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Antibiotic Susceptibility and Molecular Characterisation of *Proteus mirabilis* Isolates in Hospitals from the West Pomeranian Area of Poland

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Abstract

Proteus mirabilis isolates (n = 177), collected between 1996 and 2000 in four hospitals in the West Pomeranian area of Poland, were characterized by antibiotype and pulsed-field gel electrophoresis (PFGE). The selected isolates were collected from different wards (intensive care unit, surgery, internal medicine, and urology). The strains were cultured from various specimen types, mostly from urine, wound samples, bronchial exudates and sputa. The identification was done by biochemical test ID 32E ATB (bioMerieux). Analysis of PFGE patterns was based on comparison of the banding patterns obtained by PFGE of chromosomal DNA digested with SfiI enzyme. Among all *P. mirabilis* isolates tested three major genotypes A (A1-A7), B (B1-B4), C (C1-C5) and 71 unique patterns were identified. The same genotypes were obtained from different patients, treated in different wards and hospitals during a 5-year period. The strains which belonged to the genotypes A and B were multiresistant and most of them produced ESBL; genotype C was more sensitive to antibiotics.

Key words: *Proteus mirabilis*, antibiotic susceptibility, ESBL, nosocomial infection, PFGE

Introduction

Nosocomial infections are an important health problem worldwide and are closely related to the type of diagnostic and therapeutic procedures performed on patients. Isolating the pathogen responsible for an infection is one of the basic criteria for the identifying the type of infection, which considerably improves a chance for therapy which should be preceded by antibiogram determination (Toltzis *et al*., 2001). Urinary tract infections caused by *Proteus mirabilis* are common and often severe, leading to acute pyelonephritis, chronic inflammation, and bacteremia. The frequency of *P. mirabilis* infections in hospital patients as well as in outpatients increases the risk of endogenous infections, being infected by other patients, hospital staff, or contaminated equipment. It shows that the keeping the record of the exact characteristics of those microorganisms becomes necessary (Johnson *et al*., 1993).

Due to the increased antibiotic resistance, it becomes necessary to control the appearance of *P. mirabilis* strains isolated from infections in hospital environment. Wild-type strains of *P. mirabilis* are usually susceptible to β-lactams. However, a progressive increase in β-lactam resistance, mediated by the production of β-lactamases, has occurred in this species (Perilli *et al*., 2002).

The most recent advances in molecular biology offer promising possibilities of examining epidemiological bacteria strains in a controlled hospital environment. It is possible to determine the genetic profile of those microorganisms using pulsed field gel electrophoresis (PFGE), which can be used for analysis of chromosomal DNA restriction patterns, a gold standard in hospital epidemiology. Demonstrating evident relationship among isolated strains from different hospital wards within a few years period indicates persistence of the population of microorganisms responsible for appearance of clonal outbreaks (Fernandez-Baca *et al.*, 2001; Hennekinne *et al.*, 2003).

The aim of the present study was to characterise *P. mirabilis* strains, isolated during 5 years period in the West Pomeranian area of Poland, by molecular typing using PFGE procedure. The results obtained with the application of PFGE were then compared to antimicrobial resistance patterns.

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Experimental

Material and Methods

Bacterial isolates and clinical data. 177 *P. mirabilis* clinical isolates were collected between 1996 and 2000 from different patients in 4 hospitals in the West Pomeranian area: Clinical Hospital No 2 in Szczecin (SZC; n = 152 isolates – all isolates), 3 municipal hospitals (some resistant strains): Police (POL; 6 isolates), Choszczno (CHO; 2 isolates), Gryfice (GRY; 17 isolates). The selected isolates were collected from different wards (intensive care unit, surgery, internal medicine, and urology). The strains were cultured from various specimen types, mostly from urine (124 isolates; 70%), wound samples (39 isolates; 22.1%), bronchial exudates (6 isolates; 3.4%) and sputa (8 isolates; 4.5%). Isolates were identified to the species by the ID 32E ATB test (bioMerieux).

Antimicrobial susceptibility testing. The susceptibility to antibiotics was tested by the disk diffusion method on Mueller-Hinton agar according to the criteria of the Clinical and Laboratory Standards Institute. The following amounts of antibiotics per disc were used: ampicillin – Amp (10 μg), amoxycillin/clavulanic acid – Amc (20/10 μg), piperacillin – Pip (100 μg), piperacillin/tazobactam – Tzp (100/10 μg), cephalotin – Cf (30 μg), cefuroxime – Cxm (30 μg), cefotaxime – Ctx (30 μg), ceftazidime – Caz (30 mg), imipenem – Imp (10 mg), gentamicin – Gn (10 μg), tobramycin – Tob (10 μg), netilmicin – Net (30 μg), amikacin – An (30 μg), pipemidic acid – Pi (30 μg), pefloxacin – Pef (5 μg), norfloxacin – Nor (10 μg), ciprofloxacin – Cip (5 μg) trimethoprim-sulphamethoxazole – Sxt (1.25/23.75 μg).

