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Interlaboratory reproducibility of DiversiLab rep-PCR typing and clustering of *Acinetobacter baumannii* isolates

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We have investigated the reproducibility of DiversiLab rep-PCR fingerprints between two laboratories with the aim of determining if the fingerprints and clustering are laboratory-specific or portable. One-hundred non-duplicate *Acinetobacter baumannii* isolates were used in this study. DNA isolation and rep-PCR were each performed separately in two laboratories and rep-PCR patterns generated in laboratory A were compared with those from laboratory B. Twelve *A. baumannii* isolates processed in laboratory A showed $\geqslant$98 % pattern similarity with the corresponding 12 isolates tested in laboratory B and were considered identical. Sixty-four isolates showed 95–97.9 % similarity with their corresponding isolates. Twenty-three isolates showed 90–94 % similarity with the corresponding isolates, while one isolate showed only 87.4 % similarity. However, intra-laboratory clustering was conserved: isolates that clustered in laboratory A also clustered in laboratory B. While clustering was conserved and reproducible at two different laboratories, demonstrating the robustness of rep-PCR, interlaboratory comparison of individual isolate fingerprints showed more variability. This comparison allows conclusions regarding clonality to be reached independent of the laboratory where the analysis is performed.

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INTRODUCTION

A plethora of molecular methods are available to investigate the epidemiology of bacteria (Li *et al.*, 2009; Singh *et al.*, 2006). DiversiLab, which is one of those methods, is a commercial repetitive-sequence-based PCR (rep-PCR) typing system that amplifies strain-specific non-coding repetitive sequences. The system contains quality-controlled reagents in a kit format, automated detection and analysis using microfluidics with the corresponding information digitized in a software package that allows data archiving, retrieval and reporting (Healy *et al.*, 2005). Rep-PCR libraries can therefore be easily assembled to enable, for example, the comparison of strains over time and probably across laboratories, with a view to charting the epidemiology of isolates. Outbreaks of *Acinetobacter baumannii* have been extensively studied using DiversiLab rep-PCR typing (Carretto *et al.*, 2008; Fontana *et al.*, 2008; Kohlenberg *et al.*, 2009; Yan *et al.*, 2010; Perez *et al.*, 2010). Using DiversiLab, a snapshot emerged of the global epidemiology of carbapenem-resistant *A. baumannii*, where it was found that almost half of 492 isolates from a worldwide collection belonged to a single clonal lineage that clustered with European clone II (Higgins *et al.*, 2010; Dijkshoorn *et al.*, 1996). The majority of the remaining isolates grouped into seven distinct clonal clusters. Owing to their widespread distribution, these were termed worldwide clonal clusters 1–8 (WW1–8); WW1–WW3 corresponded to previously identified European clonal lineages 1–3 (Dijkshoorn *et al.*, 1996; van Dessel *et al.*, 2004). An in-house library representing the eight *A. baumannii* worldwide clusters, which is regularly used for

Abbreviations: KL, Kullback–Leibler; PC, Pearson correlation; rep-PCR, repetitive-sequence-based polymerase chain reaction.

our epidemiological investigations, is now established in Cologne.

To our knowledge, the interlaboratory reproducibility of rep-PCR patterns generated using the DiversiLab platform has not been demonstrated. In the present study, we investigated this by comparing rep-PCR fingerprints and clustering generated independently at two separate laboratories, in the hope that this knowledge will aid clonal investigations around the world.

METHODS

Bacterial isolates. One hundred non-duplicate, sporadic and epidemic *A. baumannii* clinical isolates were used in this study. These comprised 50 isolates each from the collections in Cologne (Germany) and Cleveland (Ohio, USA) and were numbered 1–50 (isolates supplied from Cologne) and 51–100 (isolates supplied from Cleveland) by an investigator who was unaware of the isolates' original epidemiological characteristics (Higgins *et al.*, 2010; Hujer *et al.*, 2006). The isolates from Cologne were previously assigned to each of the eight worldwide (WW) clonal clusters using an in-house library (Higgins *et al.*, 2010), and the Cleveland isolates were assigned to five clusters (Table 1).

