

Comparative Evaluation of an Automated Repetitive-Sequence-Based PCR Instrument versus Pulsed-Field Gel Electrophoresis in the Setting of a *Serratia marcescens* Nosocomial Infection Outbreak[∇]

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A semiautomated, repetitive-sequence-based PCR (rep-PCR) instrument (DiversiLab system) was evaluated in comparison with pulsed-field gel electrophoresis (PFGE) to investigate an outbreak of *Serratia marcescens* infections in a neonatal intensive care unit (NICU). A selection of 36 epidemiologically related and 8 epidemiologically unrelated isolates was analyzed. Among the epidemiologically related isolates, PFGE identified five genetically unrelated patterns. Thirty-two isolates from patients and wet nurses showed the same PFGE profile (pattern A). Genetically unrelated PFGE patterns were found in one patient (pattern B), in two wet nurses (patterns C and D), and in an environmental isolate from the NICU (pattern G). Rep-PCR identified seven different patterns, three of which included the 32 isolates of PFGE type A. One or two band differences in isolates of these three types allowed isolates to be categorized as similar and included in a unique cluster. Isolates of different PFGE types were also of unrelated rep-PCR types. All of the epidemiologically unrelated isolates were of different PFGE and rep-PCR types. The level of discrimination exhibited by rep-PCR with the DiversiLab system allowed us to conclude that this method was able to identify genetic similarity in a spatio-temporal cluster of *S. marcescens* isolates.

Concerns regarding the emergence of hospital-acquired infections, increasing antimicrobial resistance, and the increase in morbidity, mortality, and costs associated with these infections drive the need for refinement of molecular approaches to aid in the diagnosis and epidemiological analysis of nosocomial infections.

Several methods based on DNA analysis are now available that provide information about the genetic relatedness of isolates of the same species (26). These DNA-based molecular methodologies include pulsed-field gel electrophoresis (PFGE), PCR-based typing methods, and multilocus sequence analysis. Establishing clonality of pathogens can aid in the identification of the source of organisms (environmental or personnel), in distinguishing infectious from non infectious strains, and in distinguishing relapse from reinfection.

PFGE is generally considered one of the most reproducible and highly discriminatory typing techniques available and is considered the “gold standard” molecular approach for the epidemiological analysis of most bacterial pathogens (10, 28). However, PFGE requires specialized equipment and is technically demanding, labor intensive, and relatively slow, as it may take 2 to 5 days to obtain results, depending on the organism and the methods utilized.

Automation and computer-assisted pattern analysis afford several benefits for the molecular epidemiology laboratory: reduction of labor costs, standardization, decreased time to completion, and better comparison between runs. The latter is

of special importance when comparing large groups of organisms or comparing organisms submitted to the laboratory over a certain time period. Recently, two semiautomated systems for strain typing have become available on the market: (i) the Ribo-Printer Microbial Characterization System (DuPont Qualicon), which performs ribotyping of strains (8, 9, 11, 14–16, 22), and (ii) the rep-PCR DiversiLab Microbial Typing System (bioMérieux, Italy), which amplifies the regions between the noncoding repetitive sequences in bacterial genomes (12).

The goals of this study were to assess the DiversiLab rep-PCR system as a rapid method for *S. marcescens* strain identification and differentiation in comparison with PFGE results for strains from an epidemiologically well-characterized outbreak that occurred in the neonatal intensive care unit (NICU) of the Verona University Hospital and to contribute to the discussion of the use of molecular techniques to identify and differentiate bacterial isolates.

MATERIALS AND METHODS

Epidemiologic investigation. An outbreak of *S. marcescens* infection occurred in the NICU from August 2007 to December 2007. Microbiology records were reviewed to identify cases of infection and to determine the baseline rate of *S. marcescens* infection in the NICU prior to the outbreak. Case patients were defined as any neonate hospitalized in the NICU from August 2007 to December 2007 who had one or more clinical or surveillance cultures that yielded *S. marcescens*. Cultures were performed with samples from the NICU environment, including counters, sinks, bottle warmers, personnel work areas, breast milk, equipment in the breast milk room, infant formula, inhalators, medications, saline ampoules, incubators, eye examination equipment, soaps, lotions, ventilators, oxygenation and humidification devices, and other respiratory care equipment. Each member of the NICU staff was examined for the presence of artificial fingernails, and hand cultures were obtained.

