Standardization and Interlaboratory Reproducibility Assessment of Pulsed-Field Gel Electrophoresis-Generated Fingerprints of *Acinetobacter baumannii*

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A standard procedure for pulsed-field gel electrophoresis (PFGE) of macrorestriction fragments of *Acinetobacter baumannii* was set up and validated for its interlaboratory reproducibility and its potential for use in the construction of an Internet-based database for international monitoring of epidemic strains. The PFGE fingerprints of strains were generated at three different laboratories with ApaI as the restriction enzyme and by a rigorously standardized procedure. The results were analyzed at the respective laboratories and also centrally at a national reference institute. In the first phase of the study, 20 *A. baumannii* strains, including 3 isolates each from three well-characterized hospital outbreaks and 11 sporadic strains, were distributed blindly to the participating laboratories. The local groupings of the isolates in each participating laboratory were identical and allowed the identification of the epidemiologically related isolates as belonging to three clusters and identified all unrelated strains as distinct. Central pattern analysis by using the band-based Dice coefficient and the unweighted pair group method with mathematical averaging as the clustering algorithm showed 95% matching of the outbreak strains processed at each local laboratory and 87% matching of the corresponding strains if they were processed at different laboratories. In the second phase of the study, 30 *A. baumannii* isolates representing 10 hospital outbreaks from different parts of Europe (3 isolates per outbreak) were blindly distributed to the three laboratories, so that each laboratory investigated 10 epidemiologically independent outbreak isolates. Central computer-assisted cluster analysis correctly identified the isolates according to their corresponding outbreak at an 87% clustering threshold. In conclusion, the standard procedure enabled us to generate PFGE fingerprints of epidemiologically related *A. baumannii* strains at different locations with sufficient interlaboratory reproducibility to set up an electronic database to monitor the geographic spread of epidemic strains.

*Acinetobacter baumannii* is a well-recognized opportunistic pathogen that gives rise to nosocomial infections and outbreaks, in particular, in the intensive care unit setting (1). The increasing rates of resistance of *A. baumannii* to the major antimicrobial drugs make early identification and control of hospital outbreaks mandatory. Recent data indicate that several successful epidemic *A. baumannii* strains (clones) circulate in Europe, and a better understanding of the diversity within the species and the emergence of epidemic clones is urgently needed (19, 25, 29). Molecular typing plays an important role in the study of the epidemiology of *A. baumannii* and in coping with its epidemic spread.

Various genotypic methods have been developed for the typing of acinetobacters, including ribotyping (11), macrorestriction analysis by pulsed-field gel electrophoresis (PFGE) (21), randomly amplified polymorphic DNA (RAPD) analysis (13), and total genomic fingerprinting by AFLP (amplified fragment length polymorphism analysis) (33). Among these, PFGE is regarded as the “gold standard” of epidemiological typing (26). The increasing use of PFGE not only as a research tool but also as an aid in routine epidemiological analysis in clinical diagnostic laboratories has resulted in the development of a plethora of protocols for the typing of even the same species of bacteria (16). Because each laboratory uses its own techniques and protocols for molecular typing and its own designations for the resulting patterns, comparison of the results with those of another laboratory is difficult or impossible, even if both laboratories have used the same methods. This lack of comparability significantly limits the power of PFGE and hampers a more profound investigation of the epidemiology of nosocomial pathogens both for longitudinal epidemiological evaluations within a hospital and beyond the hospital level.

Moore and colleagues have recently emphasized the need for harmonization of techniques for genotyping of bacterial pathogens to be able to communicate typing results within the microbiology community (16). Such an approach has been successfully applied by using PFGE for the typing of enteric bacterial pathogens by the PulseNet system (www.cdc.gov/pulsenet/intex.html) and has also recently been proposed for the typing of methicillin-resistant *Staphylococcus aureus* (17).
The present study was performed to develop a standard PFGE typing protocol for *A. baumannii* and to assess the interlaboratory reproducibility of the PFGE-generated genomic fingerprints. The use of such a standardized typing method and the establishment of a database for web-based electronic data exchange of *A. baumannii* ApaI restriction patterns would allow isolates from different parts of the world to be compared. This approach would permit the recognition of epidemic strains and the early detection of multihospital or nationwide outbreaks, particularly those in which cases are geographically separated.