Rep-PCR and analysis. Rep-PCR typing was performed using the DiversiLab Acinetobacter kit (bioMérieux). DNA isolation and rep-PCR of the whole set of isolates were each performed separately in the two laboratories from isolates grown overnight on solid medium following the manufacturer's instructions as previously reported (Perez *et al.*, 2010; Endimiani *et al.*, 2009; Kohlenberg *et al.*, 2009; Higgins *et al.*, 2010). However, owing to problems observed in the Cologne laboratory with both DNA yield and quality using the UltraClean Microbial DNA Isolation kit (MO BIO Laboratories) that is recommended by the manufacturer, DNA isolation in Cologne was performed using the Qiagen DNeasy kit (Qiagen) whereas the Cleveland laboratory used the MO BIO kit. As part of our initial evaluation of the DiversiLab system, we compared rep-PCR fingerprints generated with the DNA template prepared using functional MO BIO and Qiagen DNA isolation kits, respectively, and found that the DNA isolation method employed had no effect on the rep-PCR patterns (data not shown). The prefixes CL- and KO- were used to denote the city where rep-PCR was performed (Cleveland and Cologne, respectively). Thus, CL-1 is the same isolate as KO-1. For this study, these are termed an 'isolate pair'. PCR was performed in a GeneAmp PCR system 9600 (Cologne) and an MJ Research Gradient Cycler model PTC 225 (Cleveland). Rep-PCR patterns generated in laboratory A (Cologne) were compared to those from laboratory B (Cleveland). The first step was to compare individual isolate patterns generated in laboratory A with their corresponding isolate patterns generated in laboratory B, i.e. isolate vs isolate (isolate pairs). In a second step we compared the clustering obtained independently at the two laboratories, i.e. if isolates that clustered together in laboratory A were the same isolates that clustered together independently in laboratory B (cluster integrity). In a third step, we compared clustering between study sites, i.e. if isolates run in laboratory A clustered with their corresponding isolates processed in laboratory B, in effect merging results from the two laboratories. The Pearson correlation (PC) and the modified Kullback–Leibler (KL) statistical methods, which are part of the analysis software, were employed for the analysis. These calculate similarity based on the relative intensity of each band; however, PC is more band-intensity based and KL is more band-presence based.

A cluster of closely related isolates was defined as isolates sharing $\geqslant$95 % similarity, and based on previous experience, for isolates to be identical a similarity of $\geqslant$98 % was used (Saeed *et al.*, 2006; Kohlenberg *et al.*, 2009). This $\geqslant$95 % similarity rule was strictly enforced, and isolates that showed $\leqslant$94.9 % similarity were classified as unrelated. DiversiLab has a function termed 'classification' whereby rep-PCR fingerprints are compared with either a pre-loaded library, or a user-generated library, to determine if an isolate clusters with a previously defined strain-type. In the fourth step, all rep-PCR patterns generated in this study were compared to the Cologne in-house library of worldwide clonal clusters to determine their epidemiological background.

Table 1. Cluster conservation (integrity) based on rep-PCR fingerprints generated at the two laboratories

The table shows the number of isolates from each centre that cluster $\geqslant$95 % similarity. Clusters A–E were assigned for isolates originating from Cleveland, and WW1–8 clustering originated from Cologne.

Cluster	Testing laboratory and no. of isolates in cluster	
	Cleveland	Cologne
A	5	5
B	4	2
C	2	2
D	4	5
E	29*	23 and 6*
Unclustered	6	7
WW1	5	5
WW2	8	8
WW3	5	5
WW4	4	4
WW5	5	5
WW6	5	5
WW7	6	5
WW8	5	5
Unclustered	7	8

*Twenty-nine isolates clustered together from data generated in Cleveland. These same isolates formed two clusters of 6 and 23 isolates when rep-PCR was performed in Cologne.

RESULTS

One hundred rep-PCR patterns generated in laboratory A were compared with the same number of patterns from laboratory B. Although we have previously used the KL method to identify worldwide clonal clusters (Higgins *et al.*, 2010), in the present study we employed both the KL and PC statistical methods.

Isolate pairs (step 1)

Analysis of isolate pairs demonstrated that the reproducibility of rep-PCR fingerprints was partially dependent on the statistical method employed. With the PC method, 12 isolate pairs were identical ($\geqslant$98 % similarity) compared

Table 2. Number of isolate pairs showing similarity between laboratories using the PC and KL methods

Percentage similarity between isolate pairs	No. of isolate pairs		Interpretation
	PC method	KL method	
⩾98	12	19	Identical
⩾95–97.9	64	43	Closely related
90–94.9	23	32	Unrelated
⩽89.9	1	6	Unrelated

to 19 isolate pairs that were identical when analysed using the KL method (Table 2). However, the PC statistical method gave an overall greater strain-to-strain similarity, with 64 isolate pairs showing closely related fingerprint patterns versus 43 closely related patterns when the KL method was used. In addition, using the KL method, a higher incidence of isolate pairs showing 90–94.9 % similarity (32 vs 23) and ⩽89.9 % similarity (6 vs 1) was observed (Table 2). Therefore few rep-PCR fingerprints were identical. Discrepancies occurred due to variability in rep-PCR fingerprints and were clearly evident as a combination of differences in band intensity and/or missing bands. For example, Fig. 1(a) shows fingerprints of isolate 17 generated at both sites. The banding patterns are nearly identical and the samples differ only in the intensity of the peaks. However, in Fig. 1(b), isolate 27 shows differences not only in band intensity but also in bands that are absent.

