

Polyclonal endemicity of *Pseudomonas aeruginosa* in a teaching hospital from Brazil: molecular typing of decade-old strains

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Abstract: *Pseudomonas aeruginosa* infections cause significant mortality and morbidity in health care settings. Strategies to prevent and control the emergence and spread of *P. aeruginosa* within hospitals involve implementation of barrier methods and antimicrobial stewardship programs. However, there is still much debate over which of these measures holds the utmost importance. Molecular strain typing may help elucidate this issue. In our study, 71 nosocomial isolates from 41 patients and 23 community-acquired isolates from 21 patients were genotyped. Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) was performed. Band patterns were compared using similarity coefficients of Dice, Jaccard and simple matching. Strain similarity for nosocomial strains varied from 0.14 to 1.00 (Dice); 0.08 to 1.00 (Jaccard) and 0.58 to 1.00 (simple matching). Forty patterns were identified. In most units, several clones coexisted. However, there was evidence of clonal dissemination in the high risk nursery, neurology and two surgical units. Each and every community-acquired strain produced a unique distinct pattern. Results suggest that cross transmission of *P. aeruginosa* was an uncommon event in our hospital. This points out to a minor role for barrier methods in the control of *P. aeruginosa* spread.

Key words: *Pseudomonas aeruginosa*, strain typing, ERIC-PCR, nosocomial infections, health care infections.

INTRODUCTION

Pseudomonas aeruginosa is a relevant pathogen in hospitalized patients, accounting for significant morbidity and mortality (1). In a recent report, it ranked second among agents of ventilator-associated pneumonia in North American hospitals. It was also among the seven most common agents of health care-acquired urinary, blood system and surgical site infections (2). This picture is worsened by the continuous, worldwide increase of multidrug resistance among *P. aeruginosa* isolates from hospitalized patients (3, 4).

Despite its high incidence, several steps in *P. aeruginosa* epidemiology remain unknown. Its reservoirs in the hospital and mechanisms of transmission have not been fully elucidated (5).

As a consequence, a debate about which measures are utmost important to control its spread within hospitals has not come to a definite conclusion (6).

In this setting, techniques of molecular epidemiology are of great value, adding precious information and helping us fulfill gaps in our present knowledge (7). The purpose of our study was to identify distribution of *P. aeruginosa* clones within a teaching hospital, and thus estimate the frequency of their cross-transmission among patients.

MATERIALS AND METHODS

Setting

The study was conducted in the teaching hospital of Botucatu Medical School (HC-FMB), a 400-bed facility that provides tertiary

care for an area with approximately one million inhabitants. At the time the bacterial strains from our study were isolated, the hospital had four areas for critical patients: high risk nursery (HRN), pediatric intensive care unit (P-ICU), medical-surgical intensive care unit (MS-ICU) and emergency room intensive care unit (ER-ICU). There were also several units for medical, surgical and pediatric non-critical patients.

***P. aeruginosa* Isolates: Collection, Storage and Susceptibility Tests**

P. aeruginosa isolates recovered from clinical cultures in the microbiology laboratory of HC-FMB from August to October 1998 were collected and stored at -70°C in 10% glycerol/BHI media. An automatic procedure (Microscan Autoscan-4, Siemens, USA) was used for antimicrobial susceptibility tests.

Epidemiological Classification of Isolates

The infection control committee data files were analyzed to identify which patients harboring *P. aeruginosa* isolates had a previous nosocomial infection. Isolates collected from those patients, as well as those cultured from previously sterile sites more than 48 hours after hospital admission were stated as nosocomial (Group N). All other strains we classified as Group C (community acquired). Group N comprised 71 isolates from 46 inpatients. Group C consisted of 23 isolates from 21 individuals, including inpatients and outpatients.

DNA Purification

DNA was purified using a method originally described for *Mycobacterium tuberculosis* (8). The final product was quantified by spectrophotometry (GeneQuant II, Pharmacia, USA).

Strain Typing

Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) was performed as described by Versalovic *et al.* (9). A single primer (ERIC2: 5'-AAG TAA GTG ACT GGG GTG AGC G - 3', Life Technologies, USA) was used. PCR solution consisted of: 100 ng of source DNA; 1.0 mM magnesium chloride (MgCl_2 , Gibco, USA); 200 mM dNTP mix (dATP, dTTP, dGTP e dCTP, Gibco); 100 pmol primer (ERIC-2); 2 U Taq polymerase (Amersham Pharmacia, USA) and 5 μL of reaction buffer

(Amersham Pharmacia, USA). Final volume was 50 μL . PCR cycles consisted of: an initial denaturation step (95°C for five minutes) followed by 35 cycles of denaturation at 90°C (30 seconds), annealing at 52°C (one minute) and extension at 65°C (eight minutes). Final extension was held for 16 minutes. Positive (ATCC 27853) and negative controls were used in every amplification.

