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Ribotyping of *Pseudomonas aeruginosa* Strains Isolated from Surgical Intensive Care Patients

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To elucidate the sources of *Pseudomonas aeruginosa* on a surgical intensive care unit, rDNA restriction fragment length polymorphism analysis (ribotyping) was applied to analyze strains isolated during a 4-month prospective study. Samples included 1635 from 153 patients, 2463 from 97 staff members, and 581 from the environment. Only 18 patients were colonized. Isolation from their animate and inanimate environment was very low, with 3 and 2 samples, respectively, being positive. Samples from tap water were negative. Ribotyping could easily distinguish 16 different digest patterns with identical follow-up isolates of the same patient. Horizontal transmission occurred only twice. The discriminatory power of ribosomal DNA in differentiating strains was dependent on the restriction enzymes used; among eight different enzymes, *Pvu*II was the most sensitive, producing 15 different patterns. Ribotyping showed high sensitivity in typing *P. aeruginosa* isolates and confirmed that colonization occurs from endogenous rather than from exogenous sources.

Pseudomonas aeruginosa is currently the fourth most frequently isolated nosocomial pathogen, accounting for 9.9% of all hospital-acquired infections, mainly surgical wound infections and gram-negative bacteremias [1]. Increasing length of hospital stay, age, gastrointestinal diseases, and prior use of antibiotics may lead to higher colonization rates with *P. aeruginosa*, especially in intensive care unit (ICU) patients [2], and colonization of patients undergoing mechanical ventilation or receiving chemotherapy for neoplastic diseases often predisposes to invasive infection. Previous typing methods such as bio-, sero-, and pyocin typing as well as newer DNA-based typing systems suggested sink drains, toilets, and showers as major sources for nosocomial infections in ICUs [3, 4]. However, although numerous investigations have been done, the routes of colonization or infection of this opportunistic pathogen remain largely a matter of speculation [2, 5].

We present an rDNA restriction fragment analysis of *P. aeruginosa* strains isolated during a 4-month prospective study on a surgical ICU of a German university hospital. Specimens were collected from both patients and personnel as well as from their inanimate environment. The incidence of colonization on admission and during hospitalization was determined, and transmission of bacteria was traced.

Materials and Methods

Study design. From 1 March to 28 June 1990, all 153 patients admitted to the surgical ICU of University Hospital, Freiburg, were screened for colonization with *P. aeruginosa*. Most were admitted after major trauma, with abdominal and skull injuries or complications of cardiovascular diseases and occasionally after organ transplantation. About 60% of the patients were directly transferred to the ICU without any previous stay on other units. Swabs from pharynx, scalp, tracheal secretions, and rectum were taken on admission and on every second day thereafter. Fifty-two percent of the patients stayed >2 days and were swabbed at least twice. Whenever found in other speci-

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mens than those mentioned, isolates of *P. aeruginosa* were included in the study.

The ICU has one four-bed room, two two-bed rooms, and one isolation room. Personnel (97: physicians, nurses, and secondary help) were screened every day for 1 week each month. Nasal swabs, two swabs from their gowns (abdominal region and outside of the pocket), and two RODAC plates from hands were taken each day at arbitrary hours (to prevent prior hand washing). Each patient was cared for by an individual nurse, but exceptions were possible when the ward was busy. Hand disinfection was usually carried out after handling a patient.

Swabs from the patients' environment were obtained at the same time. The patient monitor and the emergency button of the respirator were chosen for this purpose because of their frequent contact with hands of personnel. Additionally, water from every water tap was cultured once a week.

Culture methods. Tracheal secretions were cultured on Columbia agar with 5% sheep blood (Heipha, Heidelberg, Germany) and on cetrimide agar (Heipha), whereas nasal, scalp, and throat swabs were cultured on sheep blood agar alone. Rectal swabs were first subcultured on sheep blood agar plates and were then put into peptone water with cetrimide, which was subcultured on cetrimide agar plates the next day. All blood agars were incubated at 37°C, whereas media containing cetrimide were incubated at 42°C, both under aerobic conditions.