All isolates were recognized as ESBL producers by the double-disc test. Double-disk synergy test was performed on Mueller-Hinton agar with a central amoxycillin-clavulanic acid disk and disks of the third generation cephalosporins (cefotaxime, ceftazidime) placed 20 mm (centre to centre) from each other. The test was considered to be positive for ESBL production when the bacterial growth had a 'champagne cork' appearance. For each strain the test was repeated twice.

Molecular typing. Isolates were typed by determining PFGE SfiI DNA macrorestriction patterns with the GenePath Group 5 Reagent Kit (Bio-Rad Laboratories) according to the manufacturer's recommendation. Pulsed-field gel electrophoresis (PFGE) was performed using the GenePath System (Bio-Rad). Differences detected in band patterns analysed using Molecular Analyst Fingerprinting software (Bio-Rad). The PFGE pattern was interpreted according to Tenover *et al*., (1995) recommendations.

Results

SfiI PFGE patterns. Analysis of PFGE patterns was based on comparison of the banding patterns obtained by PFGE of chromosomal DNA digested with the SfiI enzyme. Among all *P. mirabilis* isolates in the collection, three major types A: n = 48 (subtypes: A1 – 14 strains, A2 – 16, A3 – 6, A4 – 5, A5 – 4, A6 – 2, A7 – 1), B: n = 49 (subtypes: B1 – 36 strains, B2 – 9, B3 – 2, B4 – 2), C: n = 9 (subtypes: C1 – 2 strains, C2 – 3, C3 – 1, C4 – 2, C5 – 1). The remaining strains had some unrelated PFGE patterns (more than six band differences), which were designated by roman numerals and letter P: P1 – P71 (Fig. 1).

The data for the three major types A (A1-A7), B (B1-B4), C (C1-C5) from hospitals in the West Pomeranian area are presented in Table I.

Molecular typing and antibiotic resistance. Genotype A (A1-A7) strains (n = 48) were resistant to ampicillin, amoxycillin/clavulanic acid, piperacillin, cephalotin, cefuroxime, gentamicin, tobramycin, netilmicin, amikacin, pipemidic acid, pefloxacin, norflo-

Table I
The distribution of the three major types A (subtypes A1-A7), B (B1-B4), C (C1-C4) *P. mirabilis* strains isolated from four hospitals in the West Pomerania between 1996 and 2000

Hospital (ward)	1996	1997	1998	1999	2000
Urology SZC[a]	A1, A2, A3, A4, B1, C1, C2	A1, A2, A5, B1, C4	A1, A6, B1, B2, C4	A2, B1	A3, B1, B2, B4
Surgery II SZC[a]	A1			A1	A1, A2
Surgery III SZC[a]	C2		A5, A6, B2, B3	A1, A2, A7	A1
Intensive Care Unit SZC[a]	A2, A3, C1		A3, A4, A5, B2	A2, B1	
Internal Medicine SZC[a]	A1, A2, A4, A5, C2, C3	A2, B1	B1, B4	A2, B1, C5	B1, B2
POL[b]					B1
CHO[c]		A4	A4		
GRY[d]					B1

[a] SZC – Clinical Hospital No 2 in Szczecin; [b] POL – Municipal Hospital in Police; [c] CHO – Municipal Hospital in Choszczno;
[d] GRY – Municipal Hospital in Gryfice