**MATERIALS AND METHODS**

**Bacterial strains.** The *A. baumannii* isolates selected for this study and their epidemiological details are listed in Tables 1 and 2. Identification to the species level had been confirmed previously at the participating laboratories (Cologne, Germany; laboratory A; Leiden, The Netherlands; laboratory B; and Trieste, Italy, laboratory C) by established methods, such as biochemical characterization (3), DNA-DNA hybridization (27), amplified ribosomal DNA restriction analysis (9, 30), ribotyping (11), and/or AFLP analysis (18). The isolates had also previously been characterized at the subspecies level by using genotypic typing methods, including cell envelope protein typing, AFLP, PFGE, RAPD analysis, and ribotyping. Isolates were distributed blindly to the participating laboratories. The first set of 20 *A. baumannii* isolates (study phase I) comprised three isolates each from 3 well-characterized hospital outbreaks and 11 sporadic strains that were all geographically separated.

**Consensus protocol.** The study group decided that adherence to a standard protocol should be feasible for all laboratories performing pulsed-field gel electrophoresis. This permitted, in particular, the use of different types of PFGE apparatuses as well as the use of chemicals and reagents produced by different companies, unless these were considered crucial for standardization. In a pilot phase the three study centers exchanged their current PFGE protocols and agreed on the key steps of the consensus protocol listed below, which were adapted from the PFGE protocol described by Ribot et al. (20). In addition, all steps of the procedure were rehearsed during a workshop held at one of the participating laboratories.

**Plug preparation.** Test strains were inoculated onto Iso-Sensitest agar (purchased from different suppliers) and incubated overnight at 36 ± 1°C in an ambient air. A loopful of bacteria was removed from the agar surface with a sterile plastic loop and suspended in 10 μl loop and suspended in a glass or polystyrene round-bottomed tube containing 2.5 ml of cell suspension buffer (100 mM Tris, 100 mM EDTA, pH 8.0). Each cell suspension was adjusted to give a cell density of approximately 10^6 cells/ml. This was performed by using the optical instruments available in each laboratory (turbidity meter, filter photometer, or spectrophotometer) and may be checked by the volume of the resulting pellet. For this purpose, a 500-μl aliquot of the adjusted bacterial cell suspension was transferred to a 1.5-ml screw-cap microcentrifuge tube and centrifuged at 13,000 × g for 1 min to evaluate visually the resulting pellet size and adjust the cell suspension if necessary. The pellet was resuspended by vortexing, and the cell suspension was incubated at 55°C for 10 min in a thermomixer or a water bath. An aliquot of 25 μl proteinase K (20 mg/ml stock solution in ultrapure water) was added, and the suspension was mixed gently by inverting the tube two to four times. An equal volume of melted 1% SeaKem Gold agarose (Biozym Diagnostics, Hessisch Oldendorf, Germany)–1% sodium dodecyl sulfate in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) was added to the cell suspension, and the suspension was mixed gently by inverting the tube 10 to 12 times. The agarose-cell suspension mixture was immediately dispensed into the wells of reusable plug molds (catalog no. 170-3713; Bio-Rad Laboratories, Munich, Germany). The agarose plugs were allowed to solidify at room temperature for 5 min and at 4°C for another 5 min.

**Lysis of cells in plugs and washing.** The plugs were transferred to disposable screw-cap 50-ml polypropylene tubes containing 5 ml of cell lysis buffer (50 mM Tris, 50 mM EDTA [pH 8.0], 1% sarcosine) and 25 μl of proteinase K (20 mg/ml stock solution). Lysis was performed at 55°C in a shaking water bath for 2 h with constant and vigorous agitation (150 to 200 rpm). After lysis, the buffer was carefully removed and the plugs were washed five times (15 min wash) at 55°C (two times with sterile ultrapure water and three times with TE buffer; 10 ml for each washing step) in a shaking water bath. The water and TE buffer were preheated at 50 to 55°C before each washing step. After the last wash, the TE buffer was poured off and 10 ml of fresh TE buffer (room temperature) was added to each tube. If the plugs were not used on the same day, they were kept at −20°C.