Environmental samples were inoculated on Columbia agar containing 0.5% Tween 80, 0.07% lecithin, and 7% sheep blood (Heipha) and were incubated at 37°C. Media were incubated for up to 2 days, and pigmented (pyocyanin or pyomelanin) colonies with cytochrome oxidase activity were transferred from blood to cetrimide agar. Strains were stored on trypticase soy agar at 20°C until typing was done. Water samples were aspirated through a cellulose filter membrane. One-half of this filter was placed on a blood agar plate and the other half on agar with cetrimide.

Ribosomal RNA gene (rDNA) restriction patterns. Isolates of *P. aeruginosa* were regrown on 5% sheep blood agar, and one colony each was suspended in 5 mL of Luria Bertani broth at 37°C. After incubation overnight, 1.5 mL was used for isolation of total DNA by a mini-prep procedure [6]. DNA samples were digested with eight restriction endonucleases, *Pst*I, *Sma*I, *Eco*RI, *Bcl*I, *Hin*dIII, *Cla*I, *Sph*I, and *Pvu*II (Boehringer, Mannheim, Germany), as recommended by the manufacturer. Fragments were then separated by electrophoresis through a 0.8% agarose gel in TRIS-borate-EDTA buffer (TBE; 0.089 *M* TRIS, 0.089 *M* boric acid, and 0.2 m*M* EDTA, pH 8.0), stained in ethidium bromide (1 g/mL), and photographed. Thereafter they were transferred to a nylon membrane (BiodyneA; Pall Biosupport, East Hills, NY) using a vacuum transfer apparatus (Pharmacia Biotechnology, Piscataway, NJ) at 50 mbar for 1 h with 20× SSC (3 *M* NaCl, 0.3 *M* Na citrate, pH 7.0) as transfer solution. Prehybridization and hybridization were carried out at 42°C in 5× Denhardt's solution, 5× SSC, 50 m*M* sodium phosphate buffer, pH 6.5, 250 g/mL denatured salmon sperm DNA, and 5% dextran sulfate. After prehybridization for 1 h, the heat-denatured DNA probe (a pBR322-derived biotin-labeled plasmid pKK3535 containing a ribosomal RNA operon of *Escherichia coli*) [7, 8] was added to fresh solution, and the filters were incubated overnight at 42°C. The filters were washed and hybrids visualized by a chromogenic reaction using the BluGene kit (Bethesda Research Laboratories, Gaithersburg, MD).

Results

During the 4-month period, 4679 samples were collected and cultured, 1635 from patients, 2463 from personnel, and 581 from environmental sources. Only 18 (11.5%) of 153 patients were colonized at single or multiple body sites with *P. aeruginosa* (table 1), 8 of them (44%) being positive from their day of admission. Of this group, 4 patients had been admitted directly, whereas the other 4 were transferred from other hospitals or wards. Rectal swabs yielded the organism in a significantly higher proportion than nasal swabs or tracheal secretions, both in patients colonized from the beginning of their stay on the ICU (75% vs. 63% and 37%) and in patients whose cultures were found positive only later on (70% vs. 30% and 30%).

Among the samples collected from personnel, *P. aeruginosa* was isolated three times from hands of personnel caring for different patients. Twice the organism was found on the emergency button from respirators, whereas tap water samples were always negative.

Fifty-one isolates from 18 patients and 3 isolates from hands of different nurses were investigated using restriction endonuclease analysis of rDNA. The 2 isolates from respirators could not be included because they had not survived several subculturing passages (table 1).

Discrimination between independent strains varied with the restriction enzymes used. It was lowest for *Bcl*II and very high for *Pvu*II (table 1). Two endonucleases, *Pst*I and *Sma*I, were abandoned because of their low discriminatory power. *Hin*dIII had to be excluded from the study because of incomplete digestion of the DNA and arbitrary restriction patterns that were interpretable with uncertainty only. The remaining five enzymes could easily distinguish 16 different groups of ribotypes, and 15 were separated by the use of restriction endonuclease *Pvu*II alone (figure 1). The reproducibility of ribotypes was 100%.