Microbiological method. Clinical and environmental cultures were processed using standard practices and routine media. The bacteria were iden-

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tified by means of an automated Vitek-2 system (bioMérieux, Italy), and antibiotic susceptibility was checked routinely by means of the disc diffusion method, according to the Clinical and Laboratory Standards Institute recommendations (4). The antimicrobial agents included were ampicillin, amoxicillin-clavulanic acid, aztreonam, amikacin, ceftriaxone, ceftazidime, cefuroxime, cefotetan, gentamicin, imipenem, piperacillin, piperacillin-tazobactam, cefepime, and meropenem.

Automated rep-PCR DNA fingerprinting. DNA was extracted from a 10- μ l loopful of a *Serratia marcescens* colony using an UltraClean Microbial DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA) and following the manufacturer's instructions. The extracted DNA was amplified using a DiversiLab *Serratia* DNA fingerprinting kit (bioMérieux, Italy), which includes rep-PCR master mix 1, *Serratia* primers, and kit-specific positive and negative controls, in accordance with the manufacturer's product insert. Briefly, 50 ng of genomic DNA, 2.5 U of AmpliTaq DNA polymerase, and 1.5 μ l of 10 \times PCR buffer (Applied Biosystems, Foster City, CA) were added to the rep-PCR master mix to achieve a total of 25 μ l. Thermal cycling parameters for an MJ PTC100 PCR System (Bio-Rad, MI) were as follows: initial denaturation at 94°C for 2 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, extension at 70°C for 90 s, and a final extension at 70°C for 3 min. The amplified product was stored at -20°C until detection. Analysis of rep-PCR products was implemented using a DiversiLab system in which the amplified fragments of various sizes and intensities are separated and detected using a microfluidics LabChip with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The isolates' relatedness was analyzed by the DiversiLab software, version 3.4, which uses the Kullback-Leibler distance correlation coefficient to determine distance matrices and the unweighted-pair group method using average linkages to create dendrograms, electropherograms, virtual gel images, and scatterplots. Interpretative criteria provided by the manufacturer categorized isolates as indistinguishable, similar, or different. In general, "different" was defined as <95% similarity and two or more band differences for organisms defined as homogeneous (i.e., those that include a low number of clones in nature) or three band differences for organisms defined as heterogeneous (i.e., those that include an unlimited number of different clones in nature). "Similar" was defined as >95% similarity and one band difference for homogeneous organisms or up to two band differences for heterogeneous organisms. "Indistinguishable" was defined as >97% similarity and no banding differences, including no variation in the intensities of individual bands, although overall intensities may differ. *S. marcescens* is recognized as a heterogeneous species that probably contains an unlimited number of different clones (3).

PFGE fingerprinting. Genomic DNA was prepared as described previously (24) but with some modifications. Isolated colonies were harvested from Mueller-Hinton agar plates after overnight incubation at 37°C, and the suspension was adjusted to a concentration of 10⁹ CFU/ml in saline-EDTA buffer (75 mM NaCl, 25 mM EDTA [pH 7.5]). The bacterial suspension was then mixed with an equal volume of 2% low-melting-point agarose (Bio-Rad Laboratories) and allowed to solidify in a 100- μ l plug mold (Bio-Rad Laboratories). The DNA block was incubated for 2 h at 37°C in 1 ml of lysis buffer (10 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl, 0.5% Brij 58, 0.2% sodium deoxycholate, 0.5% sodium lauroyl sarcosine, lysozyme [0.5 mg/ml]). Following this step, the lysis buffer was replaced with 1 ml of proteolysis buffer (1% sodium lauroyl sarcosine, 0.5 M EDTA [pH 9.5], proteinase K [500 μ g/ml; Sigma]) and this solution was incubated at 52°C overnight. To eliminate the lysed bacterial material and inactivate proteinase K activity, the DNA blocks were washed four times at room temperature in 10 ml of Tris-EDTA buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA). A slice of each plug was cut and incubated with SpeI (Roche, Italy). Restriction fragments of DNA were separated by PFGE with a CHEF-DRII apparatus (Bio-Rad Laboratories) through 1.2% Pulsed Field Certified Agarose (Bio-Rad Laboratories). Electrophoresis was performed at 6 V/cm and 14°C. The run time was 22 h with the pulse time ramping from 5 to 60 s. Tenover's criteria were used to interpret chromosomal DNA restriction patterns and for strain typing (28).

