related cases with reference to the time factor /synchronous PICU stay, dates of positive cultures: retrospective analysis of CML records and clinical data/ regarding the fact, that the PICU stay of the first patient could be proven to the end of the 17. week (data not shown). No clinical epidemiological relatedness by the retrospective analysis of clinical data and CML records could be proven for the only isolate from May. Although no CREC isolate was derived from the environmental screening (16. week), the unit was disinfected at the end of the 17. week, but remained open for new admissions.

Molecular epidemiological examination: ERIC-PCR, PFGE

All the clinical isolates (n=142) have been examined by ERIC-PCR. Three groups have been discriminated: all the isolates with antibiograms "a-b-c-d-e" have shared a common pattern with each of ERIC primers, respectively (ERIC type I), the group of ESBL isolates (ERIC type II), and the only isolate of antibiogram "g" (ERIC type III) have been distinguished unambiguously (Table VIII).

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Table VIII. Laboratory results by antimicrobial susceptibility testing and ERIC-PCR (n=142)

Antibiogram type	Antimicrobial resistance	ESBL	ERIC type
a (n= 103)	-	neg	I
b (n= 13)	Tri	neg	I
c (n= 14)	NalC	neg	I
d (n= 2)	NalCTri	neg	I
e (n= 2)	(Nal)(Tri)(Sxt)Te	neg	I
f (n= 7)	TriSxtTe	pos	II
g (n= 1)	NalCipCS	neg	III

n: number of tested isolates; parenthesis: intermediate susceptible isolates; neg: negative; pos: positive

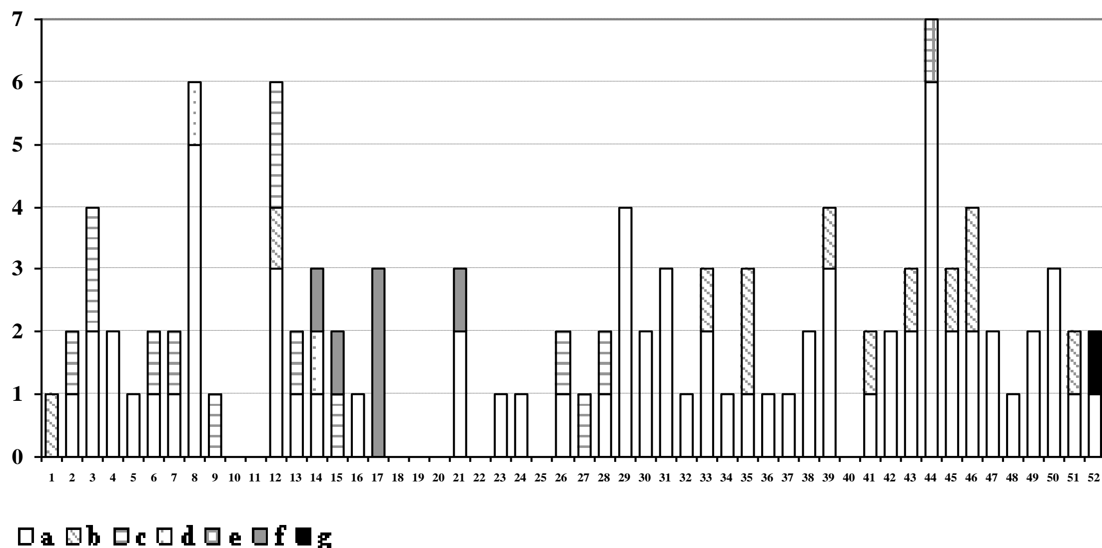

Figure XI. Distribution of CREC strains (1998) by antibiogram and date of isolation (shown in weeks)

Assortment of isolates has been made by patient and antibiogram: among the isolates identical by antibiogram only the first one of each patient has been indicated (n=106). Horizontal scale: 52 weeks of the study period. Vertical scale: number of strains. White and white-patterned columns: strains with antibiograms a-e (ERIC type I). Gray columns: ESBL cases (antibiogram: f, ERIC type II). Black column: the only strain with unique resistance pattern (antibiogram: g, ERIC type III).

Fifty-four isolates representing all the pheno- and genotypes defined by the above examinations and the 12 months were selected for PFGE. 100% corresponding to the ERIC types, three pulsotypes could be discriminated [64, 65]. Within the epidemic ERIC type, 5 pulsopatterns could be discriminated by eyes, but the groups by PFGE (pulsopatterns) did not correspond to the groups established by antimicrobial susceptibility testing suggesting hugh diversity in the background. Figures XII. a-b show the three pulsotypes, and the five pulsopatterns of the predominant pulsotype.

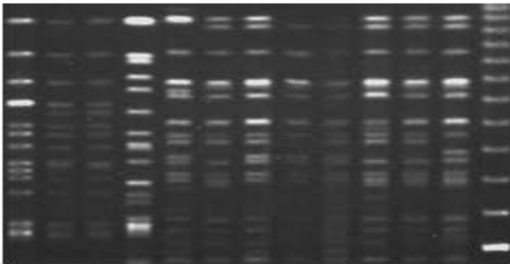

Figure XII. a

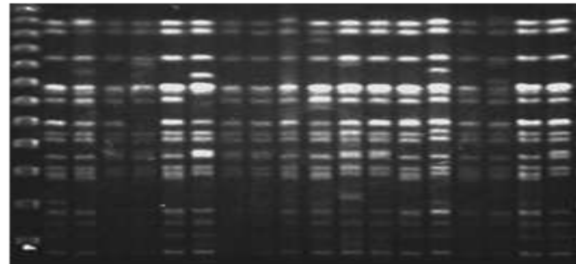

Figure XII. b

Figure XII. a The three PFGE clones of the examined period
Lanes 1-3: the ESBL clone; Lane 4: the sporadic clone; Lanes 5-12: the prevalent pulsopattern of the clone responsible for the large outbreak affecting 94 premature newborn babies; Lane 13: Lambda ladder

Figure XII. b Different pulsopatterns of the predominant pulsotype (ERIC type I)
Lane 1: Lambda ladder; Lanes 2-19: pulsopatterns of the PFGE clone responsible for the large outbreak

Phage typing

22 phage lysis patterns were present among the 118 PICU *E. cloacae* isolates that could be arranged into 12 phage types and 10 subtypes. There were two prevalent phage types (11 and 12) representing 60 and 38 of clinical isolates, respectively. 88% of similarity of them was revealed by UPGMA. Except for only one isolate these two phage types contained all the isolates of “a-b-c-d-e” antimicrobial susceptibility patterns. All the isolates of phage types 5, 6, 7, 8, 9 belonged to the prevalent antimicrobial susceptibility pattern “a”. Diversity in antimicrobial susceptibility patterns was the highest for the prevalent phage types 11 and 12; diversity in phage lysis pattern was the

highest for the prevalent pattern “a”. Altogether 41 phenotypic groups could by defined according to the results of antimicrobial susceptibility testing and phage typing.

DNA tests with ESBL positive isolates

The seven ESBL positive isolates have been further tested by class-1-integron PCR, plasmid electrophoresis, PFGE and PCR-sequencing. Isolates have been found indistinguishable regardless of their clinical relatedness by each PCR based technique and plasmid profile analysis. Computer assisted analysis of the PFGE gel has revealed 94% similarity for the single pulsopattern characteristic for the isolates from April and the pulsopattern of the only isolate from May: the only ESBL positive isolate of May has been proven to belong to the same PFGE clone [65]. All the ESBL isolates have been found positive by bla_{SHV} PCR-RFLP, indicating that the ESBL gene belongs to the bla_{SHV} family [91]. Plasmid profile analysis has revealed a plasmid of ~62Md in each of them. All the seven isolates harbored two class-1 integrons of 0.9 and 1.875 kb. PCR-sequencing has been performed (with respect to our results) on the first ESBL isolate (the outbreak strain) and has revealed the presence of a gene coding for an SHV-2a type enzyme.

VI. IV. II. *Results of the national ESBL surveillance (2002-2004) and the examination of the outbreak strain of the first reported ESBL E. cloacae outbreak in Hungary*

ESBL confirmation

Out of the 85 isolates 41 were proved to be ESBL producers. Except for two isolates, all of them were found SHV positive by PCR and were proved to harbor SHV type ESBL gene by PCR-RFLP. The remaining two isolates were found CTX-M positive by PCR. None of the ESBL producers were found TEM positive. The clinical data and the results of the lab methods performed on all the isolates confirmed as ESBL producers are summarized in Table IX.

Examining the antimicrobial susceptibility of the further three outbreak strains and 9 sporadic isolates representing all the three years of the study period for ciprofloxacin, gentamicin, amikacin, tetracycline, cotrimoxazole, carbapenems, and tigecycline, no ciprofloxacin- or carbapenem-resistant strain was found, but one outbreak strain obtained in 2004 and three sporadic strains obtained in 2002 and 2003 were found

Table IX. Clinical data and laboratory results for the confirmed ESBL isolates

Date of isolation	County/HCF ID/Dep	Patient/sample ID	Species	ERIC type	ESBL type
2002.07.02	Csongrád1/TH/neonatal-ICU	1/nasal swab/newborn	*E. cloacae*	1	SHV-2a
2002.07.08	Csongrád1/TH/neonatal-ICU	1/nasal swab/newborn	*E. cloacae*	1	SHV-2a
2002.07.08	Csongrád1/TH/neonatal-ICU	2/blood/newborn	*E. cloacae*	1	SHV-2a
2002.09.?	Somogy1/CH/?	3/blood/adult	*E. cloacae*	2	SHV-5
2002.09.?	Somogy1/CH/?	4/wound/adult	*E. aerogenes*	3	SHV
2002.09.?	Somogy1/CH/?	5/wound/adult	*E. aerogenes*	4	SHV
2002.09.?	Somogy1/CH/?	3/blood/adult	*E. cloacae*	2	SHV
2002.11.?	Pest1/MH/urol gait	6/urine/adult	*E. cloacae*	5	SHV-5
2003.03.02	Komárom-Esztergom1/?/?	7/nd	*E. cloacae*	6	SHV
2003.03.26	Bp1/TH/septic trauma	8/wound/adult	*E. cloacae*	7	SHV
2003.04.08	BAZ1/PedCH/surg,	9/wound/child	*E. cloacae*	8	SHV
2003.04.15	Bács-Kiskun1/CH/ped	10/urine/infant	*E. aerogenes*	9	SHV
2003.04.26	Somogy2/MH/ped	11/urine/infant	*E. cloacae*	10	SHV
2003.06.30	Csongrád1/TH/pediatrics-ICU	Env1/turmix/screening	*E. cloacae*	11	SHV
2003.06.30	Csongrád1/TH/pediatrics-ICU	Env2/turmix/screening	*E. cloacae*	11	SHV

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Table IX. Clinical data and laboratory results for the confirmed ESBL isolates

Date of isolation	County/HCF ID/Dep	Patient/sample ID	Species	ERIC type	ESBL type
2003.06.30	Csongrád1/TH/pediatrics-ICU	Env3/turmix/screening	*E. cloacae*	11	SHV
2003.06.30	Csongrád1/TH/pediatrics-ICU	Env4/turmix/screening	*E. cloacae*	11	SHV
2003.06.30	Csongrád1/TH/pediatrics-ICU	Env5/turmix/sreening	*E. cloacae*	11	SHV
2003.06.30	Csongrád1/TH/pediatrics-ICU	Env6/turmix/screening	*E. cloacae*	11	SHV
2003.07.04	Hajdú-Bihar1/TH/surgery	12/urine/adult	*E. cloacae*	12	SHV-12
2003.09.?	Vas1/CH/?	13/urine/infant	*E. cloacae*	13	SHV
2003.09.30	Bp2/PedTH/PIC	14/trach asp/newborn	*E. cloacae*	14	SHV
2003.10.03	Bp2/PedTH/PIC	15/ trach asp/newborn	*E. cloacae*	14	SHV
2003.10.27	Bp2/PedTH/PIC	16/trach asp/newborn	*E. cloacae*	14	SHV
2004.01.27	Bp3/MH/og-pathol	17/nph swab/newborn	*E. cloacae*	15	SHV
2004.02.13	Bp4/MH/burn-ICU	18/iv canule/child	*E. cloacae*	16	SHV
2004.03.13	BAZ2/CH/ped	19/blood/child	*E. cloacae*	17	SHV
2004.04.06	Jász-Nagykun-Szolnok1/CH/?	20/nd	*E. cloacae*	18	SHV
2004.04.30	BAZ2/CH/ped1	21/urine/child	*E. cloacae*	19	SHV
2004.05.17	Vas2/MH/haematology	22/wound/adult	*E. cloacae*	20	SHV

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Table IX. Clinical data and laboratory results for the confirmed ESBL isolates

Date of isolation	County/HCF ID/Dep	Patient/sample ID	Species	ERIC type	ESBL type
2004.06.07	Bp2/PedTH/dep2	23/umbil wound/infant	*E. cloacae*	21	SHV
2004.06.09	Bp2/PedTH/dep1	24/trach asp/screening	*E. cloacae*	22	SHV
2004.06.22	Bp2/PedTH/PIC	25/trach asp/screening	*E. cloacae*	21	SHV
2004.07.?	Zala1/CH/urol gait	26/urine/adult	*E. aerogenes*	23	CTX-M
2004.07.?	Zala1/CH/ urol gait	26/urine/adult	*E. aerogenes*	23	CTX-M
2004.09.30	Somogy2/MH/angiology gait	27/wound/adult	*E. cloacae*	24	SHV
2004.10.07	Heves1/CH/surg ICU	28/blood/adult	*E. aerogenes*	25	SHV
2004.10.12	BAZ2/CH/ped2	29/urine/child	*E. cloacae*	26	SHV
2004.10.25	BAZ2/CH/ped2	30/nd/child	*E. cloacae*	26	SHV
2004.10.25	BAZ2/CH/ped2	31/nd/child	*E. cloacae*	26	SHV
2004.12.02	Heves2/MH/ped	32/nd/infant	*E. cloacae*	27	SHV

resistant to tigecycline. All the three outbreak strains were resistant to gentamicin, two of them were resistant to amikacin, tetracycline, and cotrimoxazole as well. Among the sporadic strains, three were MDR, two of these were resistant to tigecycline as well.