**Preparation of plugs for electrophoresis.** Plugs were resuspended by vortexing and were allowed to solidify at room temperature for 5 min. Each tube was transferred to a 15-ml conical tube, and 10 ml of 1% agarose in TE buffer was added. After the plugs were completely dissolved, 1 ml of 6X TBE was added (only for the final running buffer) and the suspensions were mixed gently by inverting the tubes two to four times. The suspensions were transferred to a 1.5-ml centrifuge tube and the cell suspension was adjusted to give a cell density of approximately 10^8 cells/ml. The cell suspension was centrifuged for 1 min at 10,000 × g, and the supernatant was removed. The pellets were resuspended by vortexing with 100 μl of 1X TBE and the cell suspensions were used for the PFGE analysis.

**PFGE.** The restriction enzyme Apal was used in all laboratories. The electrophoresis was performed in a CHEF Mapper XTR apparatus (Bio-Rad). The fragments were visualized by ethidium bromide staining. Photographs were taken after exposure on Polaroid sheet film (Polaroid, Cambridge, Mass.). Results were analyzed using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium).

**TABLE 1. Characteristics of 20 *A. baumannii* isolates from hospital outbreaks as well as sporadic isolates from several countries in Europe used in study I (stains 1 to 20)**

<table>
<thead>
<tr>
<th>Straina</th>
<th>Running no.</th>
<th>Outbreak</th>
<th>Origin (specimen)</th>
<th>Yr of collection</th>
<th>City and countryb</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL 20820</td>
<td>Reference strain</td>
<td>Blood</td>
<td>1991</td>
<td>Cologne, GE</td>
<td>21–23</td>
<td></td>
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<tr>
<td>MB142</td>
<td>20</td>
<td>No</td>
<td>Skin swab</td>
<td>1981</td>
<td>London, UK</td>
<td>8</td>
</tr>
<tr>
<td>PGS189</td>
<td>16</td>
<td>No</td>
<td>Cural ulcer</td>
<td>1984</td>
<td>Odense, DK</td>
<td>8</td>
</tr>
<tr>
<td>TU133</td>
<td>8</td>
<td>No</td>
<td>Wound swab</td>
<td>1980</td>
<td>Malmoe, SW</td>
<td>8</td>
</tr>
<tr>
<td>LHU0545</td>
<td>18</td>
<td>Leiden 1</td>
<td>Catheter tip</td>
<td>2000</td>
<td>Leiden, NL</td>
<td>2</td>
</tr>
<tr>
<td>LHU1065</td>
<td>15</td>
<td>Leiden 1</td>
<td>Abdominal tissue</td>
<td>2001</td>
<td>Leiden, NL</td>
<td>2</td>
</tr>
<tr>
<td>LHU7312</td>
<td>6</td>
<td>Leiden 1</td>
<td>Sputum</td>
<td>2001</td>
<td>Leiden, NL</td>
<td>2</td>
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<tr>
<td>7B</td>
<td>7</td>
<td>Trieste 1</td>
<td>Bronchial aspirate</td>
<td>1996</td>
<td>Trieste, IT</td>
<td>12</td>
</tr>
<tr>
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<td>1996</td>
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<td>17A</td>
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<td>Wound swab</td>
<td>1996</td>
<td>Padova, IT</td>
<td>12</td>
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<tr>
<td>105B</td>
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<td>No</td>
<td>Blood</td>
<td>1997</td>
<td>Aviano, IT</td>
<td>12</td>
</tr>
<tr>
<td>110B</td>
<td>9</td>
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<td>Pharyngeal swab</td>
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<td>Udine, IT</td>
<td>12</td>
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<tr>
<td>W 8832</td>
<td>4</td>
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<tr>
<td>V 4316</td>
<td>14</td>
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<td>23</td>
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<tr>
<td>M 3317</td>
<td>19</td>
<td>No</td>
<td>Tracheal aspirate</td>
<td>1991</td>
<td>Cologne, GE</td>
<td>23</td>
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<tr>
<td>M 7968</td>
<td>1</td>
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<td>Sputum</td>
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<td>23</td>
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<tr>
<td>Col4220</td>
<td>2</td>
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<td>Blood</td>
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<td>21</td>
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<tr>
<td>Col15343</td>
<td>3</td>
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<td>Blood</td>
<td>1991</td>
<td>Cologne, GE</td>
<td>21</td>
</tr>
<tr>
<td>Col19536</td>
<td>10</td>
<td>Cologne 6</td>
<td>Blood</td>
<td>1991</td>
<td>Cologne, GE</td>
<td>21</td>
</tr>
</tbody>
</table>

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*a* Original strain designation.