Interlaboratory clustering (step 2)

To determine if interlaboratory clustering was conserved, we compared clustering (groups of isolates showing ⩾95 % similarity) generated from data in laboratory A with clustering generated in laboratory B. Table 1 summarizes cluster conservation between the two laboratories. For this comparison, rep-PCR data were separated into four separate datasets based on origin of the isolates and where the rep-PCR was performed. Each dataset therefore contained 50 rep-PCR fingerprints. We employed the PC statistical method for this analysis. Isolates that originated from Cleveland were represented by five clusters (termed A–E). Comparison of rep-PCR patterns of these strains amplified in Cleveland and Cologne showed that on the whole, clustering was conserved. For example, cluster A consisted of five isolates when tested in Cleveland, and these same isolates also clustered when rep-PCR was performed in Cologne. The only major difference was cluster E, which was not wholly conserved between laboratories: rep-PCR patterns from Cleveland had 29 isolates clustering at ⩾95 % similarity but when these isolates were tested in Cologne they formed two separate clusters of 23 and 6 isolates. Rep-PCR patterns from the isolates originating from Cologne showed a similar degree of clustering (Table 1). Therefore, cluster integrity was maintained.

Merging data from two laboratories (step 3)

When rep-PCR fingerprints generated in both laboratories were merged, clustering was found to be partly laboratory-specific (Fig. 2). For example in Fig. 2 using the PC method of analysis, isolates CL-28, CL-2, CL-25 and CL-20 (rep-PCR fingerprints generated in Cleveland) cluster together and the corresponding fingerprints generated in Cologne are adjacent to this cluster, i.e. they are not intermingled. Taken together, these eight fingerprints still form a cluster where there is ⩾95 % similarity between the samples, but the CL fingerprints show greater similarity with one another than to KO fingerprints. With few exceptions, CL clusters were adjacent to their corresponding KO

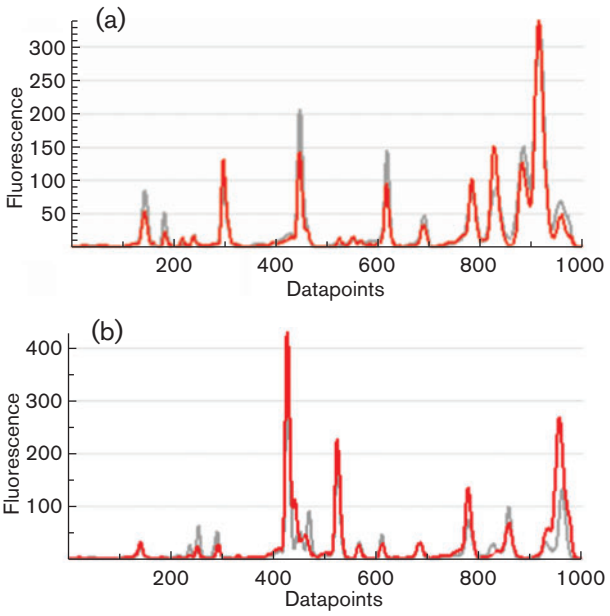

Fig. 1. Comparison of fingerprints using the PC and KL statistical methods. (a) Comparison between CL-17 (red line) and KO-17 (grey line). By the PC method the isolates show 96.6 % similarity and by the KL method 94.9 % similarity. (b) Comparison between CL-27 (red line) and KO-27 (grey line). By the PC method the isolates show 95 % similarity and by the KL method 90.9 % similarity.