RESULTS

A total of 16 patients admitted to the NICU were identified as infected and/or colonized with *S. marcescens* from August 2007 to December 2007. The baseline rate of *S. marcescens* infections/colonizations for the 24 months prior to the outbreak was 0.20 case/month, compared with 4 cases/month during the outbreak. Six patients developed infections; one expe-

rienced bacteremia, one conjunctivitis, one conjunctivitis and urinary infection, two urinary infection, and one umbilical wound infection. In 12 colonized patients, *S. marcescens* was detected in pharyngeal, nasal, and perianal swab samples for surveillance culture. Seven patient cultures from more than one body site grew *S. marcescens*. In addition, *S. marcescens* was isolated from the milk of five wet nurses and in three cases the isolates had a PFGE pattern identical to that of the outbreak isolates (Table 1).

Examination of the hands of NICU personnel revealed no lesions or artificial fingernails. Hand cultures obtained from NICU personnel did not grow *S. marcescens*. One environmental culture sample from a sink drain in the filter room yielded a strain of *S. marcescens*. Education about *Serratia* and standard infection control procedures was reviewed with NICU personnel. Cleaning and disinfection procedures were reviewed and reinforced with infection control personnel. Contact isolation precautions were implemented for all neonates with *S. marcescens* infection. NICU personnel were instructed to gather the infected neonates into one area of the unit and to dedicate staff to care only for these patients. All infected and colonized patients were assembled into a cohort. During the outbreak, admission to the NICU was drastically reduced. The first strain stored and analyzed was dated 21 September, and thus, the molecular study related to strains isolated after the restriction of outside admissions. Weekly surveillance cultures of stool and pharyngeal and nasal swabs were taken to detect whether there was any asymptomatic colonization of neonates with *S. marcescens*. These infection control measures successfully halted the outbreak.

The isolates from the NICU presented the same antimicrobial susceptibility profile: they were susceptible to aztreonam, amikacin, ceftriaxone, ceftazidime, cefotetan, gentamicin, imipenem, piperacillin, piperacillin-tazobactam, cefepime, and meropenem and resistant to ampicillin, amoxicillin-clavulanic acid, and cefuroxime.

Thirty-six epidemiologically related and eight unrelated isolates were tested by both the PFGE and automated rep-PCR typing methods. Table 1 lists the epidemiological data and the PFGE and rep-PCR patterns of each isolate. By visual inspection of the PFGE profiles, 1 major pattern (type A) and 12 unique genetically unrelated patterns (types B through O) were identified. Isolates from 15 patients and three wet nurses were genetically identical and belonged to type A. Unique unrelated PFGE patterns were identified in one patient (intestinal carriage), in two wet nurses, in an isolate collected from the NICU environment, and in the eight epidemiologically unrelated isolates (adult patients admitted to other wards at a different time of year). A selection of these PFGE types is shown in Fig. 1. The rep-PCR analysis identified three different patterns (types a, b, and c) which included all of the type A PFGE isolates and 12 unique profiles (types d through q) corresponding to unique PFGE types B through O (Table 1 and Fig. 2A). Each rep-PCR pattern clustered together isolates with 95% or more similarity. Since the similarity percentages generated by rep-PCR suggested possible genetic diversity among the isolates belonging to PFGE cluster A, a visual inspection of the sample fingerprints was performed (Fig. 2B). The fingerprints of patterns a and c were similar, whereas pattern b showed differences from patterns c and a in more

TABLE 1. Description of *S. marcescens* isolates from NICU and genomic typing results