After amplification, 5 μL of PCR products were submitted to electrophoresis in 2% agarose (50 V, six hours). The gel was stained in ethidium bromide (0.5 $\mu\text{g}/\text{mL}$) and photographed with Polaroid (USA).

Cluster Analysis

Images were visually analyzed and converted into binary sheets. Cluster analysis was performed in free software (Treecon for Windows, Bioinformatics, Belgium). Similarity coefficients of Dice (S_D), Jaccard (S_J) and simple matching (S_{SM}) were calculated (10). Unweighted pair method group with arithmetic averages (UPGMA) dendrograms were drawn for simple matching similarity.

Ethical Issues

This study was fully approved by the Research Ethics Committee of Botucatu Medical School (protocol number 0394).

RESULTS

A total of 94 isolates (71 from group N and 23 from group C) were collected. Group N isolates grew predominantly from respiratory specimens (50.1%). On the other hand, wound secretions were the source of 47.8% of community-acquired isolates (Group C).

Typing of Nosocomial Strains (Group N)

Seventy-one isolates from group N produced 40 unambiguous patterns, named N1 to N40. Similarity coefficients varied from 0.14 to 1.00 (S_D), 0.08 to 1.00 (S_J), and 0.58 e 1.00 (S_{SM}) (Figure 1).

We found the coexistence of several clones in most units (Figure 2). However, some clusters with complete similarity were found:

- High risk nursery (HRN): N4 – 15 isolates from six patients (Figure 3).
- Neurology unit: N33 – five isolates from four patients.