Molecular typing

Four small outbreaks, affecting two-times 2, and two-times 3 patients were highlighted by the ERIC-PCR, the further 23 ESBL strains belonged to unique ERIC patterns (Table IX) and so proved to be obtained from sporadic cases. The isolates of the first outbreak were further examined. The Eb101/02 (nasal swab I, patient A) and 112/02 (nasal swab II, patient A), isolates of the same patient but different days showed identical pulsopatterns, while Eb102/02 (bloodculture, patient B) isolated the same day as the second isolate of patient A, differed by one band, so, all the outbreak isolates belonged to the same pulsotype: the two cases were proved to be caused by the same PFGE strain [65]. Plasmid electrophoresis revealed a large, ~62 Md, and a small, ~5 Md plasmid in each of the three outbreak isolates. Specific PCR reactions showed *tet*A, *tet*C, and *aac*-III/2 genes in each outbreak isolate. Sequencing revealed the mutations characteristic for $bla_{\text{SHV-2a}}$ genes (35: Leu->Gln; 238: Gly->Ser) and two silent mutations (138: CTA->CTG; 267: ACC->ACG) in each isolate.

Analytical IEF

The crude extract of the clinical isolates showed two β-lactamase bands focused at pI-s of 7.6, and 5.4, respectively. Regarding the results of the above typing methods, the later β-lactamase could not be an SHV or a TEM type enzyme, but possibly a β-lactamase found in this species by IEF by Tzelepi *et al* [52].

Analysis of transconjugants

With respect to the clinical data, the results of the quantitative antimicrobial susceptibility testing, and those by the molecular techniques, the Eb101/02 and Eb112/02 isolates (obtained from two nasal specimens of one patient) were considered as one strain, and only the first isolate of each patient (Eb101/02 and 102/02) was drawn in the conjugation experiments. Conjugation efficiency was about 10^{-5}. Results of the detailed antimicrobial susceptibility testing performed on the outbreak isolates, the transconjugants, and the recipient strain for cephalosporines, AMC, aminoglycosides,

ciprofloxacin, tetracycline, and cotrimoxazole are outlined in Table X. The double disc and the synergy tests were found positive for the transconjugants as well. Addition of clavulanic acid to third the generation cephalosporines (E test) dramatically reduced the MIC values for both of cefotaxime and ceftazidime suggesting the presence of a plasmid encoded ESBL enzyme. The MIC values of the transconjugants were found to be much higher than those of the recipient strain for cephalosporines, gentamicin, tobramycin, netilmicin, and tetracycline (gray rows in Table X). By IEF, only one band was detected (at a pI of 7.6) in both of the transconjugants, while no band was detected in the lysate of *E. coli* J5-3 F $^-$ R $^-$ rif^R. Both transconjugants were SHV positive and TEM negative by PCR, while *E. coli* J-5-3 F $^-$ R $^-$ rif^R was negative by both of the PCR reactions. Also the PCR-RFLP gave positive results with the transconjugants. Sequencing revealed bla$_{SHV-2a}$ gene with the same characteristic mutations as in the clinical isolates. The transconjugants were found positive for *tet*A and for *aac*-III/2. Plasmid electrophoresis revealed a plasmid of 62 Md in the TCEC strains, while *E. coli* J5-3 F $^-$ R $^-$ rif^R did not contain any plasmid. The plasmids of the two transconjugants showed identical patterns by PREA.

Table X. MIC values (mg/L) of the three outbreak isolates, the three corresponding transconjugants (T), and the recipient strain (J-5-3)

MIC values (mg/L)	101/02	102/2	112/02	T119	T120	T218	J-5-3
CAZ	4	3	4	0.25	0.5	0.5	0.094
CTX	16	8	4	0.75	0.75	0.75	0.032
AMC	32	24	16	4	4	6	1.5
CRO	8	8	8	0.19	0.38	0.5	0.016
FEP	1.5	1.5	1	0.125	0.125	0.125	0.023
CN	96	128	32	4	4	3	0.064
TOB	12	16	6	1.5	1.5	1	0.125
NET	12	16	8	1.5	1.5	1.5	0.047
AK	1.5	1.5	1.5	0.25	0.25	0.25	0.19
CIP	0.012	0.006	0.023	0.006	0.006	0.006	0.006
TE	256	256	256	64	48	48	0.38
SXT	32	32	32	0.023	0.023	0.023	0.016

VI. IV. III. *Cyclohexane tolerance and Phe-Arg-ß-naphtylamide susceptibility of MDR Enterobacter cloacae clinical isolates, predominance of one PFGE clone in Hungary, report of a nationwide survey*

Analysis of data sheets

The total collection of isolates derived from 25 Hungarian health-care facilities (Table XI). The reported diagnoses were urinary tract infection (46 patients–38 of them with positive anamnesis for urinary tract catheter), sepsis (19 patients–4 of them with urosepsis), wound infection (19 patients), respiratory tract infection (9 patients–7 of them with well documented VAP, all of them with positive anamnesis for mechanical ventilation). Four outbreaks (involving altogether 42 patients) were reported by the senders. 81 cases were considered nosocomial according to the definition of CDC; some required clinical data were missing in further 10 cases.

Disc diffusion tests, ERIC-PCR, and PFGE

The results of tests performed on all the isolates (antimicrobial susceptibility testing, ERIC-PCR, *Xba*I PFGE) and the results of ESBL-PCR-sequencing are summarized in Table XI. The ESBL negative isolates (n=110) showed the typical resistance pattern of de-repressed AmpC mutants [47]. No carbapenem resistance was detected. Resistance rates to Ni, Ak, Gn, C, Net, Tob, Te, S, and Sxt were 30%, 32%, 63%, 65%, 68%, 71.5%, 91%, 96.5%, and 97%, respectively. Forty-three percents of isolates were resistant to at least seven of these nine drugs. Incidence rates of nitrofurantoin resistance were found to be 50% for urine isolates (n=50) and 14% for the other 63 isolates.

By the ERIC-PCR, four ERIC types (A-D) could be distinguished. Type A comprised 109 isolates. Two of the three ESBL isolates had unique ERIC profiles. The fourth type was distinguished only by the ERIC-1 primer.

Forty-three PFGE strains [64, 65] were revealed by *Xba*I PFGE. Type-A-isolates were found to belong to 40 PFGE strains ranked in four PFGE clusters by the use of the Pearson's coefficient (Table XI: clusters 1, 2, 3, 4). The further three ERIC-types were 100% concordant with three novel *Xba*I PFGE clusters (Table XI: clusters 5, 6, 7).

Table XI. Clinical data and results of laboratory tests performed on all the isolates

Isolation[1]	HF ID/dep[2]	Sample	Antibiogram[3]	ERIC-type/ *Xba*I Pearson cluster/ strain
1997	1/og	wound*	CnNetSxtS	A/c1/P1
1998	2/gm	urine*	CnTobTeSxtS	A/c1/P2
1998	2/gm	urine*	NetTeSxtS	A/c1/P3
1998	3/orth	wound*	CnTobCTeSxtS	A/c1/P1-v1
1998	2/gm	puncture*	CnTobNet(Ak)CTeSxtS	A/c2/P4
1998	4/pulm	sputum*	CnTobNet(Ak)CTeSxtS	A/c2/P5
2000-05	5/urol	urines*	TobNetAkCTeNiSxtS (n=4)	A/c1/P6-v1
2000-05	5/urol	urines*	CnTobNet(Ak)CTeSxtS (n=4)	A/c1/P6
2000-05	5/urol	urine*	AkCTeNiSxtS	A/c1/P6-v4
2000-05	5/urol	urine*	NetCTeSxtS	A/c2/P4-v1
2000-05	5/urol	blood*	TobNet(Ak)CTeSxtS	A/c2/P4-v2
2000-05	5/urol	iv canule	CnTobNet(Ak)CTeSxtS	A/c2/P4-v2
2000-05	5/urol	urine*	CnTobNet(Ak)CTeNiSxtS	A/c1/P6-v3
2000-05	5/urol	wounds	CnTobNet(Ak)CTeSxtS (n=2)	A/c1/P6
2000-05	5/urol	urines*	NetCTeSxtS (n=2)	A/c1/P6-v2
2001-02	4/pulm	sputum*	TobNetCTeSxtS	A/c2/P7

Table XI. Clinical data and results of laboratory tests performed on all the isolates

Isolation[1]	HF ID/dep[2]	Sample	Antibiogram[3]	ERIC-type/ *Xba*I Pearson cluster/ strain
2001-05	6/urol	urine*	NetSxtS	A/c1/P8
2001-05	6/urol	urine*	TobNetAkCTeNiSxtS	A/c2/P7
2001-05	6/urol	urine*	CnTobNetAkCSxtS	A/c1/P8
2001-12	7/urol	urine*	NetSxtS	A/c4/P9
2002-03	8/?	urine*	CTeSxtS	A/c2/P10
2002-09	9/?	blood*	TobNetTeNi /SHV5	B/c6/P42
2002-09	10/gm	urine, blood*	CnTobNetAkCTeSxtS (n=2)	A/c1/P11
2002-10	11/surg	wound*	CnCTeSxtS	A/c1/P12
2002-10	12/ICU	tracheal aspirate*	CnTobCTeSxtS	A/c2/P13
2002-10	13/gp	urines*	TobNetTeNiSxtS (n=2)	A/c2/P14
2002-11	2/gm	blood*	CnTobNetCTeSxtS	A/c1/P15
2002-11	14/?	urine*	CnTobNetCTeSxtS	A/c2/P16
2002-11	14/?	urine*	CnNetCTeNiSxt /SHV5	C/c7/P43
2003-04	9/?	wound*	TeSxtS	A/c1/P17
2003-04	9/emerg	urines, wound, blood*	TobNet(Ak)CTeSxtS (n=4)	A/c1/P17
2003-08	15/gm	urine*	CTeSxtS	A/c2/P18

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Table XI. Clinical data and results of laboratory tests performed on all the isolates

Isolation[1]	HF ID/dep[2]	Sample	Antibiogram[3]	ERIC-type/ *Xba*I Pearson cluster/ strain
2003-10	16/gm	urine*	CnTobNetSxtS	A/c3/P19
2003-11	16/gm	wound*	NetCTeSxtS	A/c1/P20
2003-12	9/ICU	tracheal aspirate*	CnTobNet(Ak)CTeSxtS	A/c1/P21
2004-01	17/ICU	hand contact surface	CTeSxtS	A/c2/P22
2004-01	17/ICU	throat swabs	CTeSxtS (n=3)	A/c2/P22
2004-01	17/ICU	nasopharyngeal swabs	CTeSxtS (n=2)	A/c2/P22
2004-01	17/ICU	tracheal aspirate*	CTeSxtS	A/c2/P22
2004-01	17/ICU	throat swab (nurse)	CTeSxtS	A/c2/P22
2004-04	17/ICU	drain	CTeSxtS	A/c2/P22-v1
2004-01	18/TB	transthoracal drain*	CnTobNetCTeSxtS	A/c1/P23
2004-02	19/ICU	puncture*	CnTobSxtS	A/c1/P24
2004-02	19/urol gait	urine*	CnTobNetTeSxtS	A/c1/P25
2004-02	19/urol gait	urine*	CnTobTeNiSxtS	A/c1/P24-v1
2004-06	19/urol gait	urine*	CnTobNetCTeNiSxtS	A/c1/P24-v1
2004-07,-08	19/urol gait	urines	CnTobTeNiSxtS (n=6)	A/c1/P24-v2
2004-08	19/urol gait	urine	CnTobTeNiSxtS	A/c1/P24-v3

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Table XI. Clinical data and results of laboratory tests performed on all the isolates

Isolation[1]	HF ID/dep[2]	Sample	Antibiogram[3]	ERIC-type/ *Xba*I Pearson cluster/ strain
2004-08	19/urol gait	urine*	CnTobNetTeNiSxtS	A/c1/P24-v2
2004-09	19/urol gait	urine*	CnTobTeNiSxtS	A/c1/P25-v1
2004-02	20/gm	iv canule*	TobNetAkCTeNiSxtS	A/c1/P35
2004-02	20/ICU	tracheal aspirate*	CTeNiSxtS	A/c2/P36
2004-03	20/ICU	tracheal aspirate*	CnTobNetAkCTeSxtS	A/c2/P36
2004-03	20/ICU	wound	CnTobNetAkCTeSxtS	A/c2/P36
2004-01	20/neurosurg	tracheal aspirate*	CnTobNetCTeNiSxtS	A/c1/P35
2004-02	20/neurosurg	urine*	CnTobNetCTeNiSxtS	A/c2/P36
2004-08	20/neurosurg	blood*	CnNetTe	D/c5/P41
2004-08	20/ICU	throat swab*	Ni	D/c5/P41
2004-08	20/neurosurg	blood*	CnNetTet	D/c5/P41
2004-08	20/ICU	throat swab*	Ni	D/c5/P41
2004-03	20/urol	hand contact surface	CnTobCTeNiSxtS	A/c1/P35
2004-03	20/urol	watertap*	CnTobCTeNiSxtS	A/c1/P35
2004-05	21/surg	wound*	CnNetCTeSxtS	A/c1/P28
2004-05	21/surg	wound*	CnTobTeSxtS	A/c3/P29

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Table XI. Clinical data and results of laboratory tests performed on all the isolates

Isolation[1]	HF ID/dep[2]	Sample	Antibiogram[3]	ERIC-type/ *Xba*I Pearson cluster/ strain
2004-06	21/surg	urine*	CnTobTeNiSxtS	A/c3/P30
2004-02	22/urol	urine*	CnTobNetSxtS	A/c1/P26
2004-06	22/urol	urine*	CnTobNetSxtS	A/c1/P31
2004-04	23/gp	urine*	CTeSxtS	A/c1/P27
2005-01	20/urol gait	urine*	CTeSxtS	A/c1/P32
2005-02	20/traumatol	urine*	CnNetCTeSxtS	A/c1/P34
2005-04	20/urol	blood*	CnTobNetCTeSxtS (n=2)	A/c1/P35-v1
2005-04	20/urol	urine*	CnTobNet(Ak)CTeSxtS	A/c1/P35
2005-06	20/urol	wound	CnTobNet(Ak)CTeSxtS	A/c1/P35-v2
2005-02	24/gm	sputum*	GnTobNetCTeNiSxtS /CTXM15	A/c1/P33
2005-04	25/stroke	nasopharynx* (swab)	TobNetAkCTeNiSxtS	A/c1/P37
2005-03	21/dermatol	urine*	NiSxtS	A/c1/P38
2005-08	21/urol gait	urine*	NetCTeNiSxtS	A/c1/P38-v1
2005-11	26/ICU	blood*	CnTobNetAkCTeSxtS	A/c1/P39
2005-11	26/infect gait	urine	CnTobNetAkCTeSxtS	A/c1/P39
2005-11,-12	26/ICU	bloods	CnTobNetCTeSxtS (n=5)	A/c1/P40

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Table XI. Clinical data and results of laboratory tests performed on all the isolates

Isolation[1]	HF ID/dep[2]	Sample	Antibiogram[3]	ERIC-type/ *Xba*I Pearson cluster/ strain
2005-12	26/ICU	tracheal asp, wound	CnTobNetCTeSxtS (n=2)	A/c1/P40
2005-12	26/ICU	blood, drain	CnTobNetCTeSxtS (n=2)	A/c1/P40
2005-12	26/ICU	Swang-cath, blood	CnTobNetCTeSxtS (n=2)	A/c1/P40
2005-12	26/ICU	blood*	CnTobNet(Ak)CTeSxtS	A/c1/P40
2005-12	26/ICU	bloods*	CnTobNetAkCTeSxtS (n=2)	A/c1/P40

[1]Isolation: Years/months of isolations

[2] HF ID/dep: Identity numbers for healthcare facilities, counties/ departments

HF1-6, HF10, 25: teaching hospitals, Budapest (Bp); HF7, 9, 12, 21, 22, 26: county hospitals of Nógrád, Somogy, Borsod-Abaúj-Zemplén (BAZ), Veszprém, Zala, Győr-Moson-Sopron; HF8, 11, 14-17, 19, 20, 24: municipal hospitals of Komárom-Esztergom, BAZ, Pest, Bp, Heves, Bács-Kiskun; HF13, 23: general practitioners of BAZ, Zala; HF18: TB sanatorium, Heves; og: obstetrics-gynaecology; gm: general medicine,

[3]Antibiogram: resistance patterns, parentheses: intermediate susceptible isolates

ERIC-types: A-D; *Xba*I PFGE Pearson's clusters: PFGE/c1-7; *Xba*I PFGE strains: PFGE/P1-43; *Xba*I PFGE strain-variants (for isolates with <= three bands difference): PFGE/P-v.