Each patient appeared to be colonized by 1 single strain; 14 (78%) of 18 patients harbored *P. aeruginosa* types that had not been isolated from any other animate or inanimate source except for 2 cases (table 1), and only twice did 2 patients have the same ribotype. All environmental samples and samples from personnel remained single findings.

Discussion

Epidemiologic associations of *P. aeruginosa* strains have usually been based on a combination of serotyping as a primary screen and pyocin typing for additional discrimination [9]. However, the frequency of individual O types of *P. aeruginosa* in clinical specimens has varied with the country of investigation and the source of the isolate. In addition, ~8%

Table 1. Sources and rDNA patterns of isolates from an intensive care unit.

Patient no.	Isolation site of *P. aeruginosa* from patient	Isolation site of *P. aeruginosa* from environment and personnel	rDNA pattern with restriction enzyme					Ribotype
			*Eco*RI	*Bcl*II	*Cla*I	*Sph*I	*Pvu*II	
1	Rectum (1), tracheal secretion (1)	Emergency button of respirator (1)*	a	a	a	a	a	A
2	Nose (1)	Hand of personnel (1)	a	a	a	a	a	A
3	Rectum (2), decubitus (2)	None	b	a	c	c	m	B
4	Pharynx (1)	None	b	a	c	c	m	B
5	Tracheal secretion (5)	None	d	a	c	a	e	C
6	Rectum (1), drain (2)	None	a	a	c	b	b	D
7	Rectum (2), nose (5), drain (4)	Hand of personnel (1)†, emergency button of respirator (1)*,†	c	c	c	b	f	E
8	Rectum (3)	None	c	a	c	a	k	F
9	Tracheal secretion (3), nose (1), pharynx (1)	None	e	a	a	c	g	G
10	Rectum (1)	Hand of personnel (1)	a	a	c	e	h	H
11	Rectum (1)	None	a	a	c	a	i	I
12	Nose (1)	None	c	b	d	d	p	K
13	Rectum (1), nose (1)	None	a	a	c	a	c	L
14	Tracheal secretion (1)	None	a	a	b	a	b	M
15	Rectum (1)	None	c	a	a	c	l	N
16	Rectum (2)	None	a	b	c	d	n	O
17	Rectum (1), nose (1)	None	a	a	c	f	o	P
18	Rectum (2), tracheal secretion (1), nose (2), scalp (1)	None	a	a	a	b	d	Q

NOTE. No. of isolates is in parentheses.
* Not ribotyped.
† Positive 1 month before patient's samples.

of clinical isolates react strongly with a pooled serum but fail to be agglutinated by individual ones [9]. Pyocin as well as phage typeability rates are high but of low reproducibility and discriminatory power, factors that affect the reliability of most typing methods in epidemiologic investigations of *P. aeruginosa* [10]. Biotyping is rarely helpful, and antibiograms of strains can give a useful guide to the identification of a problem strain, but specificity is low and unpredictable changes in sensitivity patterns, especially during outbreaks, may occur [9].

Novel approaches to the typing of *P. aeruginosa*, such as analysis of multilocus isoenzyme patterns [11] or molecular typing methods, have recently been developed. Genomic fingerprinting by field inversion gel electrophoresis [12] and restriction fragment length polymorphism analysis in the exotoxin A [4, 13] or pilin [14] region has been shown to significantly enhance discrimination compared with that achieved by former methods [15]. However, different serotypes, biotypes, and antibiograms may be genetically indistinguishable [10, 13]. Ribosomal DNA polymorphisms have been used successfully to distinguish between individual strains in a variety of bacteria, and recently *P. aeruginosa* strains from cystic fibrosis patients were analyzed using this method [11].

The present study used the plasmid pKK3535 probe, which encodes a ribosomal RNA operon consisting of the genes for 5S RNA, 16S RNA, 23S RNA, and $tRNA_2^{glu}$ [7]. The sensitivity of the method is highly dependent on the restriction enzymes used to digest chromosomal DNA [8]. The best discrimination among our strains was provided by restriction endonuclease *Pvu*II, which separated 15 of 18 strains (figure 1, table 1). The relatively low sensitivity of rDNA restriction patterns cited by other authors [11] is most probably a result of insufficient searching for appropriate endonucleases.