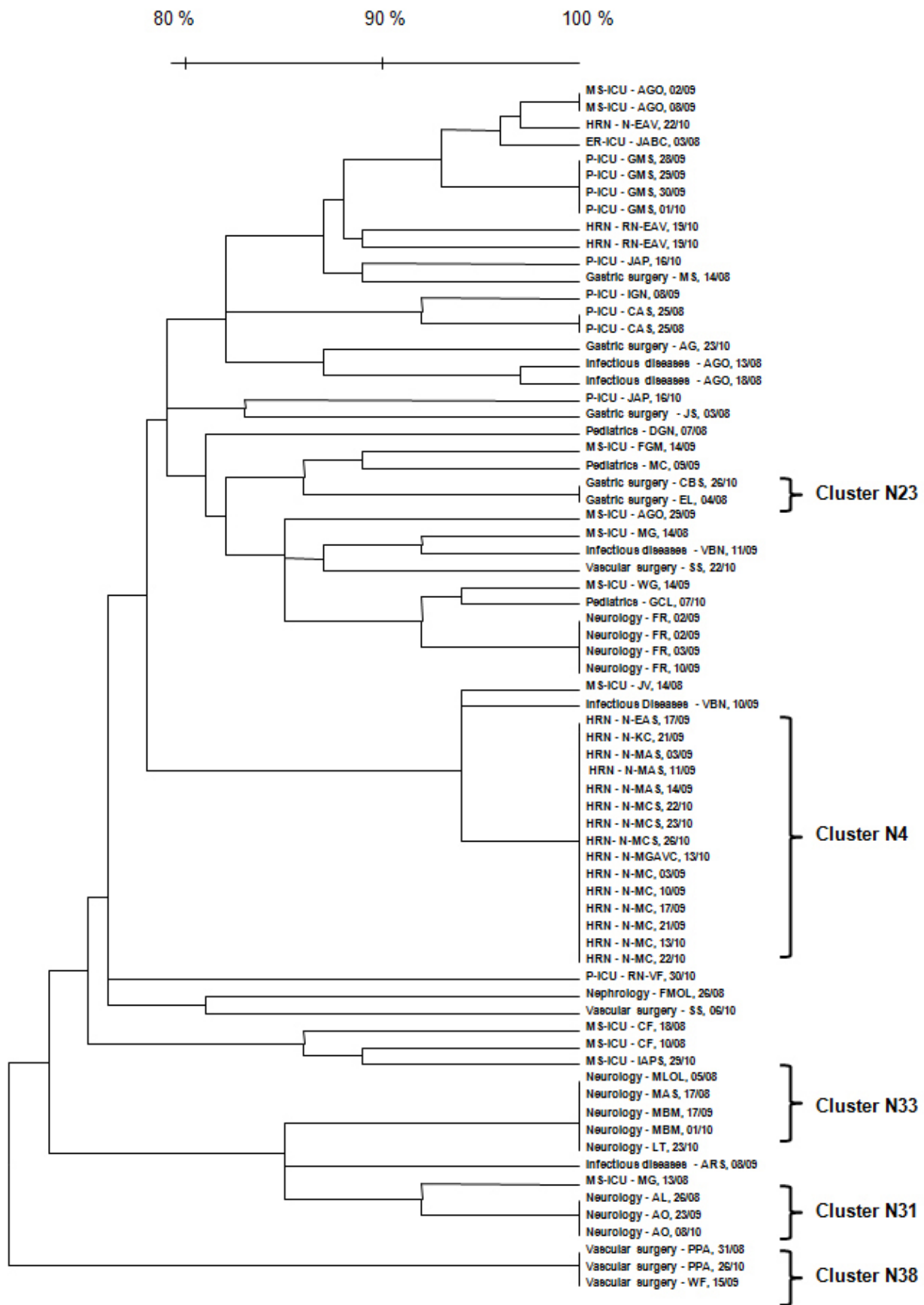


Figure 1. Dendrogram of similarity for nosocomial isolates of *Pseudomonas aeruginosa*.

Note: labels present hospital units, followed by patients' initials and date of strain isolation in clinical culture (European form, DD/MM). "N" identifies newborns, followed by mother's initials.

MS-ICU: medical-surgical intensive care unit; ER-ICU: emergency room intensive care unit; P-ICU: pediatric intensive care unit; HRN: high risk nursery.

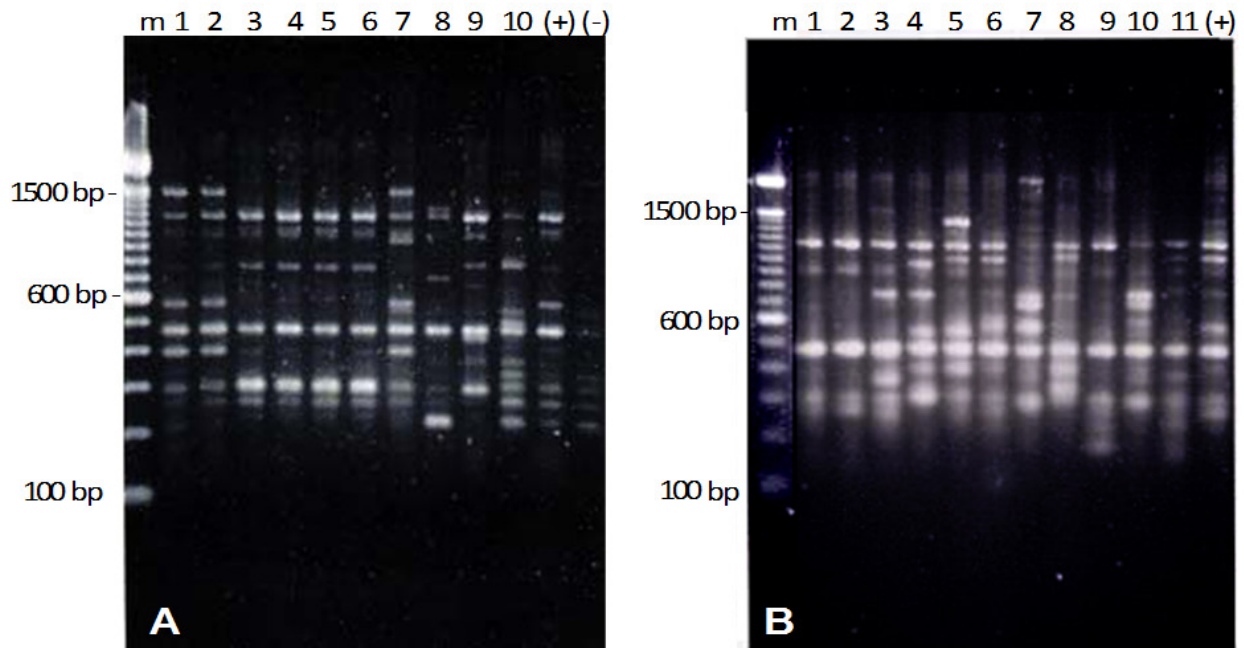


Figure 2. Coexistence of several different clones of *Pseudomonas aeruginosa* in pediatric (A) and medical-surgical (B) intensive care units. (A) Lanes 1 and 2: patient CAS, clone N5; lanes 3 to 6: GMS (N6); lane 7: IGN (N7); lane 8: JAP (N9 = 8); lane 9: JAP (N9); lane 10: N-VF (N10). (B) Lanes 1 and 2: AGO (N11); lane 3: AGO (N12); lane 4: CF (N13); lane 5: CF (N14); lane 6: FGM (N15); lane 7: IAS (N16); lane 8: JV (N17); lane 9: MG (N18); lane 10: MG (N19); lane 11: WG (N20).