*b* Isolate number used in blinded study.

**c** DK, Denmark; GE, Germany; IT, Italy; NL, The Netherlands; SW, Sweden; UK, United Kingdom.
Restriction digestion and loading of the gel. A slice from each plug (4.0 by 5.5 mm) was cut with a scalpel or razor blade and transferred to a microcentrifuge tube containing 200 µl of the restriction buffer provided with the enzyme (see below), with 100 µg/ml bovine serum albumin included in the buffer. The plug slices were incubated in this restriction buffer at 25°C for 15 min. Then, the restriction buffer was removed and replaced with 200 µl of fresh restriction buffer containing 30 U of Apal (New England Biolabs, Frankfurt, Germany, or Promega, Madison, Wis.). The reaction tubes were shaken gently, and the plug slices were incubated at 25°C for 2 h. Prior to casting of the gel, the restriction mixture was removed from each tube and replaced with 200 µl of 0.5× TBE (10× TBE is 0.89 M Tris, 0.89 M boric acid, and 20 mM EDTA, pH 8.3). If the slices were not used on the same day, they were kept overnight or for up to several days at 4 to 8°C in a refrigerator. The plug slices were allowed to stand at room temperature for 5 min, after which they were loaded into the appropriate wells of a 1% SeaKem Gold agarose gel. The wells were made by using a 15-slot comb, 5.0 mm wide, with each slot being 1.5 mm thick (catalog no. 1704324; Bio-Rad Laboratories). A slice of a bacteriophage lambda ladder PFGE marker (CHEF DNA size standard; catalog no. 170-3035; Bio-Rad Laboratories) was loaded into lane 1; and reference strain COL 20620 was loaded into lanes 2, 6, 11, and 15 to allow later normalization of the electrophoretic patterns across the gel. Isolates for overnight for direct use or were stored up to several weeks at 4 to 8°C for later use.

Electrophoresis. Electrophoresis was performed in a contour-clamped homogeneous electric field (CHEF). The gels were covered with 2,000 ml of 0.5× TBE. Different equipment was used in the participating laboratories (laboratory A, CHEF DRH; laboratory B, CHEF Mapper apparatus; and laboratory C, CHEF DRH), all equipment was purchased from Bio-Rad Laboratories). The running temperature was set at 14°C. The total run time was 19 h, with switch times ranging from 5 to 20 s and linear ramping; laboratory B, however, used switch times ranging from 5 to 35 s in study phase I. The voltage for the run was 6 V/cm or 200 V. The gels were stained for 30 min with 300 ml of ethidium bromide solution (1 µg/ml) and destained for 45 min in distilled water with gentle shaking. The gels were observed under UV illumination and photographed by using each laboratory’s imaging and documentation system. Digital images were stored electronically as TIFF files with an overall resolution of approximately 1,000 pixels per lane.

Data analysis. Local data analysis of the PFGE patterns was performed visually, and the band patterns were interpreted according to the criteria suggested by Tenover et al., with a difference of six bands or less used to define epidemiological relatedness (26). For study phase I, the local grouping of strains and the interpretation of the results as well as the TIFF files were forwarded to the central data bank manager at a national reference institute. For study phase II, only the TIFF files were submitted to the central data bank manager, who was blinded to the origin and epidemiological details of the strains, as were the investigators at the three laboratories. For central analysis of pattern reproducibility, PFGE-generated DNA profiles were entered into the BioNumerics software package, version 3.0 (Applied Maths, Sint-Martens-Latem, Belgium). Cluster analysis was performed by the unweighted pair group method with mathematical averaging (UPGMA), and DNA relatedness was calculated by using the band-based Dice coefficient with a tolerance setting of 1.5% band tolerance and 1.5% optimization setting for the whole profile. Only bands larger than 48 kb were considered for central analysis.