*The isolate was selected for CH tolerance and PAβN susceptibility tests with respect to the clinical data and the lab tests performed on all the isolates. Among the isolates listed in the same raw, only one was further tested.

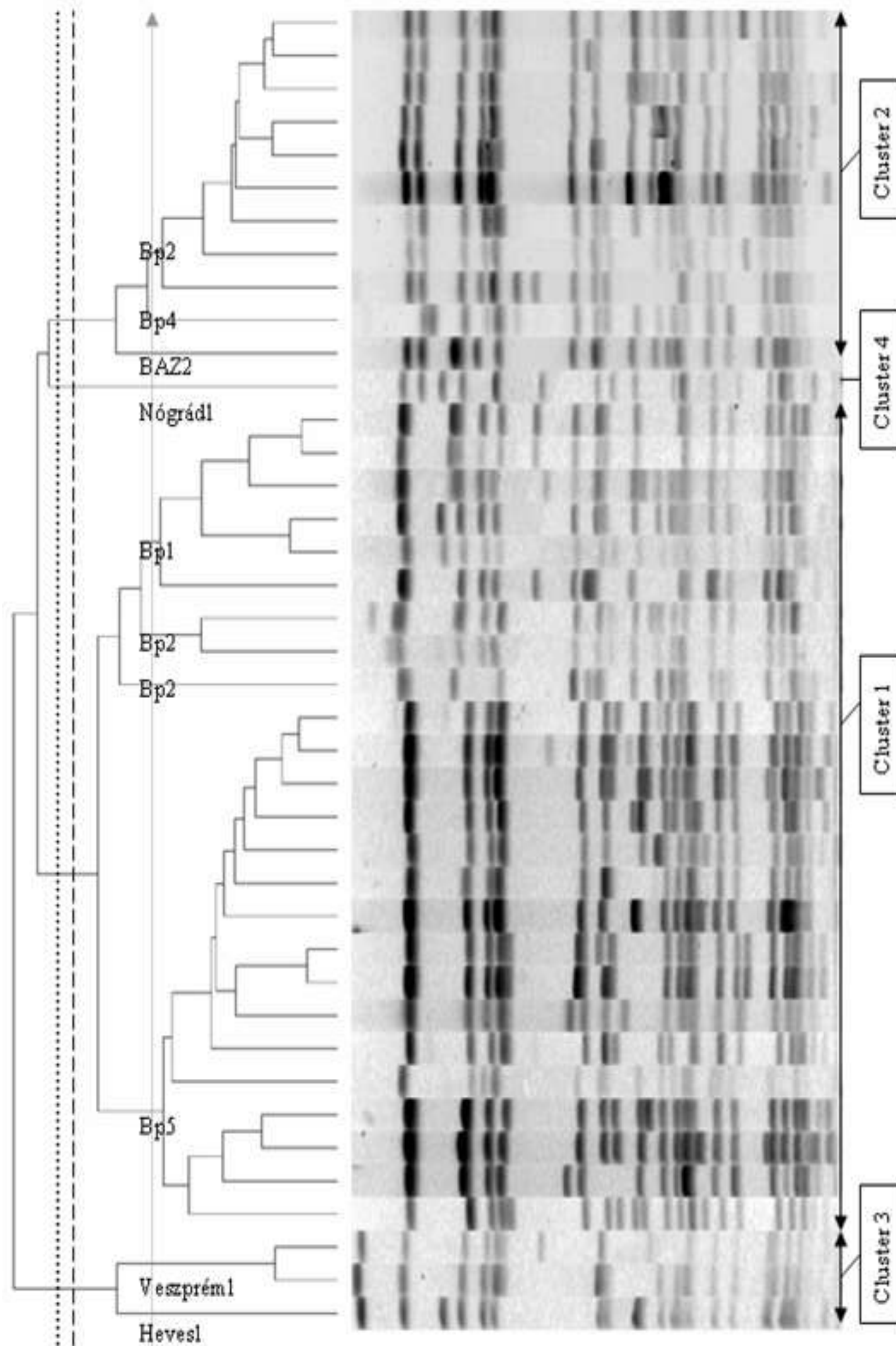

Figure XIII. *Xba*I Pearson's hierarchical tree diagram for the 40 PFGE strains of type A; Bp: Budapest, BAZ: Borsod-Abaúj-Zemplén

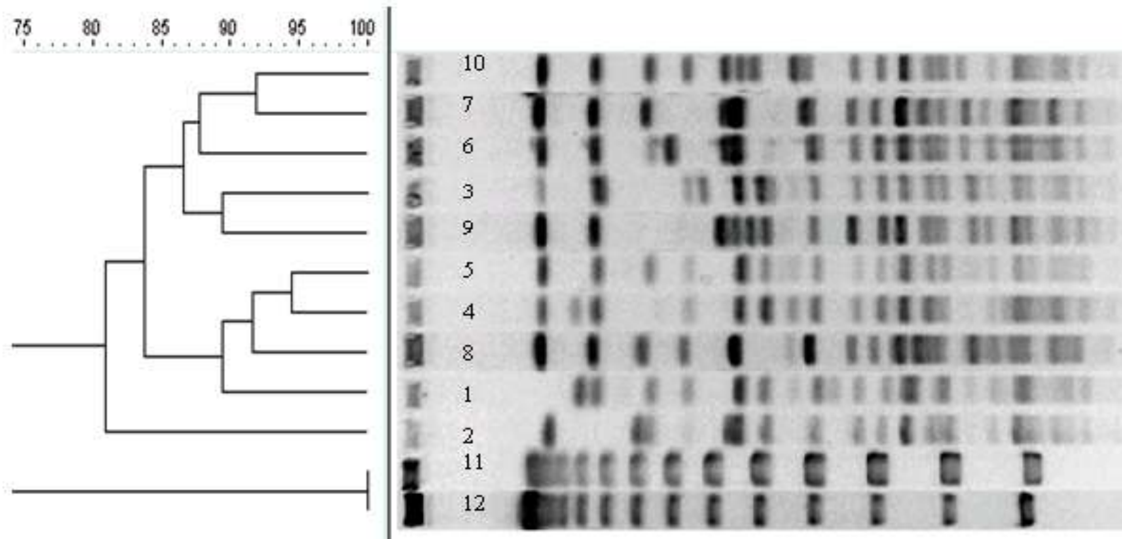

Figure XIV. *Spe*I Dice hierarchical tree diagram for the selected isolates (n=10) and the Lambda concatemers (used as standards)

Lanes 1, 2: the first and the second isolates of ERIC-type A (Bp1, Bp2, *Xba*I-Pearson's cluster1, 1997-1998); lane 3: the first provincial isolate (Nógrád1, 2001, *Xba*I-Pearson's cluster4); lane 4: the first isolate of the first cluster of cases (Bp5, 2000, *Xba*I-Pearson's cluster1); lane 5: the first isolate of the *Xba*I Pearson's cluster2 (Bp2, 1998); lane 6: the second isolate of the *Xba*I Pearson's cluster2 (Bp4, 1998); lanes 7, 8: the first and the second isolates from the *Xba*I Pearson's cluster3 (Heves1, 2003; Veszprém1, 2004); lane 9: *Xba*I Pearson's cluster2 isolate (BAZ2, 2002); lane10: *Xba*I Pearson's cluster1 isolate (Bp2, 1998); lanes 11, 12: Lambda concatemers.

Figure XIII shows the Pearson's tree diagram prepared for the 40 PFGE strains of type A. The Pearson's tree diagram prepared for the 40 PFGE strains of type A shows the three clusters that could be distinguished by the Dice analysis as well (Pearson's clusters 1-2-4), and a novel cluster, containing only 3 isolates (Pearson's cluster3) - clusters are separated by a black broken line. Although the similarity by the Dice coefficient was no more than ≥ 66% for the 40 PFGE strains, but the similarities (Dice analysis) for the PFGE strains of Pearson's clusters 1-2-4, and for the PFGE strains of Pearson's clusters 1-3 were found 74%, and ≥ 72%, respectively, indicating the monoclonal origin of type-A-isolates [65]. The black scattered line is indicative for 50% similarity (Pearson's analysis). The grey arrow shows the cut of the tree made for PFGE examinations with *Spe*I. *Spe*I PFGE was performed on the first isolate of each branch.

Places of origin of these isolates are indicated on the tree at the section points (Bp1: HF1, Bp2: HF2, Bp4: HF4, Bp5: HF5, Nógrád1: HF7, BAZ2: HF12, Heves1: HF16, Veszprém1: HF21).

The Pearson's tree was cut (Figure XIII) and the first isolate of each branch was tested with *Spe*I, and *Not*I, respectively. The Dice analysis on the *Spe*I-patterns revealed >=80% similarity (Figure XIV) confirming the monoclonal origin of isolates belonging to the epidemic ERIC-type. Restriction with *Not*I generated double the usual number of fragments on the genomes of the isolates from ERIC-type A. Sixty-seven representative isolates (see Table XI) were selected for further examinations.

The efflux pump inhibitor test with Phe-Arg-ß-naphtylamide (PAßN) and organic solvent tolerance test

Five MDR isolates and the control strain (ATCC 13047) did not grow in the presence of cyclohexane (CH-), five isolates showed visible growth (CH+/-), and a further 57 isolates (85%) showed confluent growth (CH+).

By repeated testing with PAßN, ciprofloxacin MICs of 14 isolates decreased 0-1 dilution steps (PAßN-), ciprofloxacin MICs of further 28 isolates decreased 2 dilution steps (fourfold reduction: PAßN+/-), ciprofloxacin MIC-s of the remaining 25 isolates decreased at least 3 dilution steps (at least eightfold reduction: PAßN+), respectively.

All the CH- isolates were found PAßN-. Among the CH+/- isolates, four were PAßN+ one was PAßN-. Among the CH+ isolates 8, 28, and 21 isolates were found PAßN-, PAßN+/-, and PAßN+, respectively. Concordance between the results with CH and PAßN rose from 39% to 73% when not only an eightfold or greater [59, 98], but also a fourfold reduction of the ciprofloxacin MIC in the presence of PAßN was considered notable [44, 99, 100]. Ciprofloxacin MIC_{50} values increased progressively from 1997 to 2002: 4 mg/L (n=1, 1997), 32 mg/L (n=5, 1998), 64 mg/L (n=7, 2000), 64 mg/L (n=5, 2001), 128 mg/L (n=9, 2002), 128 mg/L (n=6, 2003), 128 mg/L (n=23, 2004), 64 mg/L (n=11, 2005). Except for the one isolate from 1997, MIC_{90} value was found to be 128 mg/L for each year. Tetracycline and chloramphenicol MIC testing was performed on 39 of the 67 representative isolates, those from ERIC-type-A, collected from 1997 to the end of 2004. Chloramphenicol MIC_{50} values seemed to be depending rather on the sender facility than the year of isolation: 64 mg/L (n=1, 1997), 1024 mg/L (n=5, 1998), 512 mg/L (n=7, 2000), 32 mg/L (n=5, 2001, four of them derived from one sender),

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1024 mg/L (n=7, 2002), 1024 mg/L (n=6, 2003), 32 mg/L (n=8, 2004). Tetracycline MIC_{50} values were found to be constant (512 mg/L) from 1997 to 2004, except for 2001 ($MIC_{50\ (2001)}$ = 32 mg/L). Tables XII. a-b show the MICs plotted against the CH tolerance (a) or the PABN susceptibility (b). Statistical comparisons on MICs showed that only the differences those seen in the ciprofloxacin MICs were statistically significant (Tables XII. a-b). Ciprofloxacin (Cip), chloramphenicol (C), and tetracycline (Te) MIC values plotted against the cyclohexane (CH) tolerance (Table XII. a) and against the Phe-Arg-ß-naphtylamide (PABN) susceptibility (Table XII. b).