Key sample ID ^a	Isolate source	Date of isolation (mo/day/yr)	PFGE type	rep-PCR type
Patient isolates				
1	Blood	09/21/2007	A	a
2	Eye discharge	09/24/2007	A	a
3	Pharyngeal swab	09/27/2007	A	a
4	Pharyngeal swab	09/27/2007	A	a
5	Pharyngeal swab	09/27/2007	A	a
6	Pharyngeal swab	09/27/2007	A	a
7	Pharyngeal swab	09/28/2007	A	a
8	Nasal swab	09/28/2007	A	a
9	Nasal swab	10/01/2007	A	c
36	Stool	10/04/2007	A	a
11	Pharyngeal swab	10/05/2007	A	c
12	Stool	10/06/2007	A	c
13	Stool	10/09/2007	A	a
14	Stool	10/10/2007	A	a
15	Eye discharge	10/11/2007	A	c
16	Pharyngeal swab	10/11/2007	A	a
17	Stool	10/11/2007	A	b
18	Urine	10/11/2007	A	b
20	Stool	11/06/2007	A	b
21	Stool	11/07/2007	B	l
22	Pharyngeal swab	11/14/2007	A	b
23	Pharyngeal swab	11/15/2007	A	b
24	Pharyngeal swab	11/21/2007	A	a
25	Pharyngeal swab	11/23/2007	A	b
26	Pharyngeal swab	11/23/2007	A	b
27	Urine	11/23/2007	A	c
28	Urine	11/23/2007	A	c
29	Umbilical wound	12/04/2007	A	b
Wet nurse isolates				
10A	Milk	09/04/2007	A	a
10B	Milk	09/25/2007	A	a
10C	Milk	10/04/2007	A	a
19	Milk	10/24/2007	A	b
32	Milk	10/15/2007	C	i
33	Milk	11/08/2007	D	e
34	Milk	11/12/2007	A	a
NICU environmental isolate	Tap	10/2/2007	G	g
Epidemiologically unrelated isolates				
30	Blood	10/07/2007	E	h
37	Cutaneous swab	03/14/2008	F	f
747c	Bronchial aspirate	12/30/1999	O	d
825c	Pus	01/13/2000	H	m
1836c	Bronchial aspirate	01/21/2002	I	n
1959c	Blood	04/06/2002	L	o
2275c	Surgical swab	11/30/2002	M	p
823c	Nasal swab	01/13/2000	N	q

^a Key sample ID reported in Fig. 2.

than two bands. According to the criteria suggested by the manufacturers and the visual inspection only, isolates showing c and a profiles could be classified as similar (fingerprints that show differences in one or two bands), whereas isolates with pattern b could be classified as different but highly related (more than 80% similarity). The reason why indistinguishable or similar banding profiles gave rise to the low similarity index was investigated by comparing the electropherograms directly using the pattern overlay function of the DiversiLab software. Small difference in the fluorescence intensities of individual peaks were revealed by the analysis, since the software

weighted more intense peaks more heavily. These results indicated that types a, b, and c should be classified as highly related and confirmed the monoclonal origin of the outbreak. In comparison with PFGE, rep-PCR exhibited a higher discrimination power and evidenced the emergence of subclones within a cluster of highly related isolates.

The reproducibility of rep-PCR patterns was assessed on three different genomic extracts of three isolates and on 10 runs of the same genomic extract. The results showed virtually indistinguishable patterns within each of the three isolates and within the different runs (data not shown). A high level of

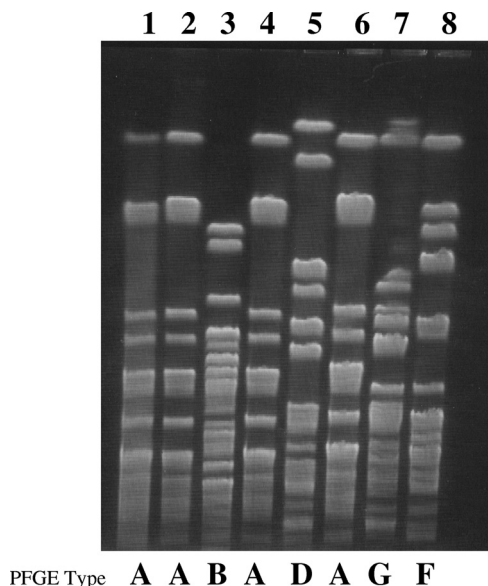


FIG. 1. Representative PFGE patterns of *S. marcescens* isolates recovered during the NICU outbreak. The same strain (PFGE type A, lanes 1, 2, 4, and 6) was recovered in 32 isolates from patients and wet nurses. Genetically unrelated PFGE patterns B, D, G, and F are also shown (lanes 3, 5, 7, and 8). Lane 1, sample ID 1; lane 2, sample ID 17; lane 3, sample ID 21; lane 4, sample ID 27; lane 5, sample ID 33; lane 6, sample ID 10A; lane 7, sample ID 35; lane 8, sample ID 37.

reproducibility of rep-PCR patterns generated by DiversiLab was also reported by Healy et al. (12).

DISCUSSION

S. marcescens is a well-recognized, hospital-acquired pathogen, and outbreaks in neonatal units have been previously described (6, 9, 17, 19, 30). Usually, one clone is associated with an outbreak and in the study by Jang et al., for instance, it was easy to determine an epidemiological link (17). However, it has also been reported that *S. marcescens* outbreaks may be polyclonal and this may be due to a mixture of new external clones, as well as evolutionary changes in a common nosocomial strain (3, 5).