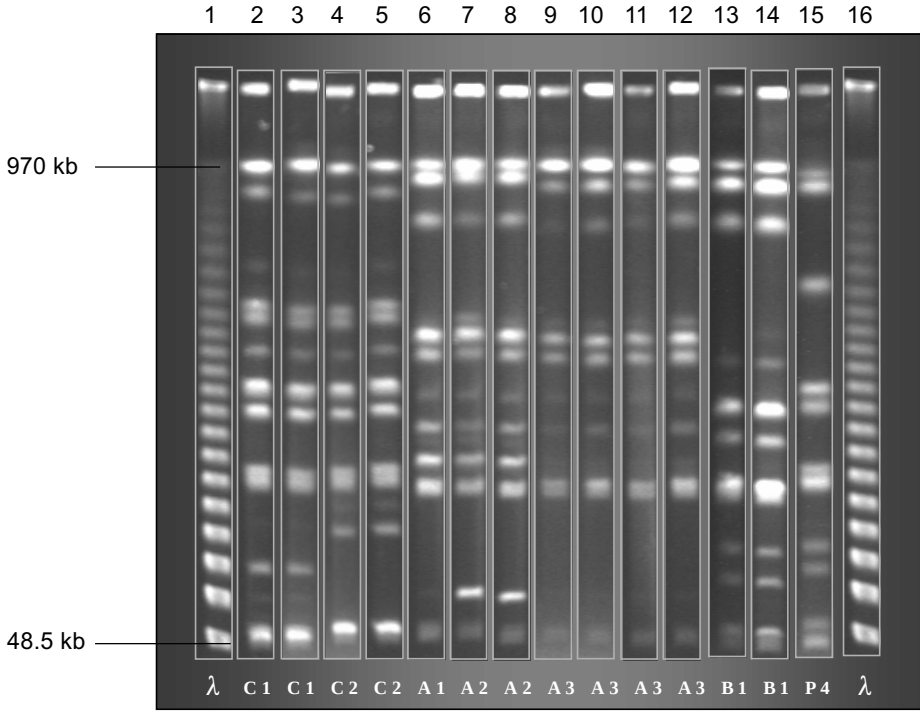

Fig. 1. Examples of pulsed-field gel electrophoresis profiles obtained for *P. mirabilis* isolates. Lanes: 1and 16, λ ladder used as molecular size (MW) markers; 2–5, subtype C1 and C2; 6–12, subtype A1 and A2, A3; 13–14, subtype B1; 15, unrelated pattern P4.

xacin, ciprofloxacin, trimethoprim-sulphamethoxazole. Only 3 isolates were sensitive to cefotaxime, 5 to ceftazidime, all strains were sensitive to imipenem. Only 5 (10.4%) isolates were resistant to piperacillin/tazobactam.

Genotype B (B1-B4) strains (n = 49), were resistant to ampicillin, amoxycillin/clavulanic acid, piperacillin, cephalotin, cefuroxime, gentamicin, tobramycin, netilmicin, amikacin, pipemidic acid, pefloxacin, norfloxacin, ciprofloxacin, trimethoprim-sulphamethoxazole. Most of the genotype B strains were resistant to cefotaxime (96%) and ceftazidime (92%), 10 strains (20.4%) to piperacillin/tazobactam. One of the isolates representing genotype B sshowed very high antibiotic resistance, including imipenem. This strain was isolated from a patient's urine in the internal medicine SZC.

All genotype C (C1-C5) strains (n = 9) were resistant to ampicillin, cephalotin and trimethoprim-sulphamethoxazole. The 3 *P. mirabilis* isolates were resistant to amoxycillin/clavulanic acid, 6 to piperacillin, 2 to cefuroxime, 1 to cefotaxime and ceftazidime, 7 to pipemidic acid, 2 to pefloxacin, norfloxacin and ciprofloxacin. All strains were susceptible to piperacillin/tazobactam, imipenem, netylmicin and amikacin.

Identification of β-lactamases. Thirty four isolates were found to be ESBL producers. The number of strains producing ESBL isolated in particular years showed an increasing trend. In 1996 only 2 (5.9%) strains showed the presence of ESBL, in 1997 – 4 (11.8%), in 1998 – 6 (17.6%), in 1999 – 7 (20.6%), and in 2000 – 15 (44.1%). All of the *P. mirabilis* ESBL producers belonged to multiresistant strains and to genotypes A1 (5 strains), A2 (7), A5 (2), A7 (1) and B1 (14), B2 (4), B3 (1).

The following antibiotic resistance patterns were observed:

AmpAmcPipCflCxmCtxCazGnTobNetPiNorPefCipSxt (20 isolates; 58.8%)

AmpAmcPipCflCxmCtxCazGnTobNetAnPiNorPefCipSxt (13 isolates; 38.3%)

AmpAmcPipCflCxmCtxCazGnPiNorPefCipSxt (1 isolates; 2.9%)

Discussion

Despite the progress of knowledge, improved preventive and scrutiny procedures, hospital infections still pose a serious clinical, therapeutic and epidemiological problem. The most common are urinary tract infections that comprise 35–45% of all hospital infections and often cause dangerous diseases such as septicemia, pyelonephritis or wound infections. The most common pathogen isolated in urinary tract infections is still *Escherichia coli.* However, other bacteria including representatives of *Proteus* genera (especially *P. mirabilis*) begin to play ever greater role in pathogenesis of hospital infections, especially in urology wards (Chippendale *et al.*, 1994; Clapham *et al.*, 1990).