Table XII. a Ciprofloxacin (Cip), chloramphenicol (C), and tetracycline (Te) MIC values plotted against the cyclohexane (CH) tolerance

Cip	CH-(n=5)	CH+ (n=57)
MIC range (mg/L)	4-32	2-512
MIC_{50} (mg/L)	16	64
MIC_{90} (mg/L)	nd	128

C	CH-(n=5)	CH+ (n=31)
MIC range (mg/L)	4-1024	8-1024
MIC_{50} (mg/L)	256	1024
MIC_{90} (mg/L)	nd	1024

Te	CH-(n=5)	CH+ (n=31)
MIC range (mg/L)	<2-1024	8-1024
MIC_{50} (mg/L)	256	512
MIC_{90} (mg/L)	nd	512

nd: not defined

All the PABN-CH- isolates (n=5), seven of the PABN-CH+ isolates (n=8), sixteen of the PABN+/- isolates (n=28, all of the PABN+/- isolates were found CH+), three of the

PABN+CH+/- isolates (n=4), and eight of the PABN+/CH+ isolates (n=21) were drawn in C/Te MIC testing. One-tailed P values (Mann-Whitney test) were:
$P(Cip)_{CH-vsCH+}$=0.0005; $P(Cip)_{PABN-vsPABN+/-}$=0.0015; $P(Cip)_{PABN+/-vsPABN+}$=0.0003;
$P(C)_{CH-vsCH+}$=0.267; $P(C)_{PABN-vsPABN+/-}$=0.2786; $P(C)_{PABN+/-vsPABN+}$=0.1983;
$P(C)_{PABN-vsPABN+}$=0.4141; $P(C)_{PABN-vsPABN+}$=0.132;
$P(Te)_{CH-vsCH+}$=0.1728; $P(Te)_{PABN-vsPABN+/-}$=0.0806; $P(Te)_{PABN+/-vsPABN+}$=0.4306.

Table XII. b Ciprofloxacin (Cip), chloramphenicol (C), and tetracycline (Te) MIC values plotted against the Phe-Arg-ß-naphtylamide (PABN) susceptibility

Cip	PABN- (n=14)	PABN+/- (n=28)	PABN+ (n=25)
MIC range (mg/L)	4-128	64-128	64-512
MIC_{50} (mg/L)	32	64	128
MIC_{90} (mg/L)	64	128	128

C	PABN- (n=12)	PABN+/- (n=16)	PABN+ (n=11)
MIC range (mg/L)	4-1024	32-1024	16-1024
MIC_{50} (mg/L)	256	1024	256*
MIC_{90} (mg/L)	1024	1024	1024

Te	PABN- (n=12)	PABN+/- (n=16)	PABN+ (n=11)
MIC range (mg/L)	<2-1024	16-1024	8-1024
MIC_{50} (mg/L)	256	512	512
MIC_{90} (mg/L)	512	512	1024

* For the tested PABN+CH+ isolates (n=8), the C MIC_{50} value was found 1024 mg/L.

PCR-sequencing of QRDRs

The strains showing ciprofloxacin MICs ≥4 mg/L in the presence of PAßN exhibited mutations leading to Ser-83→Tyr and Asp-87→Asn changes (five PAßN-negative

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isolates from genotype A with different Cip MICs, ranging from 4 to 64 mg/L in the presence of PAßN) or Ser-83→Tyr and Asp-87→Ala changes (isolates of types B, and C) in gene encoding GyrA, and a single mutation generating a Ser-80→Ile change in gene encoding ParC (the same isolates from types A, B, and C) The strains of type D (showing 0.5-1 mg/L ciprofloxacin MICs in the presence of PAßN) had a single mutation generating a Ser-83→Phe change in *gyr*A, and no mutations in the *parC* gene.

VII. Discussion

MDR pathogens that cause serious nosocomial infections and which are more and more difficult to treat include MDR Gram-negative bacilli like *E. coli*, *K. pneumoniae*, *Enterobacter spp*, *A. baumannii* or *P. aeruginosa*, and Gram-positives like MRSA or MDR *S. aureus*, PRSP, and VRE. According to the last data published by the CDC collected in 2006-2007 [101] 84% of the healthcare associated infections (HAIs) are caused by the following 10 groups of pathogens in the USA: *S. aureus* (15%), CNS (15%), *Enterococcus spp*. (12%), *Candida spp*. (11%), *E. coli* (10%), *P. aeruginosa* (8%), *K. pneumoniae* (6%), *Enterobacter spp*. (5%), *A. baumannii* (3%), *K. oxytoca* (2%). In the same data branch, the incidence of multidrug resistance was found 16% by the CDC. The incidences of MRSA, VRE, carbapenem-resistant *P. aeruginosa*, and ESBL *K. pneumoniae* were found 8%, 4%, 2%, and 1%, respectively. ESBL *E. coli* gave the half of the remaining 1%, the missing 0.5% was given by carbapenem resistant *A. baumannii*, and carbapenem-resistant *Klebsiella spp*. and *E. coli*. Reviewing the scientific literature, namely the results of the large surveillance studies conducted in the second half of the 1990's we can conclude, that many of the most important nosocomial drug-resistant bacterial pathogens of the last decade emerged or became frequent in the hospital environment (and moreover, some of them spread beyond the walls of the acute-care hospitals) yet in the 1990's, while spread of carbapenem resistance in Gram-negatives, and the spread of vancomycin-nonsusceptible staphylococci are the most threatening news of the last decade.

The national nosocomial surveillance system operated by the NCE in Hungary was started in 2004. So, it is not surprising, that only a few analyzed data are available about the incidence of HAIs in Hungary. The data collected between 2004 and 2008 for surgical site infections (SSI) were outlined in the thesis book of the PhD work by Szilágyi Emese [102]. The incidence of SSIs (for operations performed on the bile-tract, the colon and for the section caesarean, mammal, heart, hip-prosthesis, and knee-prosthesis operations) changed between 0.8% and 10.9% in the study period. In this period, a notable decrease could be seen in the hospitals participated in the surveillance study in the incidences of HAIs for the knee-prosthesis operations, and for the CVC associated BSIs. The teen most frequent pathogens were *S. aureus* (36%), *E. coli*,

Enterococcus spp., *Enterobacter spp*., *Klebsiella spp*., *Streptococcus spp*., *Acinetobacter spp*., *P. aeruginosa*, *Proteus spp*., and CNS. Thirty-five and fifty-eight percent of *S. aureus* and CNS strains were found resistant to methicillin, respectively.

The global emergence and spread of penicillin and MDR pneumococcal strains was one of the news in the field of emerging resistance of Gram positive microbes for the nineties. The emergence and rapid dissemination of antibiotic-resistant pneumococcal strains in areas of southern and eastern Europe, North America, South America and Asia in the 1990s was associated with an increase in antibiotic consumption [103, 104]. As the number of the drug-resistant isolates, and so, the therapeutical problem caused by *S. pneumoniae* strains increased, more studies were performed looking for risk factors for resistance in pneumococci and have identified antimicrobial use as a risk factor, especially the following aspects: ongoing, recent, repeated, frequent, and prophylactic antibiotic use. The effect of individual classes of antimicrobials has not been studied in detail but use of beta-lactam antibiotics and cotrimoxazole has been associated with increased risk [105]. Furthermore, the list of risk factors [106] for having pneumococci resistant to frontline antimicrobials (beta-lactams, macrolides, respiratory tract fluoroquinolones) show, that many of these cases might have been healthcare associated. Risk factors for colonization with PNSP include prior antibiotic use, age <5 years, and attendance in day-care centers. Risk factors for PNSP among invasive pneumococcal disease (IPD) include prior antibiotic use, residence in long-term care facilities (LTCFs), recent hospitalization, HIV infection, chronic pulmonary disease, recent respiratory tract infection(s), underlying immunosuppressive disease, and non-bloodstream isolates. Transmission of resistant strains from carriers in day-care centers, hospitals, LTCFs, jails, homeless shelters, and crowded conditions may facilitate spread. Risk factors for cephalosporin resistance are similar to PNSP and include age <5 years, residence in a day-care center, isolates from the middle ear or nasopharynx, recent hospitalization, and penicillin resistance. The dominant risk factor for macrolide resistance is prior antibiotic use. Several population-based studies noted correlations between the prevalence of macrolide resistance among *S. pneumoniae* and overall macrolide consumption in the region or country. Exposure to both macrolide and non-macrolide antibiotics increases the risk of macrolide resistance as well. The incidence of macrolide resistance is higher among PNSP or MDR strains. Prior

exposure to fluoroquinolones (FQs) is the major risk factor for selecting FQ resistant strains of *S. pneumoniae*. COPD, residence in LTCFs, and older age are common associations. Patient-to-patient transmission of FQ-resistant *S. pneumoniae* may occur in hospitals or LTCFs. Global surveillance studies have shown that β-lactam-nonsusceptibility rates increased worldwide during the 1990s and 2000s. The Alexander Project monitored resistance in *S. pneumoniae* from 1992 to 2001, and reported increases in the levels of non-susceptibility to penicillin from 24.9% in 1992 to 30.2% in 2001 in Spain, from 7.7% to 35.8% in France, and from 5.6% to 20.4% in the USA, whereas in Italy, Germany, and the UK, resistance rates remained below 5% during this period [107]. Macrolide-resistant pneumococci were first detected in 1967 in Canada, but rates of macrolide resistance among pneumococci remained low worldwide (<5%) during the 1970s [108]. By the early 1980s, the highest prevalence of erythromycin-resistant pneumococci was found among pneumococci isolated from hospital carriers in South Africa (63%) in 1983. The majority of these strains showed multidrug resistance [108]. Thereafter, in the 1990s a rapid worldwide increase in the prevalence of macrolide resistance associated with an increase in macrolide consumption, especially of long-acting macrolides such as clarithromycin and azithromycin, was observed. Global surveillance studies have shown that macrolide resistance rates increased during the 1990s. The Alexander Project gave a global rate of macrolide resistance of 16.5–21.9% in 1996–1997, increasing up to 24.6% in 1998–2000 [107]. The *ermB* gene is the major cause of macrolide resistance in most European countries, the M phenotype isolates predominate in the USA, Canada, the UK, Germany, and Norway. The emergence of pneumococci that carry both *ermB* and *mefE* macrolide resistance genes is a cause for concern, especially in Asian countries, Russia, South Africa, and the USA [109, 110]. The *mef*-class and *erm*-class resistance determinants are carried by a variety of mobile genetic elements including transposons (e.g. Tn917), conjugative transposons of the Tn916 family, and derivatives of chimeric prophage-tranposon composite elements that often carry also additional resistance genes. These highly promiscuous elements contribute to the dissemination of the carried resistance determinants not only among pneumococci but also among other streptococci and enterococci by different mechanisms (transformation, conjugation and/or phage transduction) [111]. The increase in the proportion of pneumococcal isolates with combined non-susceptibility to

penicillin and erythromycin was related to the spread of classic penicillin-resistant clones that have acquired determinants of macrolide resistance, mostly carried by transposons of the Tn916 family [112, 113]. In *S. pneumoniae*, pandemic clones such as ST81, ST90, and ST156 represent major invasive and MDR isolates that have spread globally [114]. The allelic profiles of these clones, however, are highly diverse, which suggests that they are genetically unrelated and do not constitute a single genetic lineage, as does *E. faecium*. Furthermore, the serotype of *S. pneumoniae* seems a more important marker of invasiveness than the overall genotype [94]. Most of the resistant strains belong to only a limited number of serotypes, which are also among the most common causes of pediatric infections (so called "pediatric serotypes"). The highest penicillin and erythromycin resistance proportions worldwide were associated with serotypes 6B, 6A, 9V, 14, 15A, 19F, 19A, and 23F, the so-called 'pediatric serotypes'. The isolates of drug-resistant serotypes belong to a small number of pneumococcal clones worldwide whose nomenclature is standardized by the Pneumococcal Molecular Epidemiology Network (http://www.sph.emory.edu/PMEN/) [115]. The most important pneumococcal clones involved in the global spread of antibiotic resistance in the 1980s and 1990s were Spain23F-ST81, Spain6B-ST90, Spain9V-ST156, England14-ST9, Taiwan19F-ST236, Taiwan23F-ST242, Poland6B-ST315, Sweden15A-ST63, and Colombia23F-ST338. Although Spain23F-ST81 and Spain6B-ST90 were well-established clones in the 1980s and 1990s, their prevalence decreased after the introduction of PCV7 [116, 117]. Although the so called serotype-switching (the genetic event which results in a new serotype variant of the same genetic clone) was described to establish a new epidemic type (PRSP 14) from an old (PRSP 9V) clone [118], but serotype switching was found to be uncommon in the famous clones [119]. Although laboratory detection and identification of *S. pneumoniae* may be based not only on culture, but also on microscopic examinations, antigen-detection by quick-tests, or PCR based methods, emergence of drug-resistant pneumococci is a fundamental reason for enhanced efforts toward culturing pneumococci. Many studies have provided that the knowledge of local and global antibiotic resistance patterns and the clinical relevance of the level of beta-lactam resistance should be the key consideration directing the empiric therapy of respiratory tract infections caused by *S. pneumoniae*. This, together with the rapid, but careful report of the results of penicillin susceptibility testing for the

clinicians could extend the use of penicillin G and postpone the emergence of resistance to other groups of antimicrobial agents among pneumococci. Therefore it was suggested by Thornsberry *et al* [120] that the surveillance programs should be designed to collect information about: a) the specimen source that affects the clinical relevance of penicillin MICs; b) the patients' age; c) the associated resistance varying widely in the different countries. Ad a) The history of PRSP has been accompanied with doubts about the clinical relevance of penicillin resistance from the beginning to nowadays. The mechanism of penicillin resistance in *S. pneumoniae* involves structural changes in the penicillin targets, the penicillin-binding proteins 1A, 2X, and 2B [121]. These changes result in reduced affinity for penicillin as well as for other beta-lactam antibiotics. However, ceftriaxone, cefotaxime and carbapenems are less affected, and are generally the most active compounds [122]. Previous studies have demonstrated that PNSP (MICs ≥0.12 mg/L) can be associated with treatment failures in patients with meningitis, because the cerebrospinal fluid levels achieved with penicillin or the standard dosage of third-generation cephalosporins are insufficient to eradicate the infecting organism. The classical NCCLS breakpoints for penicillin (susceptible, ≤0.06 mg/L; intermediate, 0.12–1 mg/L; and resistant, ≥2 mg/L) were established in the late 1970s in order to prevent failures in patients with pneumococcal meningitis caused by PNSP. In contrast, *S. pneumoniae* strains with penicillin MICs of 0.12–2 mg/L had little effect on outcome in patients with pneumonia and other non-meningeal systemic pneumococcal infections that were treated with parenteral penicillin, amoxycillin, cefotaxime, or ceftriaxone. According to these observations, the CLSI breakpoints for ceftriaxone and cefotaxime were modified in 2002, distinguishing between meningeal infections (susceptible, MIC ≤0.5 mg/L; intermediate, MIC 1 mg/L; and resistant, MIC ≥2 mg/L) and non-meningeal infections (susceptible, MIC ≤1 mg/L; intermediate, MIC 2 mg/L; and resistant, MIC ≥4 mg/L). However, penicillin breakpoints were not modified until 2008, when the site of infection and route of administration were considered. The current penicillin parenteral breakpoints for non-meningeal infections are MIC ≤2 mg/L (susceptible), MIC 4 mg/L (intermediate), and MIC ≥8 mg/L (resistant), whereas MICs ≥0.12 mg/L for strains causing meningeal infection are considered to reflect resistance [123]. Inspite of the alarming penicillin resistance levels, pneumococci with penicillin or cefotaxime/ceftriaxone MICs ≥4 mg/L are rarely described worldwide. If the revised