+: positive control; -: negative control; m: molecular weight marker.

- Neurology unit: N31– three isolates from two patients.
- Vascular surgery unit: N38 – three isolates from two patients.
- Gastric surgery unit: N23 – two isolates from two patients.

Other similarity clusters consisted of several cultures from the same patient: N5, N6, N11 and N32. We noticed some variation in antimicrobial susceptibility profiles within the same cluster (Table 1).

In six occasions, two or more different clones were cultured from the same individual. In one

case there was a significant similarity between strains (N28-N29 – $S_D = 0.91$). Mostly in these occasions, antimicrobial susceptibility patterns were quite divergent.

Typing of Community-Acquired Strains (Group C)

Each and every isolate in Group C produced a unique unambiguous pattern (Figure 3). Similarity results varied (S_D : 0.20-0.86; S_I : 0.11-0.75; S_{SM} : 0.55-0.94). In two occasions, different patterns were observed in isolates from the same patient. In both cases, the difference was only one band.

Table 1. Antimicrobial susceptibility variation within the same clone.

Cluster	Antimicrobials with susceptibility variation
N4	Ticarcillin, ceftazidime
N31	Ceftazidime, cefepime, carbapenems
N33	Ticarcillin, aztreonam, ciprofloxacin, carbapenems
N23	Carbapenems

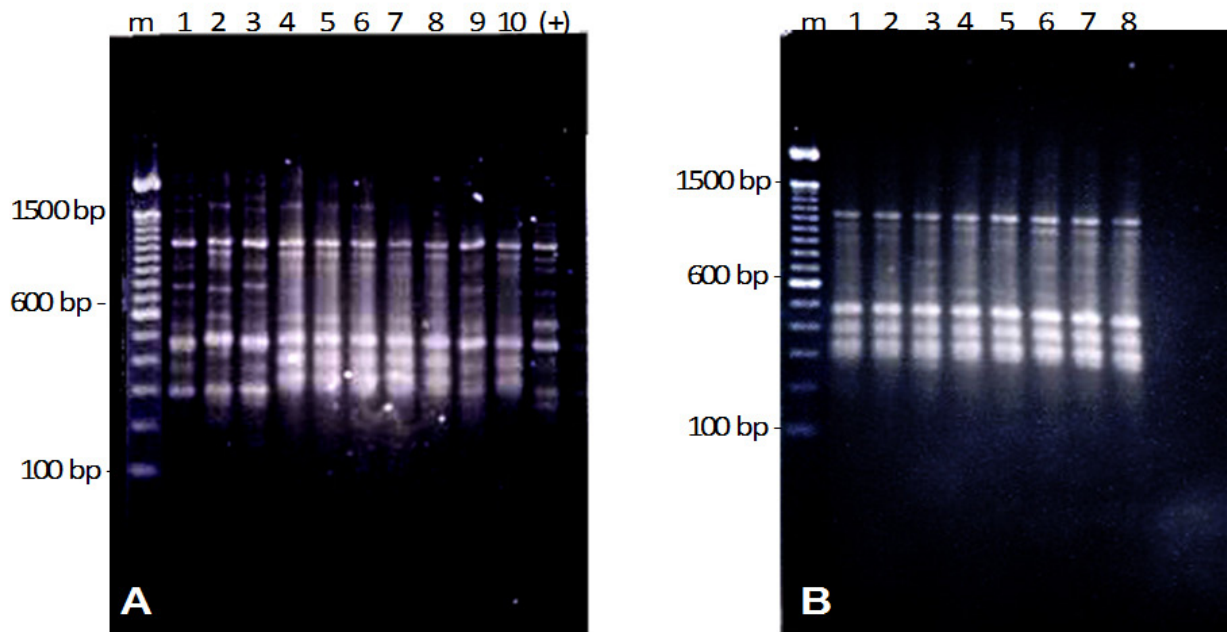


Figure 3. Spread of clone H4 among patients from the neonatal intensive care unit (high risk nursery). (A) Lanes 1 to 3: N-EAV (clones N1, N2 and N3). All others: clone N4 (lane 4: N-EAS; lane 5: N-KC; lanes 6 to 8: N-MAS; lanes 9-10: N-MCS). (B) All isolates belong to the clone N4 (lane 1: N-MCS; lane 2: N-MGAV; lanes 3-8: N-MC).