P. mirabilis is the second most common cause of urinary tract infections, and it is a frequent cause of nosocomial infections as well. It was confirmed in our own research, as much as 70% isolates isolated from urine of patients suffering for urinary tract infections were classified as *P. mirabilis*, and only 30% from other materials. The existence of multiresistant *P. mirabilis* strains in hospital environment makes constant monitoring for presence of those microorganisms in specific hospital wards a necessity. It is also necessary to monitor emerging new resistance mechanisms as well as transmission of strains between patients and wards as it is commonly done in case of other bacteria (Bonnet *et al.*, 2002; Mammeri *et al*., 2001).

In our research we used PFGE as a method for analysing restriction patterns of chromosomal DNA of *P. mirabilis* strains in order to classify them into particular genotype. It has been shown that up to 60% of *P. mirabilis* strains isolated from hospital infections belong to the three main genotypes A, B, C.

Type A and B showed high antibiotic resistance to all tested aminoglycosides and chinolons, and most of β-lactam antibiotics. One strain belonging to genotype B also showed resistance to imipenem. Subtypes C1-C5 differed from types A and B in regard to antibiotics susceptibility. All those strains showed higher sensitivity to antibiotics, but the diversity of antibiotic profiles was observed among subtypes.

Demonstrating clear genetic relationship between *P. mirabilis* strains isolated from different environments within a few years period indicates existence of outbreak clones in wards of the clinical hospital as well as the transmission of those strains to other hospitals in the region. β-lactamases producing *P. mirabilis* strains with extended spectrum, ESBL, pose additional therapeutic problems in treatment of infections. The selection of ESBL producers occurs most frequently in surgery, urology and neonatal wards (Goering 1993; Saladin *et al*., 2002). In our research seven-fold increase of ESBL producing strains was observed: from 5.9 % in 1996 to 44.1% in 2000 year.

ESBLs are functionally differentiated. Some of them efficiently hydrolyse cefotaxime and ceftriaxon but not ceftazidime, while others clearly prefer ceftazidime and aztreonam. These differences may cause problems, particularly in interpretation of sensitivity to combinations of penicillins with inhibitors. ESBL are inhibited by β-lactamase inhibitors, but some β-lactamases hydrolyse penicillin to a considerable degree and so the inhibition effect may not suffice for the strain to be sensitive to a combination of penicillin with and inhibitor. Genes encoding for ESBL are often located on plasmids which in a short time may cause a spread of resistance genes among different bacteria through conjugation and exchange of plasmids (Neuwirth *et al.*, 2001; Pałucha *et al.*, 1999). The results concerning aminoglycosides resistance confirm that genes responsible for aminoglycoside resistance are often located on the same plasmids, where genes encoding ESBL reside.

Our research has shown constant increase ESBL producers among *P. mirabilis* strains, which indicates proliferation of plasmid-coded resistance mechanism in the given environment, a disconcerting tendency. The phenomenon of proliferation of plasmid located resistance genes does not seem to be related to other genetic traits of the tested strains. ESBL producers belonging to multiresistant genotypes A and B, not to more sensitive to antibiotics genotype C. No unique pattern among ESBL producing strains has not been found.

Appearance of *P. mirabilis* strains with the same restriction patterns in different wards of the same hospital as well as in different hospitals in the West Pomeranian area proves the spread of the strains in hospital environment. In addition to the strains classified as particular genotypes, in our research 71 unique strains characterized by a singular restriction pattern were found. It indicates a relatively great variety among *P. mirabilis* strains present in our environment. The unique strains usually showed high sensitivity to antibiotics. They were probably "patients own strains" that caused endogenous infection. Appearance and remaining of the same clones of strains in different wards, their transmission between wards and their appearance in other hospitals may certainly cause the problems. It is also an irrefutable proof for circulation of hospital strains in the environment and poses danger to potential patients. Hospital strains are characterized by much higher resistance to antibiotics than patients' indogenous strains and frequently require treatment with expensive antibiotics of wide spectrum of antibacterial activity. It results in the prolongation of the time of hospitalisation and in increasing the cost of treatment. Scarce information appearing in the literature concerning increasing number of clonal strains among *P. mirabilis* is probably due to the fact that until recently these bacteria have not been considered to be particularly dangerous in hospital environment, contrary to MRSA or *Klebsiella pneumoniae* strains which are commonly known to be alert-pathogens (Fiet *et al.*, 2000; Traub *et al.*, 1996). Our results indicate that also *P. mirabilis* may turn out be a dangerous pathogen in hospital environment causing dangerous clinical infections. This fact should make us aware that greater attention must be paid to the situation. Periodic surveillance tests for *P. mirabilis* presence should be regularly performed as it is done with other bacteria in a properly managed, modern hospital. It would certainly aid determining the frequency and type of hospital infections in the relation to a given ward's specific function and sanitary conditions.

Moreover, more intensive efforts of research teams working on hospital infections could contribute to a decrease in infection rates caused by Gram-negative rods of *Proteus* genus, especially hospitals isolates.

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