CLSI breakpoints for parenteral penicillin are applied to pneumococci isolated from non-meningeal infections, more than 95% of invasive pneumococcal isolates collected worldwide are currently susceptible to penicillin and third-generation cephalosporins [123]. These antibiotics should continue to be first-line therapy for these infections. Cefotaxime and ceftriaxone are the most active cephalosporines against pneumococci. Ad b) The incidence of penicillin resistance has been reported to be much higher in children with lower ages, than in adults, and the nasopharyngeal region of children was found to be the main source of PRSP [105, 124]. In Hungary, the epidemiology and incidence of penicillin resistance of pneumococci were studied by Marton *et al* in the 1990s [125, 126]. Extremely high incidence of penicillin resistance (44-58% in adults and 70% in children), and associated resistance (70% of PRSP strains was reported as resistant to tetracyclines, erythromycin, and cotrimoxazole and 30% of PRSP strains resistant to chloramphenicol), but no resistance to third generation cephalosporins was reported in 1991 [125]. Only a few data were published about the antimicrobial susceptibility of Hungarian pneumococci isolated in the 1990s. During the nineties, the incidence of penicillin resistance was reported to have been decreased to 40% [126]. There were two small outbreaks in two children's hospitals in the mid-1990s (reported by Marton *et al* [127]) in which MDR (penicillin-, erythromycin- and cotrimoxazole-resistant) and cefotaxime nonsusceptible strains were isolated from 9 children. Hungary participated in the Alexander project from 1996 to 2000. The penicillin resistance percentages were found much lower in this study [128] testing 127 Hungarian strains isolated in 1996 (11.8% resistant, 24.4% intermediate), macrolide resistance was notably high in isolates collected from Hungary (39.4%) [128]. In the present study, examining the prevalence of penicillin, macrolide, cotrimoxazole, tetracycline and chloramphenicol susceptibility of Hungarian clinical isolates of *S. pneumoniae* (isolated in the year of 2000 in the capital) by disc diffusion test the followings were found: (i) the incidence of penicillin susceptibility was found a little higher: 64% (n=327); (ii) in general, 54% of the strains but 89% of PRSP isolates were proved to be resistant to erythromycin; (iii) the cotrimoxazole resistance remained at the same level among PRSP isolates - it is about 70% even nowadays; (iv) no chloramphenicol resistant strain was found among the PRSP isolates. After MIC testing, the incidence of penicillin susceptibility increased to 70% (n=327). The overall incidence of penicillin resistance

was found to be about 9% in our study, but it was four times higher in children, than in adults: 12% and 3%, respectively. Most of PRSP strains were isolated from children with lower ages. Following the suggestions by Thornsberry *et al* [120] and the instructions given by the EARSS, and the NCCLS, our surveillance study (cefotaxime and levofloxacin MIC testing) was performed on the PNSP respiratory tract isolates which took more than 90% (96/101=0,95) of the PNSP strains isolated during the study period. So, it is not surprising that calculating the incidence of penicillin resistance for the respiratory tract isolates separately, it comes about 9%, too. The 1 μg oxacillin disc diffusion test has overestimated the prevalence of penicillin resistance, and so, we advise that penicillin MIC determinations are carried out on all clinically significant *S. pneumoniae* isolates as the first susceptibility test. In our study, penicillin resistance in *S. pneumoniae* was strongly associated with resistance to macrolides, and cotrimoxazole. The incidence of penicillin resistance associated multidrug resistance took only a few percentages taking the susceptibility data for all the examined isolates as a whole (6%), examining the susceptibility data for PRSP isolates separately, it comes to 75% (21/28). Sixty-four percents (18/28) of PRSP isolates were resistant to erythromycin and cotrimoxazole as well. Whereas no resistance to the third generation cephalosporins was detected by Marton *et al* in the early nineties, in our study, in the year of 2000, 3% of the PISSP, and 64% of the PRSP strains were found cefotaxime resistant. Our present study indicates a considerable change in the resistance of Hungarian *S. pneumoniae* strains to currently used antimicrobials. Cefotaxime-resistant *S. pneumoniae* strains have emerged in Hungary; this may be attributable to the introduction, and frequent administration of oral cephalosporins in general practice as well as ceftriaxone use. Smith and Klugman [129] analyzed the penicillin-binding proteins (PBPs) of some other high-level resistant Hungarian isolates and found them to be clonal. In the hospitals where our patients were treated, penicillin and piperacillin have been used frequently and may be selectors for altered PBP 2B, which leads to high-level cefotaxime resistance [129]. The fact that all but two of the 28 PR strains showed intermediate or full resistance to cefotaxime, and that most of the penicillin and cefotaxime highly resistant isolates were also resistant to carbapenems indicates potential for the selection of penicillin-cephalosporin-carbapenem cross-resistant *S. pneumoniae* strains. *S. pneumoniae* strains with extremely high level cefotaxime and /or

penicillin resistance (MIC ≥8 mg/L) with values of penicillin and cefotaxime MIC up to 32 mg/L have not been reported yet from any other geographical area of the world. In our study, significant differences have been found in the meropenem susceptibility of pneumococci with extremely high level resistance to penicillin, thus, it can be concluded that MICs of meropenem, that was reported to be effective against penicillin-resistant *S. pneumoniae* previously [130] should be examined in infections caused by *S. pneumoniae* strains with extremely high level resistance to penicillin and/or cefotaxime. Vancomycin retained its in vitro activity also in this group of isolates. *S. pneumoniae* strains with extremely high level penicillin resistance have been found resistant to cephalosporins, macrolides, more than half of them have been resistant to carbapenems and four of them have been found non-susceptible for levofloxacin as well, leaving few effective antimicrobial agents to treat serious infections caused by these strains. In our study, all the pneumococci with extremely high level penicillin resistance were isolated from children younger than 6 years of age. In agreement with previous findings, in our study, strong positive correlation was found between the penicillin and the cefotaxime MICs. On the other hand, in contrast to George *et al* [131], weak positive correlation was found between the beta-lactam and levofloxacin MIC values. Considering the correlation studies performed previously on the levofloxacin and the beta-lactam susceptibilities of Pneumococci, the activity of levofloxacin on *S. pneumoniae* isolates had been found to be not associated with penicillin susceptibility examining either the MIC or the bactericidal index for penicillin and levofloxacin [131], so, the question of the associated resistance to β-lactams that had been examined by others as well [132] is yet of great concern. Fluoroquinolones have been widely used in Hungary in hospitals and general practice; therefore, it is not surprising that in as little as 6 months after introducing levofloxacin in the Department of Pulmonology resistant strains have appeared. Thus, in agreement with Heffelfinger [133] and others it can be concluded that the use of new fluoroquinolones should be restricted to adults with penicillin allergy or highly drug-resistant Pneumococci to retain the activity of the newer fluoroquinolones as long as it is possible preventing the emergence of fluoroquinolones-resistant strains. Little or no levofloxacin resistance had been reported from Asia and Europe previously (before 2000), but high level levofloxacin resistance had already been detected among some US isolates belonging to the Spanish/USA clone that had

already been reported spread in several countries before 2000 [134]. In the SENTRY study from 1999 to 2003, five epidemic clusters of levofloxacin-resistant pneumococci were noted in Italy, but FQ resistance rates did not increase significantly in other countries in Europe, Latin America, or the United States [106]. Intraspecific or interspecific recombination of mutated QRDR domains following transformation may be responsible for the dissemination of quinolone resistance among different pneumococcal clones [135]. The need for annual surveillance of antimicrobial resistance in *S. pneumoniae* was concluded by Sahm *et al* in the year of the present study [136]. In Hungary, the NCE made a suggestion in 2001 for the examination of penicillin MIC value of each *S. pneumoniae* isolate with a smaller than 20 mm diameter inhibition zone around the 1 μg disc. In the national microbiological surveillance operated by the NCE, penicillin susceptibility data of 1031 *S. pneumoniae* strains, isolated mainly in the laboratories operated by the NPHMOS were collected between January and March of 2002, and the incidence of penicillin resistance was found 9.7%, although, the previous year, with the 1 μg disc method, 25.4% of isolates, and, in the year before, 33.2% of isolates were found resistant to penicillin [137]. Of the 3061 strains isolated in the first five months of 2003 and examined following the suggestions made in 2001 by the NCE, the rate of penicillin resistance was found 9% again [137]. On the other hand, by the new protocol (MIC testing of nonsusceptible isolates), the rate of intermediate-susceptible strains was found 30% [137], similarly to the results of the present study. Some upper respiratory tract *S. pneumoniae* isolates (obtained from children) which had extremely high penicillin MICs (16-64 mg/L) were reported by Nagy *et al* a few years later [138]. The incidence of high-level penicillin resistance (MIC ≥2 mg/L) using the agar dilution technique was found low (2%) by Dobay *et al* [139], in contrast with other's results got with disk diffusion technique in Hungary (~15% and ~35% measured by others in 2000 [139]), although the percentage of PNSP isolates was found 39%, and the percentage of macrolide-resistant isolates was ~40%. The macrolide resistance was significantly higher among the penicillin non-susceptible isolates, than among the penicillin sensitive ones (~54% vs ~29%) [139], just like in our study. The other β-lactam antibiotics showed very good efficacy (97-99% sensitivity), five and two strains were found with 4 mg/L and 2 mg/L ciprofloxacin MICs, respectively (~5 and ~2%) [139]. Nowadays, PNSP are widespread worldwide,

although with notable temporal and geographical variation (e.g. from <5% in Northern Europe to over 60% in South Africa [104]. Despite marked escalation in PNSP, rates of resistance to cefotaxime (MICs ≥2 mg/L) globally remain low (1 to 7%). However, a few highly resistant clone-expressing cefotaxime MICs up to 32 mg/L have been noted in Asia [106]. For the macrolide resistance, the PROTEKT study showed an overall rate of 31% in 1999–2000, increasing to 37% in 2003–2004, but there was marked geographical variability [140]. Macrolide resistance in Europe was notably high in isolates collected from Belgium (31.5%), Spain (33.5%), Hungary (39.5%), Italy (41%), Greece (51.5%), and France (55.5%). In the National Bacteriological Surveillance (NBS), operated by the NCE, the antimicrobial susceptibility of *S. pneumoniae* isolates has been examined and documented by a standard protocol since 2005 [141]. Based on the data published in the annual reports by the NBS team [141], there has been a continuous decrease in the incidence of PNSP isolates in the outpatients' samples since 2006 (from 36.7% to 19.8%, n≈3000/year), in the inpatients' samples since 2005 (from 36.1% to 26.4%, n≈1500/year), and in the samples of patients with IPD since 2008 (from 25.3% to 15.8%, n≈150/year). Incidence of PRSP isolates changed between 2.4% and 5% (n≈3000/year) in the outpatients' samples, little bit higher incidences were found for inpatients (between 4.1% and 5.7%, n≈1500/year). Relatively higher incidence values of PRSP isolates have been found in the samples of patients with IPD since 2008 (2005-2007: between 1.2% and 3.8%, 2008-2010: between 4.9% and 5.6%, n≈150/year). Incidence of ampicillin-resistance remained below 5% throughout the study period (2005-2010) [141]. The highest incidences were found in the samples of inpatients. Incidences of resistance to antipneumococcal extended spectrum cephalosporins, namely Ctx and Cro remained below 2.0% (n≈1000/year and ≈1500/year, respectively) all along the study period, except for the TRI-resistance rate in 2007 measured in the samples of patients with IPD (2.4%, n≈100). On the other hand, threefold and twofold higher incidences of Ctx- and Cro-resistant isolates have been detected in the outpatients' samples since 2008, respectively. The incidence of erythromycin-resistance was found to be high all over the study period (changed between 32.5% and 40.6% in the outpatient's samples, n≈3000/year; and between 33.6% and 38.4% in the inpatient's samples n≈1500/year), but a continuous decrease have been found in the outpatient's samples since 2008, and in the inpatient's samples

since 2007 as well. The incidence of erythromycin resistance was found 20.6% - 34.6% in the samples of patients with IPD (n≈150/year). Over the 1990s, an increase in the proportion of pneumococcal isolates with combined non-susceptibility to penicillin and erythromycin was also observed [107]. The last EARSS report (2008) noted that the overall rate of dual non-susceptibility remained below 5% in Europe. Of concern is the increase observed over the last 4 years in the level of dual non-susceptibility in Ireland (from 3% to 12%), Hungary (from 13% to 21%), and Turkey (from 10% to 23%) [142]. Rates of FQ resistance remains low (< 2%) globally [102], but higher rates (5 to 14%) have been noted in some geographic regions or hospitals: it is above 10% in some Far East areas [135, 143]. Resistance to levofloxacin remains rare (< 0.5%) in most northern European countries. However, increasing trends have been observed due to the extensive use of these antibiotics, with treatment failure in infections caused by FQ-nonsusceptible pneumococci. A multi-center study performed in Europe in 2004–2005 involving community-acquired respiratory tract infections [144] showed a low level of quinolone resistance in the majority of European countries, with some exceptions, higher rates were also detected recently in Canada (7.3% in 2006) [145]. In this country, the increase in ciprofloxacin resistance was strongly associated with an increase in fluoroquinolone consumption [146]. To avoid therapeutic failures, it is very important to detect strains with first-step mutations, which usually have low-level ciprofloxacin resistance, and frequently appear to be levofloxacin-susceptible, with MICs of 1–2 mg/L [146, 147]. These strains could become highly resistant under selective fluoroquinolone pressure. By the NBS team of the NCE, the resistance rates for levofloxacin were found low recently, but it rose above 2% in the years of the study period (2005-2010) in the samples of the patients with IPD [141]. The epidemiology of drug-resistant pneumococci on a global scale was last reviewed by Lynch and Zhanel [106]. Globally, rates of MDR pneumococci are also highly variable. The global SENTRY program (1999 to 2002) cited rates of MDR ranging from 36.3% in Asia to 2.9% in Brazil. A survey of 15 European countries in 2004–2005 cited overall prevalence of MDR of 15.8%, with a wide variability between countries (e.g., 0% in Denmark, 40.8% in France, 42.9% in Greece) [106]. In the United States, surveillance studies from 1994 to 2006 cited MDR in 9 to 24% of pneumococci [106]. Resistance to vancomycin has not been found among pneumococci. However, tolerance (the ability of

bacteria to survive but not proliferate in the presence of a bactericidal antibiotic) has been described rarely [106]. The efficacy of the most important new antimicrobials for drug-resistant pneumococci and the therapeutic options for vancomycin-tolerant pneumococci was reviewed also by Lynch and Zhanel [106]. Data about the rates of resistant strains for further drugs in Hungary are available on the homepage of the NCE [141]. Proportions of PNSP, macrolide-, tetracycline-, or Sxt-resistant *S. pneumoniae* isolates are above 10% in Hungary, but ampicillin, it's derivatives, and the antipneumococcal cephalosporines (in the cases of meningitis) can be the first choice in pneumococcal infections also in Hungary. No data are available about the proportion of MDR pneumococci in Hungary in the last five years [141].