The manual rep-PCR technique has been shown to be useful for typing prokaryotic and eukaryotic organisms, including those of epidemiological significance (1, 2, 3, 15, 27, 31) and the semiautomated rep-PCR system that has been introduced recently (12) was employed for typing *Staphylococcus aureus*, enterococci, *Neisseria meningitidis*, *Acinetobacter*, *Aspergillus*, and *Candida albicans* (2, 7, 13, 18, 21, 23, 25, 29). Differences in discriminatory power between PFGE and semiautomated rep-PCR are dependent on the bacterial species being studied. Rep-PCR was found to be less discriminatory than PFGE for *S. aureus* and enterococci (21, 23, 25, 29) and concordant for *Acinetobacter* and *Escherichia coli* (2, 7, 20).

Our study is the first to assess the reliability with which the DiversiLab rep-PCR system, in comparison with PFGE, identified the genetic similarity of *S. marcescens* isolates during a well-characterized outbreak which occurred in a NICU. We clearly documented the spread of infections due to *S. marcescens* in the NICU by tracing *S. marcescens* isolation in colo-

nized and infected patients, as well as in wet nurses, and by matching clinical and molecular typing data. The PFGE analysis clearly suggested that the outbreak was monoclonal since 32 of the 38 isolates collected during the outbreak showed identical fingerprints. The rep-PCR results apparently suggested a higher level of genetic variability, and both the dendrogram and the similarity matrix generated by the DiversiLab software (Fig. 2) indicated that not all of the samples in the cluster of PFGE type A isolates showed the same level of similarity. In particular, the rep-PCR type b isolates showed a lower degree of similarity than did those of types a and c. Visual inspection of fingerprints and analysis of normalized sample graphs resolved this discrepancy and suggested that type b isolates could be classified as similar to type a and c isolates. It is possible that in *S. marcescens* the marker used for the rep-PCR analysis (the region between the noncoding repetitive sequences in bacterial genomes) is genetically less stable than the one used for PFGE (the target sequence of the SpeI restriction enzyme). As a consequence, the variability shown by rep-PCR likely represented evolutionary changes of the same clone that could not be detected by PFGE. Interestingly, rep-PCR allowed us to track the molecular evolution of the epidemic clone. Type a was isolated in September, at the beginning of the outbreak, and gave rise to type c in October. Type b emerged in November and circulated in the NICU together with types a and c until the end of the outbreak in December. The origin of the outbreak could be attributed to the use of breast milk samples collected from wet nurses and contaminated with *S. marcescens*. The babies of two of these wet nurses were colonized by isolates sharing the same type a isolates from the milk, but it was not possible to document the supply of milk from these wet nurses to other infected babies since the breast milk was pooled.

Automated systems for microorganism typing constitute a compelling tool for the identification and management of nosocomial infections. They assess outbreaks in real time, provide comprehensive surveillance or epidemiological data, and possess data-archiving capability, all of which are required to build libraries and share data among laboratories.

The main limitation of automated systems is the lack of validated interpretative criteria. Without criteria for bacterial strain typing interpretation, no method can be used alone for studies of ongoing outbreaks. Typing results make it possible to categorize isolates as (i) identical, (ii) highly related but not identical, (iii) moderately related, and (iv) unrelated. Since 1995, criteria for bacterial strain typing interpretation have been available but validated only for interpreting chromosomal DNA restriction patterns produced by PFGE (28). In this case, validation was done by investigators who correlated epidemiological data from several outbreaks with strain typing results produced by PFGE. If a system provides results that are too discriminatory, the potential association between the isolates is completely missed and the mistaken conclusion that multiclonal outbreaks have occurred with multiple sources of infecting isolates is generated. This conclusion could be correct if the diversity reflects poor genetic relatedness and not the rapid genetic evolution of the same clone that could occur in a very short time period. Conversely, a method with low discriminatory power could generate the wrong conclusion that outbreaks are monoclonal and thus that only one source is involved.