The fact that 16 ribotypes were obtained from 18 unrelated patients (table 1) suggests that except for 2 cases, no cross-infections took place. We presume that gastrointestinal carriage of *P. aeruginosa* with numbers below the threshold of detection was present before hospital admission, as suggested also by others [2]. Antibiotic pressure and total digestive decontamination, which alter the barrier effect of the normal intestinal flora [12], were therefore probably the reason for endogenous acquisition of *P. aeruginosa*.

Only twice we assumed that a strain was passed from one patient to another by an undetected carrier. These nondistinguishable strains were harbored by patients who followed each other in the same room (patients 1 and 2) or who were cared for at the same time on the ward (patients 3 and 4). Since standards of hygiene are more likely to fail when the unit is busy, cross-infection may have taken place. Environmental contamination could be proven in the first case, as *P. aeruginosa* was isolated from the nurse's hand as well as from

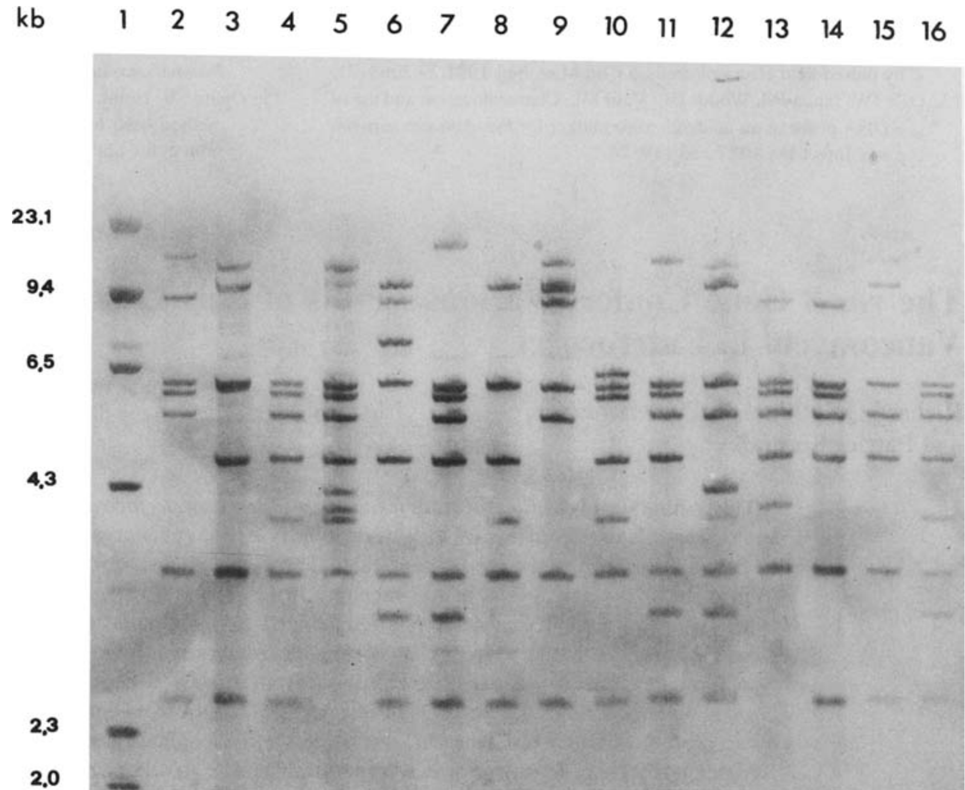

Figure 1. rDNA patterns of *P. aeruginosa* strains (digestion with *Pvu*II). Lane 1: Molecular weight marker, λ DNA digested with *Hin*dIII; lanes 2–16: 15 different ribotypes after digestion with *Pvu*II.

the respirator (table 1). Longitudinal sampling of *P. aeruginosa* from patients, personnel, and the environment of hospitals in other studies has revealed that up to 42% of the personnel may have positive hand cultures and that in some cases *P. aeruginosa* genotypes were isolated from sinks before they were isolated from hands of personnel or patients' specimens [4]. As the staff is generally considered responsible for transmission of bacteria within the ICU, the number of positive specimens (3) from personnel as well as from other environmental samples during this study was surprisingly low. Equally, the organism could not be isolated from water sources at all, although water reservoirs have been shown to harbor most nosocomial strains [2, 4].