Glycopeptide-resistant enterococci had been first described in France and UK in 1988 [148, 149] and the year after, in the USA. Although the national nosocomial surveillance system of antimicrobial resistance (NBS) has been working since 2005, there are some earlier data available about the occurrence of VRE in Hungary. The first cases confirmed by phenotypic examinations were reported by Füzi *et al* [150]. These isolates were recovered from urine and fecal samples taken by a hospital hygienic screening in a hematological unit. There were some sporadic cases of BSIs caused by VRE in Hungary before 2000 also by the annuals published by the NCE before 2000, and in the year of 2000, Ghidán *et al* reported the first case confirmed by PCR [151]. The first reported human clinical VRE isolate was detected in Hungary in 1998, in a senies (toe ulcer) of a 64 year old diabetic patient. The isolate was an *E. faecalis* strain showing VanA phenotype: the vancomycin and teicoplanin MICs were >256 and 96 mg/L, respectively. The carriage of the *vanA* gene was proved by PCR [151]. In 2002, Konkoly-Thege *et al* [152], and after than, in 2003, Knausz *et al* [153] reported isolates of *vanA* harboring *E. faecalis* recovered from human clinical samples in Hungary. By the annuals published by the NCE between 2000 and 2004, there were henceforward only sporadic cases of BSIs caused by VRE in Hungary. Some of these isolates were sent to the NCE and confirmed as VRE. Bacteriological surveillance data in Hungary showed that, in 2003, vancomycin-resistant *Enterococcus spp.* isolates were less than 1% of all the enterococcus isolated in Hungary that year (n=15933). According to the data collected by the NCE about the VRE strains isolated in 2004, the rate of isolation of VRE was below 1% in Hungary yet in 2004 (in more than teen thousand isolates as

well). According to the National Bacteriological Surveillance Database [141] the prevalence of vancomycin non-susceptible *E. faecium* was 1.6% (teen of the 634 strains) in 2005 among *E. faecium* clinical isolates in Hungary. The ratios of the number of *E. faecium* and *E. faecalis* strains reported in the NBS between 2005 and 2010 was between 12% and 20% indicating that the CC-17 was not widespread in Hungary yet in the last five years. These figures are not available for Serbia. Sporadic *vanA* positive *E. faecalis* human clinical isolates had previously been reported not only from Hungary but also from Serbia [151, 154]. The presented report was the first report of a molecular epidemiological study performed on Hungarian and Serbian outbreak strains isolated from the first detected and reported healthcare associated outbreaks in these countries. The first healthcare-associated VRE outbreak in Hungary - occurred at the hematology and stem cell transplantation unit of a tertiary care hospital - was caused by VanB *E. faecium*. All the VanB *E. faecium* outbreak isolates belonged to a single PFGE type showing the close epidemiological relatedness of these isolates. In contrast to the situation in the Hungarian hospital, the cluster of cases at the MMA in Belgrade was caused by VanA VREF isolates and affected several different departments. Also, in Belgrade, the VREF isolates were proved to belong to two different PFGE clones and the vanA gene cluster was identified in *E. gallinarum* isolates as well indicating an endemic situation and suggesting that VanA VREF might have been present in the hospital for months or years and [155]. VRE have been found in clinical isolates at the MMA prior and also after the 2005 outbreak with prevalence rates of 0.6%, 2.6%, and 3.2% among *Enterococcus spp*. clinical isolates in 2004, 2005, and 2006, respectively (B. Tomanovic, unpublished data). The detection of two different PFGE types among VanA VREF isolates at the MMA may indicate horizontal transfer of the vanA gene cluster. To confirm the mobility of the vanA gene cluster, mating out assays were performed. The transferability of the vanA cluster from the *E. faecium* isolate indicates that horizontal transfer also might contribute to the dissemination of vanA among *Enterococcus spp*. strains in the MMA. Phenotypic tests and PCR experiments showed that the vanA gene cluster was co-transferred with the *ermB* resistance determinant suggesting that these genes are likely harbored by the same conjugative plasmid [156, 157]. VanA positive *E. gallinarum* is considered a rare pathogen with limited epidemiological importance. However, between August 2000 and February 2001, 15

highly glycopeptides-resistant *E. gallinarum* isolates, one from blood and the remaining isolates from rectal swabs, were recovered in a general hospital in Buenos Aires Province, Argentina [158]. Considering that the vanA carrying *E. gallinarum* isolates examined in our study belonged to one single PFGE type and were isolated from blood samples of two patients, the epidemiological situation can be interpreted as a small outbreak caused by this type of enterococci. Reviewing the data above it can be inferred that the clinical importance of this pathogen would be re-evaluated in the future. All the representative VREF isolates examined in our study were found to belong to the pandemic MLST type CC-17 and the corresponding MLVA cluster 1. The occurrence of CC-17 in these two countries is proved first in this study, so, with the molecular characterization of the first reported Hungarian and Serbian VRE outbreak isolates, the documented occurrence of CC-17 was extended by our study to a novel region in Europe. It was recently shown that the CC-17 associated Esp protein variant might play a role in the response of *E. faecium* to the changing environmental conditions in the early stages of infection [159]. This study confirmed the linkage of the Esp variant with epidemic isolates and so, underlines the need for extensive experimental studies to identify the determinants that contribute to the special characteristics of the pandemic CC-17. Comparing the different typing methods, MLVA was found to be superior to PFGE for large scale epidemiological studies, especially for global scale studies, but PFGE was found to be better again for hospital epidemiological examinations showing smaller differences giving a better opportunity to identify independent clusters of cases and so, to differentiate between small and large epidemics, and moreover, to highlight endemics in hospitals. The evolution of a single epidemic and clinically relevant genetic complex, as seen with *E. faecium*, seems to differ from the evolution of other gram-positive pathogens like *S. pneumoniae* and *S. aureus*. In Willems opinion, the high genetic diversity of the major epidemic clones seen in *S. pneumoniae* or *S. aureus* have not yet evolved in the much younger *E. faecium* epidemic populations [19].

Epidemiological typing of acinetobacters became available and widespread following the introduction of modern molecular techniques, as old typing techniques were not suitable for typing the isolates of this genus. The ERIC-2 AP-PCR and the DAF-4 RAPD primers were found to be good for typing acinetobacters yet in the nineties [29, 59, 160], the integron PCR was found to be useful in the recognition of types with

epidemic potential at the turn of the century [30, 161]. Just like antimicrobial agents for empirical therapy, evaluation of a new technique for local needs must be based on local data. The aim of the presented study was the molecular epidemiological characterization of MDR *Acinetobacter spp*. isolates derived from clinical and hygienic samples of an intensive care unit in 2002 and 2003. The study was initiated and performed first of all with hospital hygienic purposes to investigate a hospital outbreak by molecular epidemiological techniques, first in Hungary. According to the results by the molecular typing techniques used in our study, there were two outbreak strains spread in 2002 in the ICU where the isolates had been collected. According to the results by PCR-based typing techniques, one of these two outbreak PCR types persisted in the environment and caused the small outbreak in 2003. On the other hand, considering the results by our PFGE examinations, the small outbreak in 2003 was independent from those weltered in 2002 as the outbreak strains from 2002 and 2003 belonged to different PFGE types (A and B vs. D). The environmental isolates belonged to the same PCR and PFGE types as one of the two outbreak strains found in 2002 (type B), the two isolates obtained from the two colonized patients belonged to the same PCR and PFGE types as the two isolates obtained from the two infected patients recognized in 2003. Our results confirmed both the prolonged persistence of *A. baumannii* strains in the hospital environment and the spread of an *A. baumannii* strain from patient to patient. These possibilities had been found to help raising hospital outbreaks many times previously by others [162]. Examining the class-1 integron patterns of the collected isolates we found that all the isolates contained a ~700 bp nonspecific sequence and further two non-coding sequences. On the other hand, although the number of isolates was low, similarly to others [30, 161] we found some aminoglycoside resistance genes in some of the isolates which had epidemic potential. The isolates obtained in 2002 were indistinguishable from each other by this technique, but were the isolates obtained in 2003: these contained one more integron gene cassette with coding regions for streptomycin and kanamicin resistance genes. Although the isolates from 2003 and the isolates from 2002 could be distinguished from each other by this technique as well just like by the PFGE, but the three PCR and PFGE types identified among the isolates from 2002 could not be distinguished by this technique. Summarizing our results, among the molecular epidemiological techniques used in our study, for hospital epidemiological

purposes, PFGE was found to be the best. And moreover, in our study, PFGE was found to be not only useful in identifying causes of bacterial infection, but, according to our results, it can aid a hospital in its infection control measures, highlighting areas of weakness, and to identify the source of hospital-acquired infections. Although *A. baumannii* strains examined in our study was found to be typable by all the three molecular epidemiological techniques, just like in our previous studies performed on *E. cloacae* and vancomycin-resistant *E. faecium* isolates, lower discriminatory power was proved for PCR based techniques, than for PFGE. That's why the question has arisen whether there have been some epidemic clones found in our study (PCR type B, PFGE types B and D). Examining further 91 Hungarian *A. baumannii* isolates obtained from eight healthcare facilities of five counties, the B and D PFGE types were found epidemic (caused at least to times at least to related cases) as (NCE, 2002-2003, unpublished data) the D type was found among the isolates from the counties of Komárom-Esztergom, Pest, and Hajdú-Bihar; the B type was found among the isolates from the counties of Budapest, Pest, Heves, and Hajdú-Bihar (data not shown). Applying the methods by Turton [163] on Hungarian strains isolated in 2009 and 2010 and examined in the NCE (n=183) [164], the B type strains (and so, the D type strains as well) which were isolated frequently yet in 2009 in Hungary (this type was the second most prevalent type, namely, the AC002 type [164]) belong to the EU I clone [163]. Consequently, the EU I clone was present yet in 2002 in Hungary, and the hospital outbreak detected in an intensive care unit and investigated in our study was caused mainly by these, the first identified Hungarian isolates of the EU I clone. Reviewing the literature, we can find that application of various methods has led to the recognition that a limited number of widespread clones are responsible for hospital outbreaks in many countries. Comparisons based on cell envelope protein profiling, ribotyping and AFLP genomic fingerprinting of epidemic and non-epidemic *A. baumannii* strains from geographically distinct European hospitals first delineated two major groups of epidemic strains, which were named European clones I and II [34]. A third pan-European outbreak clone (clone III) was subsequently distinguished based on ribotyping and AFLP [165]. The three 'European' clones should now more appropriately be called 'international clones', as they were associated with infection and epidemic spread not only in Europe, but in other parts of the world as well [34, 165-

169]. Multidrug resistance is often associated with isolates that belong to these international clones [34, 167, 169]. Clone I was found in Belgium, UK, Netherlands, Italy, Spain, Poland, South Africa, Czech Republic, USA, Pakistan, Argentina, Bulgaria, India, Puerto Rico, and Singapore [34, 164, 165, 168]; Clone II in UK, The Netherlands, Denmark, Spain, Portugal, France, Greece, Turkey, South Africa, Czech Republic, Poland, Germany, Belgium, Sweden, USA, Australia, China, Israel, Pakistan, Singapore, and Taiwan [34, 165, 166, 168, 169]; Clone III in France, The Netherlands, Italy, Spain, USA [165, 169]. MLST is the current standard for epidemiological typing on a global scale and for investigating evolutionary biology, or population structure of bacterial species [170]. MLST is a highly discriminative method of typing microorganisms [171] and has been applied successfully for the epidemiologic characterization of a variety of clinically important bacterial pathogens. Examining the genetic relationships of 154 selected *A. baumannii* isolates with MLST, among MLST genotypes (STs), using the MS-tree method, only five clonal complexes were found epidemic all over the world, three of which corresponded to international European clones I–III [172]. Summarizing the results of our study and those from the international scientific literature, it can be concluded, that the identification of epidemic (widely distributed) clones by building databases on different scales is essential not only for global epidemiological but also for hospital epidemiological purposes seeking for the most suitable molecular epidemiological technique. *Acinetobacter spp*. began to be recognized as a significant hospital pathogen in the late 1970s, but at that time it was easily treated as it was susceptible to commonly used antimicrobials. As with all other non-fermentative Gram-negative bacilli, *A. baumannii* can develop clinical resistance to all classes of antimicrobials. The acquisition of resistance mechanisms by *A. baumannii* has been estimated as a recent phenomenon that started in the 1970s. This extremely rapid development of antimicrobial resistance is due to the widespread use of antimicrobials in the hospital environment and to the ability of *A. baumannii* to respond rapidly to challenges issued by antimicrobials. Specifically, the influence of the wide use of extended-spectrum cephalosporins and quinolones has been reported in the development of resistance [173-176]. There have also been reports of panresistant *A. baumannii,* which are essentially resistant to every marketed antibiotic [177, 178]. Since it is largely a nosocomial pathogen, *A. baumannii* is bombarded by the selective