+: positive control; m: molecular weight marker.

DISCUSSION

Our study did not find evidence of extensive cross transmission of nosocomial *P. aeruginosa* clones. Instead, multiple clones coexisted in most units. This pattern was noticed even in units dealing with critical care and advanced life support (P-ICU, CA-ICU, MS-ICU), where high occurrence of cross transmission is expected. Also, no cluster was identified among community-acquired strains.

Results from strain typing of *P. aeruginosa* performed during outbreaks usually report extensive dissemination of one or a few clones (11-13). On the other hand, studies that focused on endemic clones reported two different epidemiological patterns of *P. aeruginosa* infections. In the first, few clones infect a great number of patients (14-18). This picture points out to extensive cross transmission of *P. aeruginosa* strains within the hospital. However, other authors report a different pattern, in which multiple clones coexist (19-22). Those findings are coherent with ours.

The explanation for this “polyclonal endemicity” remains controversial. However, Kropec *et al.* (23) points out the gastrointestinal tract as the most important reservoir of *P.*

aeruginosa. These authors speculate that small, undetectable community-acquired carriage becomes significant after exposure to antimicrobials and invasive procedures in the hospital setting. Otherwise, it has been suggested that strains implicated in nosocomial infections arise from water and other environmental sources (24).

In our study, clonal dissemination – probably through cross transmission – was found in four units: HRN, neurological, gastric surgery and vascular surgery. In HRN, N4 clone was isolated from six out of ten patients with positive cultures for *P. aeruginosa*. This clone has persisted in the unit for at least two months, showing a considerable variability in antimicrobial susceptibility patterns. Clonal dissemination of *P. aeruginosa* in neonatal units is expected, since newborns do not carry community-acquired strains. Other authors have described this event (25, 26). In other units, clones were also found to persist for long intervals: N23 and N33 were isolated in specimens collected in August and October.

Although it was not the main objective of this work, variation in antimicrobial susceptibility within the same clone was frequently noticed. To what extent this may be ascribed to

reproducibility problems in automatic testing is a matter of concern (27). It is well known, however, that *P. aeruginosa* shows a strong tendency to change susceptibility patterns under environmental pressure. Several antimicrobials (some penicillins and cephalosporins) may induce the overproduction of AmpC beta-lactamase, leading to resistance to currently used anti-pseudomonal drugs (ceftazidime, cefepime). Changes in outer membrane porin proteins and expression of carbapenemases account for carbapenem resistance (28).

Overall, ERIC-PCR was useful for epidemiological studies with *P. aeruginosa*. As a PCR-based method, it can provide faster results than pulsed-field gel electrophoresis (PFGE) (29). In a study by Lau *et al.* (30), both techniques had a similar discriminatory power. Using long primers directed to known repetitive elements in the genome of gram-negative bacteria, ERIC-PCR has a greater reproducibility than methods based on arbitrary primers (31).

Contrary to PFGE, no easy method was proposed for interpretation of ERIC-PCR generated patterns (32). Cluster analysis can overcome this problem, providing numerical results that can be interpreted in the light of epidemiological findings. An interesting approach to our results is to compare clusters using three different cut-points in similarity (Table 2). A polyclonal pattern results even after grouping every strain with Dice similarity above 0.75. In that case, six clusters group 24 out of 41 patients. Still, strains from different clusters coexisted in most units.

Finally, one should ask if there is anything we can learn from the typing of isolates that are more than a decade old. We believe they are valuable for understanding the epidemiology of *P. aeruginosa* in health care settings. In 1998, *P. aeruginosa*

was the bacteria most frequently recovered from clinical cultures from inpatients in our hospital, accounting for 16.1% of all positive cultures. That was the peak of hyperendemicity. The fact that it was mostly polyclonal suggests that cross transmission was infrequent. These findings are consistent with the hypothesis that strain selection from inner reservoirs is the main route for *P. aeruginosa* colonization and infection. There is a good possibility that antimicrobial control policies, rather than isolation precautions, should be effective in reducing *P. aeruginosa* incidence. Further epidemiological studies are required to strengthen our findings.

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CONFLICTS OF INTEREST

There is no conflict.

FINANCIAL SOURCE

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ETHICS COMMITTEE APPROVAL

The present study was approved by the Research Ethics Committee of Botucatu Medical School (protocol number 0394), UNESP, Brazil.

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Table 2. Clusters generated with three different cut-points for Dice similarity index (clusters grouping only isolates from the same patient were excluded)

	Clusters	Patients in clusters
$S_D = 1.00$	5	15
$S_D > 0.90$	6	16
$S_D > 0.75$	6	24

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