RESULTS

Study phase I. The rationale for the first part of the study was to test the interlaboratory reproducibility of the PFGE-generated fingerprint patterns of a set of both epidemic and sporadic A. baumannii isolates and to compare the epidemiological interpretations derived from these patterns at different institutions. The diversity of the PFGE patterns of the A.
baumannii isolates investigated and generated in one laboratory is illustrated in Fig. 1. Figure 2 shows an example of the results obtained at the three laboratories for five strains analyzed by the standardized protocol with COL 20820 as the reference standard. Of note, the CHEF mapper (laboratory B; Fig. 2B), which used a switching time of 5 to 35 s, provided profiles for high-molecular-weight fragments of increased resolution compared to those of the profiles generated with the CHEF DRII and CHEF DRIII systems. However, this difference had no impact on the grouping of the strains. Therefore, in further experiments, switching times of 5 to 20 s were also used in laboratory B.

The local groupings of the strains in each participating laboratory were identical and allowed the identification of three clusters of epidemiologically related isolates and identified all unrelated isolates as distinct (data not shown). Central analysis showed 95 to 100% matching of the outbreak strains processed at each local laboratory (data not shown) and a similarity value ≥87% for corresponding isolates that were processed at different laboratories (Fig. 3). For this reason, a grouping level of
87% was chosen as the threshold level for the establishment of clonal relatedness of unknown isolates.

Study phase II. The second part of the study was performed to explore whether *A. baumannii* isolates that are representatives of a common outbreak strain but that were investigated by PFGE at different locations (Fig. 4) would be assigned to the same strain by central data analysis of the electronically submitted TIFF files. Central computer-assisted analysis of the 30 profiles generated at the three participating laboratories correctly identified the isolates according to their corresponding outbreak. Comparison of corresponding isolates across gels showed that all isolates clustered at ≥87%, which corresponds to the grouping level of the profiles of identical strains generated at the different laboratories in study phase I. In addition, strains from the Newcastle, United Kingdom, outbreak clustered together with strains from a Cologne, Germany, outbreak. A dendrogram depicting the *A. baumannii* macrorestriction patterns is shown in Fig. 5.

**DISCUSSION**

*A. baumannii* has emerged as an important nosocomial pathogen. Recent data from the National Nosocomial Surveillance System showed a substantial increase in the incidence of *Acinetobacter* in the United States from 1.4% of gram-negative bacilli in 1975 to 6.2% of gram-negative bacilli by 2003. The most common sites of infection were the respiratory tract and surgical wounds (10). This organism is known for its propensity for epidemic spread, which has been attributed to its multidrug resistance coupled with its ability for long-term survival in the hospital environment (14, 31). An ever increasing number of hospital outbreaks caused by *A. baumannii* has been reported from numerous countries around the world. Molecular epidemiologic studies mainly based on AFLP and ribotyping have identified among these outbreak strains a limited number of successful *A. baumannii* epidemic clones in northwestern Europe, including the so-called European clone I and clone II (8, 19). More recently, another geographically widespread multiresistant *A. baumannii* clone (clone III) has been observed in various hospitals in France, Spain, and The Netherlands (29). In addition, the interhospital spread of multiresistant *A. baumannii* isolates has been observed in metropolitan areas such as New York and London (15, 28). Other researchers, in contrast, have reported great diversity among epidemic *A. baumannii* strains without evidence of interregional spread (21, 23, 25, 32). The relative contribution of widespread clones to the overall burden of epidemic *A. baumannii* strains remains largely unknown because epidemiological typing of *A. baumannii* is primarily based on comparative typing methods such as RAPD analysis, AFLP, and PFGE; and the typing data generated at the local level are difficult to compare with similar data obtained at a different laboratory. Moore and colleagues have recently emphasized the requirement of adopting a common language in the form of a “molecular Esperanto” of a harmonized and standardized methodology to be able to communicate typing results within the microbiology community (16). The exchange of typing data, preferably via an electronic platform, would facilitate our understanding of the molecular epidemiology of *A. baumannii*. Another advantage of the use of a standard procedure is that it can also be used locally to set up a local database to monitor the longitudinal occurrence of endemic or epidemic strains. Once the procedure is implemented, it can also be adapted for other pathogens and provide a tool for local hospital epidemiological evaluations and disease control.
In the present study we developed a standard PFGE procedure that was achieved by a consensus approach, similar to the PFGE protocol for epidemiological typing of methicillin-resistant *Staphylococcus aureus* proposed by the Harmony group (17). Use of an external reference standard, which in our study was the restriction fragments of a control strain extending over a major part of the gel, is of utmost importance for normalization and comparison of fragment patterns across gels and between different centers. We found that the use of identical PFGE equipment was not necessary to obtain comparable results, thus saving laboratories from purchasing new and expensive equipment to be able to participate in this form of data exchange. Apart from the agarose, the use of reagents purchased from different suppliers was permitted for local economical reasons. In fact, even though different running conditions, i.e., different switching times, altered the migration patterns of the top bands, they did not affect clustering or outbreak strain identification.