pressure of a broad variety of antibiotics. Considering that the majority of *A. baumannii* recovered from hospitalized patients are MDR, treatment of these infections is challenging. Of concern, most of the current reported *A. baumannii* outbreaks are due to MDR isolates, with limited therapeutic options available [179-184]. Comparing the results of the antimicrobial susceptibility testing performed in our study to the data about the antimicrobial susceptibility of Hungarian *A. baumannii* ICU isolates obtained in the NBS, the followings can be concluded. According to the data by the NBS, the incidence rate of ceftazidime-resistant strains increased from 55% to 93% between 2006 and 2009 among the Hungarian ICU isolates, so, the extremely high incidence of ceftazidime resistance found in our study might be explained by the low number of isolates and genotypes examined, but, on the other hand, in the background of the spread of a ceftazidime-resistant clone, there could be the regular use of ceftazidime what was usual in the Hungarian ICUs at the turn of the century. Regarding the carbapenems, the rate of meropenem resistance was found notably higher than the rate of imipenem resistance not only in our study but also by the NBS (2.7% vs 1.4%, data by the NBS [141]). From 2006-2007, but the great difference between the rate of imipenem and meropenem resistance disappeared in Hungary to 2008 [141]. Comparing the aminoglycoside susceptibility data obtained in our study to those obtained by the NBS [141] we might conclude, that the netilmicin-amikacin resistance found characteristic for the epidemic clone in our study might reflect the local antibiotic consumption (just like the extremely high rate of ceftazidime resistance), as, in the corresponding NBS data branch (ICU isolates), the gentamicin, tobramycin, netilmicin, and amikacin resistance rates were found 61%, 33%, 20%, and 18%, respectively in the first year when AMR of ICU isolates were first examined separately in the NCE (2006). On the other hand, according to the data collected in the NBS a few years later (in 2008 or 2009), the rate of tobramycin resistance was the lowest among the aminoglycosides listed above. That might reflect the aminoglycoside consumption in Hungary: gentamicin, netilmicin, and amikacin are widely used for empiric therapy, while tobramycin is used only for antibiogram directed therapy. The rate of ciprofloxacin resistance among *A. baumannii* ICU isolates was found really high in the NBS as well (70-80%), just like the incidence rates of sumetrolim or tetracycline [141]. The rate of tazobactam-piperacillin, cefepime, or levofloxacin was above 60% for each of these

drugs, the rate of amikacin-resistant strains was above 50%, and the rate of the resistant strains was above 30% for each of the best drugs (sulbactam-ampicillin, carbapenems, tobramycin) as well in 2009 [141]. In the years studied by the NBS (2005-2009), the rates for all the drugs examined increased permanently year by year, and moreover, carbapenems and sulbactam-ampicillin, previously good for empirical use in *A. baumannii* infections in Hungary were lost in the study period as the proportions of resistant strains exceed 10% in the last five years (the rate of carbapenem resistant strains was below 3%, the rate of sulbactam-ampicillin resistant strains was at about 10% in 2006 in Hungary [141]). Reviewing the data by the NBS, both the total number of strains and the number of ICU strains increased permanently between 2005 and 2010. The ratios of the numbers of ICU strains and the numbers of all the strains reported annually were between 29% and 43% (2006-2010), which values are unusually high: this ratio reached a similar value (44%) only in the case of *K. pneumoniae* in the study period, when (according to the data by the NBS) *K. pneumoniae* BSI outbreaks weltered in Hungarian ICUs might have been in the background. As similar conclusions can not be drawn for *A. baumannii* reviewing the data by the NBS, the vast majority of infections caused by *A. baumannii* in the Hungarian ICUs could not be BSIs. On the other hand, *A. baumannii* infections can be told the most usually "ICU associated" bacterial pathogen in Hungary as the value of this ratio was ~1.5% for *Enterococcus spp*., remained below 4% all along the study period for *E. coli*, was 6.5%-8% for *S. aureus*, 12.5%-15% for *Enterobacter spp*., and 19-20% for *P. aeruginosa*. As with *P. aeruginosa*, carbapenem use is a powerful selector for carbapenem-resistant *Acinetobacter* and *Klebsiella* clones, once established in the hospital setting, can prove very difficult to eliminate. Similar to *P. aeruginosa* multidrug-resistance is a common feature among hospital acquired *Acinetobacter spp.* and *Klebsiella spp.* clones and panresistance sometimes occur. It is now clear that with these Gram-negative pathogens we are close to the end of therapeutic options. The results of our study, and the data by the NBS and the international scientific literature call the attention to the importance, and moreover, to the need for the follow up of local antimicrobial susceptibility data, to the implementation of infection control rules recommended to prevent the spread of multidrug-resistant bacteria in hospitals, and last but not least, to the importance of molecular epidemiological examinations in hospital epidemiology. The CDC reports

that from 1982 to 2005, more than 100 reports have been published documenting the success of various control interventions to lessen the burden of not only *A. baumannii* but MRSA and other MDR pathogens [94]. The teen interventions found to be the most effective in controlling the transmission of MDR hospital pathogens in the meta-analysis by the CDC were the followings: education of staff, patients, and visitors; emphasis on hand washing; use of antiseptics for hand washing; contact precautions and glove use; segregation of cases; change in antimicrobial use; surveillance cultures of patients; surveillance cultures of staff; environmental cultures; extra cleaning and disinfection; dedicated equipment. Reviewing the literature of the last two decades we can add two more steps to the list by the CDC which made the nosocomial infection control better: (i) better financing (UK); (ii) and operation of a nosocomial infection surveillance system in the hospital as a stipulation of accreditation (Switzerland).

While many studies were performed on *P. aeruginosa*, *A. baumannii*, or *K. pneumoniae* strains, *Enterobacter spp*. are underinvestigated. Three studies performed by the author on *E. cloacae* strains are presented here. *Enterobacter* species are widely distributed in the environment, while in humans *E. cloacae* is a saprophytic member of the normal flora in the gastrointestinal tract. In the nineties this species has emerged as an important nosocomial pathogen because of the intrinsic resistance to ampicillin and the frequent selection of mutants resistant to extended spectrum cephalosporins and aminoglycosides, especially in intensive care units where these drugs have been used extensively. When unusual number of multi-resistant isolates of *E. cloacae* is noticed in a hospital ward the question arise: whether there was one or a few related clones spread (nosocomial clusters of cases and outbreaks as the consequence of administration of contaminated pharmaceutical products, patient-to-patient cross infection via hospital materials, etc.) what was reported many times or the extremely high number of cases was caused by many unrelated clones subsequently to the regular use of some antibiotics at the particular unit – there are some reports for the later situation as well [185, 186]. Performing genotyping techniques on the more than one hundred MDR *E. cloacae* strains isolated in a Hungarian PICU in 1998 by genotyping techniques have shown that there was a large outbreak in the PICU responsible for the plethora of cases, for the vast majority of cases affecting 94 premature newborn babies. Eight percents of the patients died. An *E. cloacae* outbreak had previously been reported to be related to

understaffing, overcrowding, and poor hygiene practices [187]. The PICU where the MDR strains examined in our study were isolated in an unusual number also suffered from understaffing, overcrowding, but poor hygiene practices might have been the most important factor leading to a large outbreak considering that the staff was undereducated, and there was no hospital hygienic professional affiliated by the hospital in which the PICU worked, and furthermore, the laryngoscopes were not taken to pieces before sterilization. On the other hand, regular use of ceftazidime might have contributed to the emergence and persistence of third-generation-cephalosporine-resistant strains. The results of this study showed that the ERIC patterns for *E. cloacae* remained unchanged for a long period, although the large outbreak clone spread to more than 90 patients. ERIC and PFGE techniques were proved to be excellent tools for molecular epidemiological follow up in a one year long outbreak period as well: methods of lowest risk for misinterpretation although phenotypic and genetic heterogeneity of the outbreak clone was highlighted. PFGE was useful not only to confirm the spread of a single clone but also to corroborate that the long outbreak period gave the opportunity for the microbes to diversify. Three PFGE clones were found to be present in the period examined: one of them was found to be predominant, and one of them an ESBL clone. As so as it can be declared in a retrospective analysis: there was a small outbreak in April caused by the ESBL clone, the sporadic isolate of May has been proven to be clonally related and, summarizing our laboratory results, can be considered an outbreak related strain. The same ESBL clone was responsible for cases of colonization and infection. The severe therapeutic problem caused by ESBL strains is enhanced by the potential co-resistance to other antimicrobial agents can be explained by the frequent occurrence of ESBL genes on large, conjugative plasmids carrying resistance determinants also for aminoglycosides, tetracycline, sulphonamides and chloramphenicol [42]. Our results have shown the multidrug–resistance of isolates and the presence of a large plasmid in each ESBL strain. The ESBL clone has been proven to contain class-1 integrons as it was found to be usual for hospital acquired MDR bacterial strains in previous studies. The worldwide distribution of ESBL positive strains is of special concern for epidemiological follow up over time and geographical areas trying to prevent and control infections. The emergence of SHV-2a positive *E. cloacae* in Hungary in a period of high clinical incidence by MDR CREC strains has

been proven in our study. While SHV-5 and SHV-12 positive *E. cloacae* strains were reported from Hungary in 2005 [89], emergence of SHV-2a positive *E. cloacae* in Hungary (as early as 1998) is described first here. SHV-2a producing *E. cloacae* have been reported only from Far East - Thailand [188], Korea [189] before 1998, so, the origin of this clone is a question of great concern, but remained unknown. Neither importation of an SHV-2a positive *E. cloacae* clone, nor transmission of a $bla_{SHV\text{-}2a}$ positive ESBL plasmid from another species by conjugation can be precluded. The first well characterized SHV-2a positive Hungarian *K. pneumoniae* isolates with a conjugative, 62 Md ESBL plasmid (NCE, 2004, unpublished data) were derived from a PICU about 100 km far away, the same year but four months later. SHV-2a positive *K. pneumoniae* isolates were reported from many different European countries before 1998, SHV-2a producing *E. cloacae* was reported from a Slovak hospital in 2005 [190], and from Greece in 2007 [191]. The emergence of SHV-2a producing *E. cloacae* strains again in Hungary in 2002 is reported in the second study performed on *E. cloacae* strains by the author and presented in this work. SHV-2a producing *E. cloacae* strains had been reported only from Korea up to 2002, but were found to be predominant in the particular country [189]. Presence of SHV-2a in other species had already been published by German [192], Swiss [193], Korean [194], French [195], Croatian [83], Spanish [196], Italian [196], Polish [197] and Canadian [198] authors previously (from 1991 to 2002). In the present study, SHV-2a gene was detected by PCR sequencing, while SHV-2a enzyme production was proved by the IEF. The cefotaximase-like activity that can be seen reviewing the cephalosporine hydrolysis pattern also suggests the production of SHV-2a [199]. The genes encoding for TetC and the β-lactamase with pI 5.4 were probably not located on a transmissible plasmid as nor a band at similar pI values in the IEF assay, neither a product by the tetC PCR could be detected in cefotaxime-selected transconjugants. On the other hand, the conjugative plasmid found in the isolates of both patients harbored not only the SHV-2a gene but the resistance determinant conferring resistance to three aminoglycosides (gentamicin, tobramycin, netilmicin), and *tet*A. While *tet*C is widely distributed among *E. cloacae* strains, *tet*A is unusual in this species. tetA is widely distributed in many other species of the *Enterobacteriaceae* family, also in *K. pneumoniae* [200]. Considering the clinical epidemiological data (namely that SHV-2a positive *K. pneumoniae* strains were isolated

in the same hospital the same month), this plasmid might have been originated from *K. pneumoniae*. Although SHV-5 was the only ESBL enzyme had been published being present in Hungary till 2002 [201, 202], but SHV-2a producing *K. pneumoniae* strains were isolated in the county of Bács-Kiskun in 1998 [203] and in the county of Csongrád in 2002 [203]. Here we report the first recorded small outbreak caused by ESBL *E. cloacae* strains in Hungary as well. Virulence of the outbreak strain was proved unfortunately in vivo as patient B had the clinical signs of sepsis and a positive blood culture. Nosocomial cross contamination of patient B from the nasopharynx of patient A calls the attention to the importance of nasal carriage [204]. Both patients were cared in a neonatal intensive care unit. The hospital settings, first of all the regular use of amoxicillin and gentamicin in combination might have facilitated the spread of bacteria carrying the ESBL gene and the aminoglycoside-resistance determinant on a horizontally transmissible plasmid. In spite of these facts, the clone disappeared; it might have been the consequence of the infection control measures taken immediately. On the other hand, examining the results for the ESBL strains collected in the Hungarian ESBL surveillance in a whole, we can conclude the same as Roberta Coque [84] - that all the ESBL clones remained ephemeral. Although tigecycline, the new glycylcycline introduced in 1999 was not a widely used drug in 2003-2004, and, in 2000, only 10% of more than 1000 enterobacterial strains were shown to be resistant to tigecycline [205], tigecycline resistance was detected in more Hungarian ESBL *E. cloacae* strains in our study. The rarity of SHV-2a producing *E. cloacae* makes the question of the origin of the Hungarian outbreak strains interesting. Horizontal transmission of the particular plasmid from *K. pneumoniae* as an epidemiological reservoir is a feasible hypothesis, although the "de novo" genesis by mutation can not be precluded either and might have been facilitated by the regular use of third generation cephalosporines which is usual at neonatal intensive care units in Hungary. Expression of the cephalosporine, the aminoglycoside, and the tetracycline resistance determinants (found on a conjugative plasmid in our study) in a foreign species, just like *E. coli* supports the hypothesis of the interspecies spread. Similar events were published had happening not only in vitro but also “in vivo” (in the hospital environment) in terms of *E. coli* and *K. pneumoniae* [206], and the hybrid character for the promoter of some Korean plasmid encoding ESBL gene has also been published [207]. Direct import of