Many studies have implicated horizontal transmission as an important problem. However, the detection of a wide spectrum of different ribotypes, the fact that 44% of the strains were already present on admission, and their low presence in the environment suggest that endogeneous colonization rather than nosocomial acquisition had occurred.

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The *vanB* Gene Confers Various Levels of Self-Transferable Resistance to Vancomycin in Enterococci

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Thirty-nine strains of *Enterococcus faecium* and *Enterococcus faecalis* resistant to vancomycin and susceptible to teicoplanin on disk susceptibility testing (phenotypic class B) were isolated in 15 hospitals in Europe and the United States. The MICs of vancomycin for these strains ranged from 4 to 1024 μg/mL. Part of the vancomycin resistance gene *vanB* from *E. faecalis* V583 hybridized with a single but variably sized *Hin*dIII-*Kpn*I fragment of total DNA from all 39 strains. This indicates that a single class of resistance determinants accounts for the VanB phenotype. No hybridization was detected with DNA from intrinsically resistant *Enterococcus gallinarum* or *Enterococcus casseliflavus*. Hybridization with DNA from enterococcal strains susceptible to or with acquired resistance to vancomycin and teicoplanin was not observed. The genes conferring resistance to vancomycin were self-transferable to other *Enterococcus* strains in 14 of the 39 strains. It thus appears that *vanB* confers various levels of conjugative vancomycin resistance in enterococci.

Glycopeptide antibiotics (vancomycin and teicoplanin) bind to the peptidyl-D-alanyl-D-alanine termini of peptidoglycan precursors and block their incorporation into the bacterial cell wall [1]. Resistance to glycopeptides in clinical isolates of enterococci can be classified by the level of resistance to vancomycin and susceptibility or resistance to teicoplanin [2]. Three classes of resistance (A, B, and C) have been distinguished.

The class A phenotype comprises strains of *Enterococcus faecium* and *Enterococcus faecalis* with high-level resistance to vancomycin (MIC ≥64 μg/mL) and teicoplanin (MIC ≥16 μg/mL). Resistance is inducible by vancomycin or teicoplanin and is often mediated by self-transferable plasmids [2]. High-level glycopeptide resistance in these strains is associated with the acquisition of genes related to *vanA* [2]. This gene encodes a D-alanine–D-alanine ligase of modified specificity that synthesizes peptidoglycan precursors with reduced affinity for glycopeptide antibiotics [3].

Class B consists of strains of *E. faecalis* and *E. faecium* inducibly resistant to low levels of vancomycin (MIC 8–64 μg/mL). These strains remain susceptible to teicoplanin (MIC ≤1 μg/mL), although vancomycin induces resistance to this antibiotic. Resistance of this class is thought not to be transferable, with the corresponding determinant tentatively assigned a chromosomal location [2].

Class C includes the majority of *Enterococcus gallinarum* and *Enterococcus casseliflavus* isolates constitutively resistant to low levels of vancomycin (MICs ≥8 and ≤32 μg/mL) and susceptible to teicoplanin (MIC ≤1 μg/mL). Resistance in these species appears intrinsic, as all the isolates examined to date are resistant [4]. Resistance is not transferable by conjugation and is thought to be chromosomal [2]. Vancomycin resistance in *E. gallinarum* is associated with the presence of nucleotide sequences related to *vanC* [4], a gene that shares similarity with *vanA* [5].

Recently, another class of glycopeptide resistance in enterococci isolated in the United States has been suggested [6]. Strains of *E. faecium* and *E. faecalis* with this phenotype are highly resistant to vancomycin (MIC ≥128 μg/mL) but remain susceptible to teicoplanin. The resistance is inducible by vancomycin but not by teicoplanin and, in certain isolates, is transferable by conjugation [7].

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