The rationale for the first part of the study was to compare the results of the analysis of strain relationships performed at each laboratory, to validate the interlaboratory reproducibility of PFGE-generated genomic fingerprints of *A. baumannii*, and to establish a similarity threshold at which corresponding isolates processed at different laboratories could be grouped together by central analysis. Identical strains as well as multiple isolates from the same outbreak all clustered at \( \geq 87\% \), indicating that the 87% level can be used to identify representatives of the same epidemic strain.

The aim of the second phase of the study was to test the hypothesis that epidemiologically related *A. baumannii* isolates, i.e., isolates representing an epidemic strain, recovered and processed at different locations can be recognized as representing the same strain by central analysis of independently generated PFGE profiles by using the standard laboratory protocol. Multiple isolates from each of the 10 outbreaks, which were processed at different laboratories, grouped at \( \geq 87\% \), the threshold level for strain recognition that had been established in study phase I. This exercise was a simulation of the ultimate aim of our project, i.e., realization of a network for surveillance of circulating strains based on electronic comparison of fingerprints generated at different locations. Of note, we also found that the Newcastle outbreak strain, which originated in 1989 and which was previously identified as belonging to the European clone II (8), clustered with the Cologne 1 outbreak strain, which was isolated in 1991. The high similarity indicates a clonal relatedness of these strains that do not have any known epidemiological link.
Percentage similarity

Isolate-laboratory

Outbreak

Newcastle

Cologne 1

Leiden

Eschwege

Cologne 2

Cologne 3

Cologne 4

Venlo

Cologne 5

Warsaw

25-B

50-A

48-A

38-C

32-C

27-B

41-A

36-C

22-B

30-B

29-C

42-A

23-B

47-A

38-C

43-A

33-C

24-B

26-B

31-C

45-A

46-A

34-C

28-B

29-B

44-A

37-C

21-B

46-A

40-C

A standardized PFGE protocol like the one proposed here can be used both at a local level, which would ensure high-quality gels for longitudinal epidemiological studies within a laboratory, and for electronic data exchange if the comparison of isolates recovered at different locations seems appropriate. This approach obviates the limitations usually seen with the use of a so-called comparative typing system, such as PFGE, which is mainly based on a side-by-side comparison of the molecular fingerprint patterns of a limited number of strains to determine the possible transmission of a nosocomial pathogen and which is less suitable for large-scale epidemiological and population studies. Our study indicates that comparison of the profiles generated at different laboratories for the identification of epidemic A. baumannii strains is feasible. Further studies are required to assess whether more distantly related strains (i.e., those with a relatively recent common ancestor but spread in time and space) can be recognized as well and whether a central database can be used to monitor the spread of epidemic strains and to assess the population diversity of this important bacterial species.

In conclusion, the standard procedure enabled us to generate PFGE fingerprints with excellent reproducibility and can be used to set up a database for the Internet-based electronic data exchange of genomic fingerprints to study the geographic spread of epidemic A. baumannii isolates.

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FIG. 5. Computer-assisted analysis of PFGE patterns generated at three different laboratories in study phase II correctly identified 3 isolates from each of 10 outbreaks among the isolates investigated.