SHV-2a producing *E. cloacae* strains from Korea can not be excluded either, as the hospital gloves are imported from the Far-East to the Hungarian hospitals. The presence of *tet*A 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 as *tet*A is unusual in *E. cloacae* strains, but widespread in other species of the *Enterobacteriaceae* family [200]. In our study, the frequency of conjugation was found much higher (about hundredfold more frequent) with *K. pneumoniae* strains, than with *E. cloacae* strains (data not shown). Although the number of ESBL *E. cloacae* is increasing worldwide, ESBL *K. pneumoniae* is isolated much more frequently than ESBL *E. cloacae* [208] (terms *Klebsiella pneumoniae* and ESBL vs *Enterobacter cloacae* and ESBL). Although the question whether the Hungarian *E. cloacae* outbreak strains were the donors or the acceptors for the SHV-2a encoding ESBL plasmid arise, but this question is only of hypothetic concern. As a consequence of globalization, ESBL strains or plasmids can spread easily. It is exemplified by the allodemic caused by ESBL plasmids (mainly SHV plasmids), international spread of successful clones, and the CTX-M pandemic. On the other hand, epidemic spread of *E. coli* or *K. pneumoniae* clones is more frequently reported than that of *E. cloacae* clones. *E. cloacae* has been far more rarely involved in large hospital outbreaks or inter-hospital spreads than in clusters of cases confined to single hospitalization units. In the third study performed on *E. cloacae* strains by the author and presented here, the epidemic spread of one single clone (ERIC-type-A) in 11 Hungarian counties (that was found highly probable analyzing the results by *Xba*I PFGE) was confirmed by *Spe*I PFGE. The restriction site of *Not*I (GC/GGCCGC) harbors a complementary sequence of a Variable Number Tandem Repeat (VNTR)/Short Sequence Repeat (SSR): (-GCC-) [209]. The results with *Not*I may indicate the presence of a VNTR/SSR sequence in an unusually high number in type-A-isolates. Comparing resistance patterns and MICs of type-A-isolates year by year show that the persistence of a common source somewhere in Hungary up to 2005 is feasible when no loss of antimicrobial resistance through the study period is postulated. That source could be the teaching hospitals or a secondary/tertiary care hospital. The only CTX-M 15 positive *E. cloacae* strain of this study was isolated in a hospital where some CTX-M 15 positive *K. pneumoniae* strains had been isolated a few weeks' earlier

(data not shown). Summarizing the results of the presented studies performed on *E. cloacae* strains we can conclude that examining the epidemiological background of emerging of ESBL *E. cloacae* strains, in two of the three studies *K. pneumoniae* strains producing the same ESBL type enzyme were found in the same hospital close in time, and, in the third study, patients were transmitted from the hospital where the ESBL *E. cloacae* strains were isolated to the hospital where ESBL *K. pneumoniae* strains producing the same type of ESBL enzyme were isolated a few month later. High-level ciprofloxacin resistance of multidrug-resistant enterobacterial strains was reported many times through accumulation of various resistance mechanisms in a step-by-step process (target gene mutations, increased efflux activity, and alteration in porin synthesis resulting in decreased membrane permeability). Mutations in the QRDRs of the *gyr*A, and *par*C genes and the role of the AcrAB efflux pump were shown to be involved in ciprofloxacin resistance also in *E. cloacae* [44, 210, 211]. Cyclohexane tolerance was frequently associated with multidrug resistance involving AcrAB efflux-pump system in *Enterobacteriaceae* species [43], but little is known about CH tolerance of *E. cloacae* strains. In two consecutive *E. cloacae* isolates, the increased expression of MarR and AcrB resulted in emergence of CH tolerance, fourfold increase of ciprofloxacin, and twofold increase of chloramphenicol and tetracycline MICs [44]. In our study performed on MDR isolates with high-level ciprofloxacin resistance, ciprofloxacin and chloramphenicol MIC_{50} values of the CH- strains were found fourfold, tetracycline MIC_{50} values twofold lower, than those of the CH+ strains. Twenty-two clinical isolates of different ERIC patterns (collected between 1991 and 2004 by the NCE) lacking resistance to third-generation-cephalosporins, ciprofloxacin, or a third, non-ß-lactam, non-fluoroquinolone drug tested in our study were found CH- (data not shown). In addition to the MarA/SoxS/Rob family [43], RamA was identified as a transcriptional regulator of the AcrAB efflux pump in many species [212]. In a recent publication, mutation in *ram*R and the consequent over-production of RamA, and AcrB in one mutant laboratory salmonella strain selected with ciprofloxacin has been associated with cyclohexane tolerance and statistically significant, fourfold increases in ciprofloxacin, chloramphenicol, and tetracycline, twofold increase in triclosan MICs [213]. Good correlation of over-expression of RamA with the increased expression of the AcrAB efflux pump has already been found also in *E. cloacae* isolates with decreased

susceptibility to tigecycline [214]. PAβN is a widely used efflux-pump inhibitor capable of at least partially reversing the MDR phenotype in *Enterobacteriaceae*. Depending on species, fourfold or eightfold reduction in fluoroquinolone MICs in the presence of PAβN was considered as an indicator of efflux-pump over-expression phenotype [44, 59, 98, 99, 100, 211]. Ciprofloxacin MIC of an *Enterobacter cloacae* clinical isolate over-expressing the *acr*A gene was found eightfold lower in the presence of PAβN [211]. In our study, a notable (fourfold or greater) reduction in ciprofloxacin MICs was found in the presence of PAβN in 79% (isolates from clones A, C, and D), an eightfold or greater reduction in 37% (isolates from clones A, and C) of tested isolates. Our results may point to a role in the decreased influx/efflux ratio for high-level ciprofloxacin resistance and the MDR phenotype in these isolates. The increased efflux and the biocide resistance mechanisms that may be indicated by cyclohexane tolerance may prolong bacterial survival [59, 95, 215] to let a bacterium accumulate other resistance mechanisms. In our study, CH-tolerant isolates derived from three of the four MDR clones. All the isolates in which fourfold or greater reduction of ciprofloxacin MICs were seen in the presence of PAβN showed slender or confluent growth in the presence of CH. The high-level ciprofloxacin and multidrug resistance of isolates can only partially be explained by our results. All the five isolates from type A, had identical mutations in the QRDRs of both of the *gyrA*, and the *parC* genes, respectively, regardless of their ciprofloxacin MICs differed in the presence of PAßN (4-64 mg/L). CH tolerance or at least eightfold reduction in ciprofloxacin MICs in the presence of PAβN was detected in altogether 91% of the tested isolates. Although the first isolate of the epidemic type was found negative by both of the cyclohexane-tolerance and the efflux-pump-inhibitor tests, and had the lowest ciprofloxacin MIC (4 mg/L) among the isolates of the epidemic type, a 4 mg/L or a lower ciprofloxacin MIC value could be achieved by the use of PAβN in only one isolate from type A, while 85% of the tested isolates from type A had ciprofloxacin MICs in the presence of PAβN significantly higher than 4 mg/L (≥16 mg/L). The isolates showing slender growth in the presence of cyclohexane had 8-16 mg/L ciprofloxacin MICs in the presence of PAβN, as did 79% of CH tolerant, and four of the five CH-sensitive isolates. These results may indicate the presence of a common, PAβN-independent resistance mechanism raising the ciprofloxacin MICs to 8-16 mg/L in the majority of isolates regardless of their

cyclohexane tolerance. In our study, 11 isolates showed 32-64 mg/L ciprofloxacin MICs in the presence of PAβN. An important role of AcrAB-efflux-pump-independent resistance mechanisms conferring resistance to chloramphenicol or tetracycline can be concluded too. Further molecular studies would be needed to highlight the resistance mechanisms.

VIII. Conclusions

The global diffusion of antibiotic resistance, with the emergence and spread of MDR, sometimes panresistant bacterial clones, is now a matter of major concern with Gram-positive and Gram-negative pathogens of healthcare associated infections. Examination of antimicrobial resistance pattern in each of these cases is essential as the number of HAIs caused by MDR organisms is increasing and the AMR patterns and frequency of them is changing over time, geographical regions, and healthcare facilities. Systematic monitoring of AMR patterns in hospitals in a surveillance system is also essential not only to help choosing drugs for an adequate empirical therapy, but also preventing the dissemination of MDR clones and/or plasmids carrying multiple resistance genes. Molecular epidemiology is useful in hospital epidemiology as the molecular epidemiological techniques can aid a hospital in its infection control measures highlighting areas of weakness and identifying the source of hospital-acquired infections. On a global scale, use of molecular epidemiological techniques can aid the authorized persons and communities in developing control strategies to prevent the spread of drug-resistant pathogens by recognizing successful, epidemic, international clones or easy spread mobile genetic elements. Both of antimicrobial susceptibility testing, and molecular epidemiology are essential in development of new drugs and treatment options as well.

Our results underline the great importance of rationalized use of antimicrobials or biocides and the tight control of health-care associated infections by multidrug-resistant bacteria in limiting the spread of multidrug-resistant bacterial clones.

IX. Summary

Healthcare-associated infections are of great concern as these are diseases with increasingly limited therapies. Rates of antimicrobial resistance vary by geographical region, by time, and by patient characteristics, that's why local data are essential. Bacterial strain typing is extremely important in hospital epidemiology to uncover the genetic diversity and the genetic background of important phenotypic characteristics.

In the presented studies, the author examined the antimicrobial resistance and/or the molecular epidemiology of drug-resistant *Streptococcus pneumoniae* (*S. pneumoniae*), vancomycin-resistant *Enterococcus spp.*, multidrug-resistant *Enterobacter spp.*, and multidrug-resistant *Acinetobacter spp.* strains isolated in Hungary.

The author showed the emergence of extremely high penicillin and cefotaxime resistance, high level levofloxacin resistance, and week positive correlation between β-lactam and levofloxacin MIC values in clinical *S. pneumoniae* isolates.

The author participated in the molecular epidemiological examination of the first reported outbreak caused by vancomycin-resistant *Enterococcus faecium* in Hungary. The author took part in the evaluation of molecular epidemiological techniques for MDR Hungarian *A. baumannii* strains and found an epidemic type described previously in other countries, and up to date in many countries (EU I clone).

The author reported the earliest European SHV-2a positive *E. cloacae* strain.

Examining the confirmed ESBL *Enterobacter spp.* strains obtained in the ESBL surveillance, more than 2/3 of cases were found sporadic, the outbreak clones were found to be ephemeral. The ESBL plasmid isolated from the first outbreak strain harbored *tet*A which resistance determinant is widely distributed in many species of the *Enterobacteriaceae* family, but unusual in *E. cloacae* suggesting the interspecies spread of ESBL plasmids in vivo between *E. cloacae* and other species.

The author reported first widely distributed cyclohexane-tolerant or PAβN+ *E. cloacae* strains. These features are indicators of adaptive mechanisms which help bacteria to survive in the hospitals and might have contributed to the nationwide spread of such strains.

The presented studies show that local antimicrobial susceptibility testing and the use of molecular epidemiological techniques help controlling HAIs and preventing the spread of drug-resistant pathogens in the hospitals and over the hospital-walls.

IX. Összefoglaló

A nozokomiális fertőzések nagy jelentőségét napjainkban a terápiás lehetőségek folyamatos szűkülése adja. Az antimikróbás szerekkel szembeni rezisztencia előfordulási gyakorisága változik nem csak időben, hanem földrajzi régiók, sőt betegcsoportok szerint is, ezért nélkülözhetetlenek a jó helyi adatok. A baktérium törzsek tipizálása pedig különösen fontos a kórházi epidemiológiában, mivel így deríthető fel a törzsek egymáshoz való genetikai viszonya és a fontos fenotípusos jellemzők genetikai háttere.

Az ismertetett vizsgálatokban a szerző Magyarországon izolált antibiotikum-rezisztens *Streptococcus pneumoniae* (*S. pneumoniae*), vancomycin-rezisztens *Enterococcus spp.*, multirezisztens *Enterobacter spp*., illetve multirezisztens *Acinetobacter baumannii* törzsek antibiotikum érzékenységét, illetve molekuláris epidemiológiáját vizsgálta.

A szerző igazolta extrém magas penicillin és cefotaxim MIC értékű, valamint levofloxacin rezisztens *S. pneumoniae* törzsek jelenlétét Magyarországon, és gyenge pozitív korrelációt talált a β-lactam antibiotikumok MIC értékei és a levofloxacin MIC értékek között.

A szerző részt vett az első ismert, vancomycin-rezisztens *Enterococcus spp*. törzsek által okozott hazai esethalmozódás molekuláris epidemiológiai vizsgálatában.

A szerző részt vett az első magyarországi *Acinetobacter baumannii* törzsek molekuláris epidemiológiai vizsgálatában és felvetette egy más országokban már leírt epidémiás típus (EU I klón) jelenlétét Magyarországon.

A szerző írta le a legkorábbi európai SHV-2a *E. cloacae* törzset, vizsgálta a nemzeti ESBL surveillance keretében 2002 és 2005 között az OEK-be küldött ESBL termelő *Enterobacter sp*. törzsek molekuláris epidemiológiáját. A törzsek csak kis járványokat okoztak: a klónok kérészéletűnek bizonyultak, sőt a törzsek 2/3-a sporadikus típushoz tartozott. Cefalosporin és fluoroquinolon rezisztens *E. cloacae* törzseket vizsgálva, az esetek nagy részének hátterében egyetlen PFGE klónt talált Magyarországon. Leírta ezen törzsek olyan fenotípusos sajátságait, amelyek hozzájárulhattak a törzsek túléléséhez kórházi környezetben, és terjedésükhöz országszerte.

Az ismertetett vizsgálatok rámutatnak az antibiotikum érzékenységi, illetve a molekuláris epidemiológiai vizsgálatok jelentőségére a rezisztens baktériumok okozta fertőzések infekció-kontrolljában a kórházakon belül és kívül.

X. References

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XI. List of publications by the author

Parts of the presented studies were published:

Glatz K, Szabó D, Szabó G, Boriszova D, Rozgonyi F. (2001) Emergence of extremely high penicillin and cefotaxime resistance and high-level levofloxacin resistance in clinical isolates of Streptococcus pneumoniae in Hungary. J Antimicrob Chemother, 48(5): 731-734.

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Glatz K, Tóth A, Pászti J. (2011) The cyclohexane tolerance and Phe-Arg-β-naphtylamide susceptibility of multidrug-resistant Enterobacter cloacae clinical isolates, and the predominance of one PFGE clone in Hungary. Clin Microbiol Infect, 7(8): 1254-1261.

Further publications by the author

Jeney C, Banizs B, Dobay O, Glatz K, Huszár T, Adám E, Nász I. (2002) The endosomal epsilon-coatomer protein is involved in human adenovirus type 5 internalisation. Acta Vet Hung, 50(4):481-489.

Glatz K, Danka J, Kucsera I, Pozio E. (2010) Human trichinellosis in Hungary from 1965 to 2009. Parasite, 17(3): 193-198.

Glatz K, Danka J, Tombácz Zs, Bányai T, Szilágyi A, Kucsera I. (2012) An outbreak of trichinellosis in Hungary. Acta Microbiol Immunol Hung, 59(2): 225-238.

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