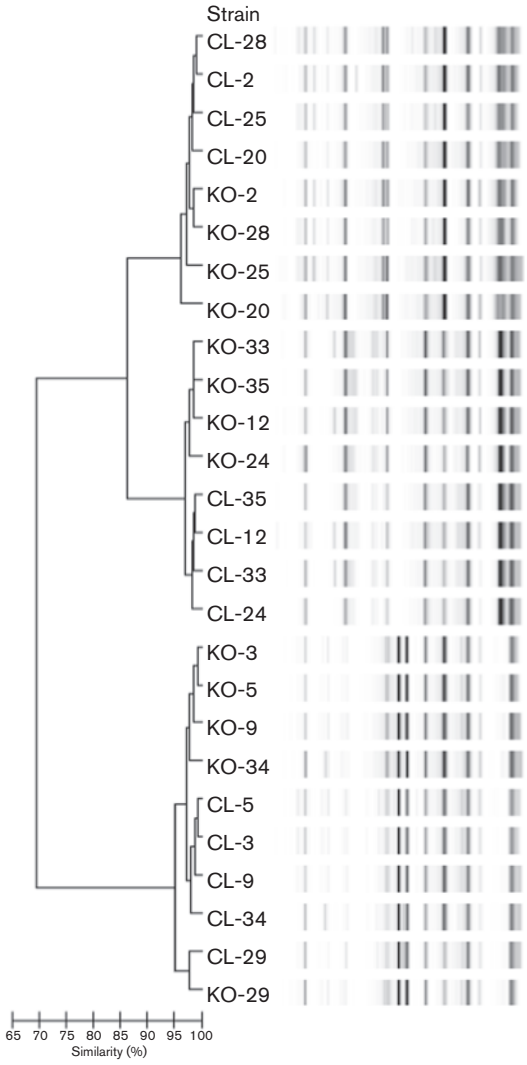

Fig. 2. Rep-PCR analysis. Dendrogram and computer-generated image of rep-PCR banding patterns showing clustering between fingerprints of corresponding isolate pairs generated in Cleveland (CL) and Cologne (KO) using the PC statistical method.

clusters, irrespective of the statistical method used for analysis (Fig. 2).

Comparison with library (step 4)

One further analysis was performed with these merged data. We used the classification report of DiversiLab with the 'top match' function using our in-house library of WW clusters. This revealed that 84 isolate pairs were in agreement, with both isolates clustering in the same epidemiological group with a similarity of $\geqslant$95 % using the PC method (Table 3). If this threshold was lowered to $\geqslant$93.5 % a further 11 isolate pairs clustered in the same epidemiological group. The KL method showed less agreement.

Table 3. Number of isolate pairs clustering within the same worldwide clonal cluster using the PC and KL methods in the centrally performed pattern analysis

Percentage similarity	No. of isolate pairs	
	PC method	KL method
$\geqslant$95	84	62
93.5–94.9	11	22
$\leqslant$93.4	5	16

DISCUSSION

Our results show that although DiversiLab fingerprints are fairly well conserved, they were not identical between laboratories. Discrepancies in banding patterns are most likely related to the technology employed, although factors such as DNA template concentration could also play a role. While it could be argued that differences in the DNA extraction methods may also play a part, we have previously found that there was little or no difference in rep-PCR fingerprints when we compared DNA prepared using functional Qiagen and MO BIO kits. The DiversiLab system uses a very precise protocol and standardized reagents to reduce the effect of outside influences (e.g. primer and dNTP concentration) that have the potential to affect rep-PCR fingerprints. However, amplification of PCR products is also dependent upon annealing temperature, and differences in the heating block between PCR machines may lead to higher or lower numbers of amplicons, or in some cases loss of amplicon. As part of our initial evaluation of DiversiLab we tested the effect of different PCR machines on rep-PCR patterns with *A. baumannii* and *Staphylococcus aureus*. Using the same template DNA and PCR reagents, samples were amplified in three different PCR machines and we found that for some strains, similarities were as low as 95 % (unpublished data). Therefore it is highly likely that differences in rep-PCR fingerprints reported here result primarily from the use of different PCR machines and not from other factors.

To our knowledge, studies have not been done on the interlaboratory reproducibility of DiversiLab rep-PCR typing. In a recent publication Carretto *et al.* (2011) found intralaboratory reproducibility to be 98.6 % when the procedure was tested in triplicate, but it was not described how their replicates were performed: three independent DNA samples, three independent rep-PCRs or three different DNA chips. Recent comparisons of DiversiLab rep-PCR typing have been made against multi-locus sequence typing, PFGE and spa-typing (Church *et al.*, 2011; Brolund *et al.*, 2010; Ben-Darif *et al.*, 2010), with the authors concluding that DiversiLab rep-PCR typing is a useful tool for identifying outbreaks. However, using isolates with previously determined *Salmonella enterica* serotypes, Ben-Darif *et al.* (2010) found that 10 % of their isolates failed to cluster with the correct serotype in the DiversiLab *Salmonella* serotype library. This may mean that

there is a small but significant problem with comparing fingerprints generated in different laboratories.

In summary, we have shown that rep-PCR clustering using DiversiLab is reproducible, demonstrating the robustness and broad applicability of the method. However, given that a small but significant proportion of isolates did not cluster when compared to their corresponding fingerprints generated in another laboratory, care should be exercised in the interpretation of every isolate. We recommend that individual rep-PCR libraries should also be generated in house to serve as reference standards for local analysis of outbreaks since centrally hosted libraries are probably not able to correctly assess all strain identities, e.g. for outbreak delineation. Despite these limitations, our data show that conclusions regarding clonal relatedness, while dependent on the statistical method used, can be reached independent of the laboratory where the analysis was performed.

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