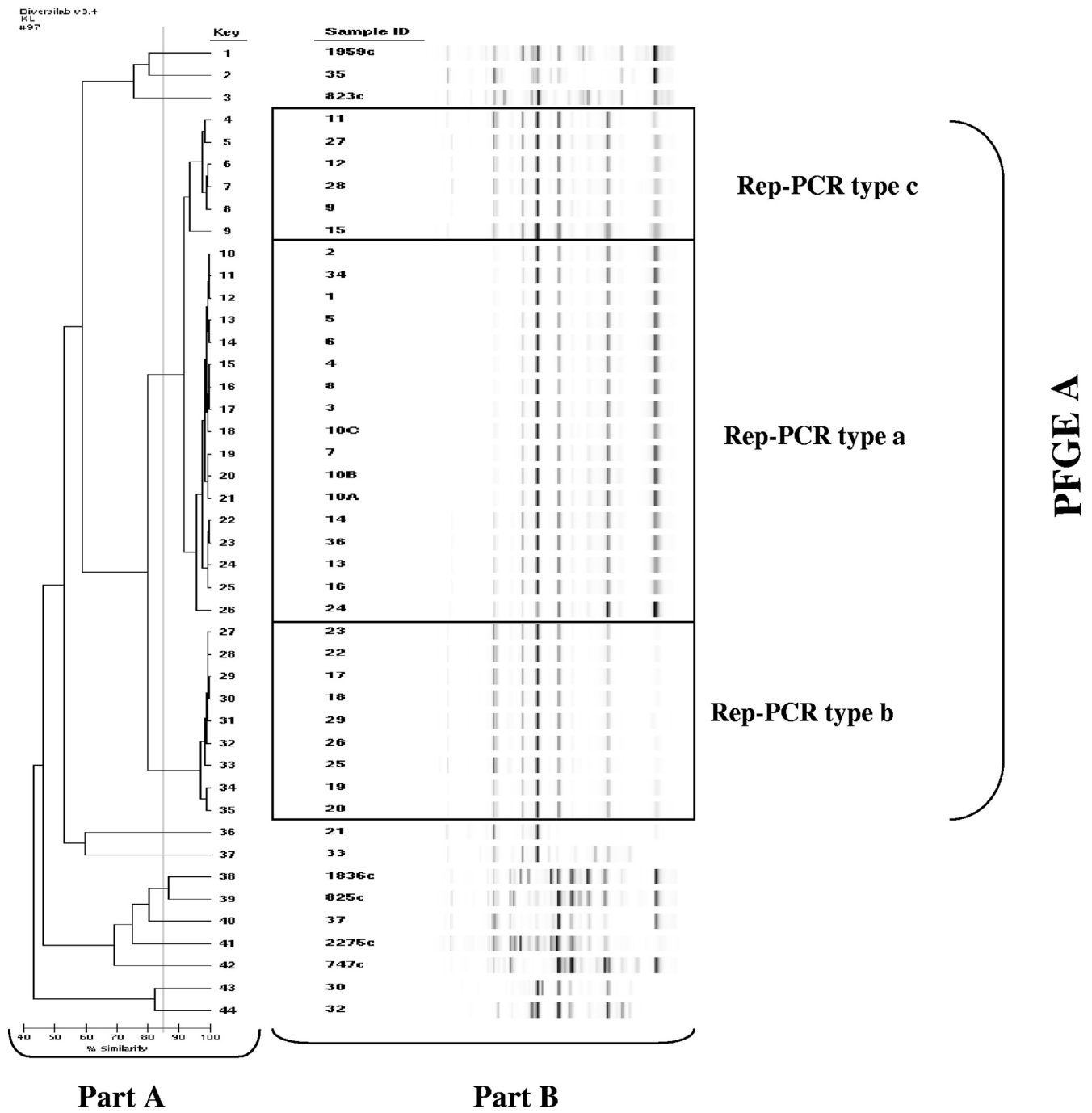


FIG. 2. A dendrogram (A) and virtual gel images (B) representing the rep-PCR fingerprint patterns of the *S. marcescens* isolates described in Table 1, generated by the DiversiLab software, version 3.4. Clonal isolates are boxed.

Close cooperation between clinicians and microbiologists is important for resolving an epidemic event. Microbiologists play a primary role in the study of isolates circulating in a hospital. At the same time, microbiologists must also be able to rapidly confirm or exclude the possibility of an epidemic cluster by fingerprinting of isolates. In conclusion, our data suggest that the automated rep-PCR system is a reliable tool for epidemiological investigation. The DiversiLab fingerprinting rep-PCR provides an option to clinical laboratories to perform

molecular typing and will benefit infection control procedures with the rapid introduction of control measures and significantly improve patient care; however, the study of more outbreak events is required to validate it as a method to be used alone in ongoing outbreaks.

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