International Multicenter Evaluation of the DiversiLab Bacterial Typing System for *Escherichia coli* and *Klebsiella* spp.

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Internationally successful multi-drug resistant clones are globally increasing in prevalence, which makes the ability to identify these clones urgent. However, adequate easy-to-perform and reproducible typing methods are lacking. We investigated whether DiversiLab (DL), an automated repetitive-sequence-based PCR bacterial typing system (bioMérieux), is suitable to compare isolates analyzed at different geographic centers. A total of 39 *Escherichia coli* and 39 *Klebsiella* spp. isolates previously typed by the coordinating center were analyzed. Pulsed-field gel electrophoresis (PFGE) confirmed the presence of one cluster of six isolates, three clusters of three isolates, and three clusters of two isolates for each set of isolates. DL was performed in 11 centers in six different countries using the same protocol. A total of 425 *E. coli* and 422 *Klebsiella* spp. DL profiles were obtained. DL showed a lower discriminatory power than PFGE for *E. coli*. Local DL data showed a low concordance as indicated by the adjusted Rand’s and adjusted Wallace’s coefficients (*E. coli*: range 0.132-0.740 and 0.070-1.0; *Klebsiella* spp.: 0.091-0.864 and 0.056-1.0, respectively). Central analysis showed significantly improved concordance (*E. coli*: 0.473-1.0 and 0.290-1.0; *Klebsiella* spp. 0.513-0.965 and 0.425-1.0). Misclassification of profiles for individual isolates was mainly due to inconsistent amplification, which is most likely due to variations in the quality and amount of the isolated DNA used for amplification. Despite local variations, DL may have the potential to indicate the occurrence of clonal outbreaks in an international setting, provided there is strict adherence to standardized, reproducible, DNA isolation methods and analysis protocols, all supported by a central database for profile comparison.
The prevalence of internationally successful multidrug-resistant clones, e.g., *Klebsiella pneumoniae* ST258 and *Escherichia coli* ST131, is increasing globally (1-4). The spread of these high-risk clones is aided by an increase in international travel, medical treatment abroad, and repatriated patients (5, 6). The ability to identify these epidemic clones is of importance to understand the epidemiology of these isolates and may alert hospitals of the emergence of epidemic strains. This requires a reliable typing method capable of identifying epidemic clones that can be performed at different centers together with an internationally accessible database for comparisons (7). Pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) have been used for this purpose. The main drawback of PFGE, however, is poor reproducibility due to technical variation and the time consuming nature of the method, whereas MLST lacks sufficient discriminatory power. Multiple-variable number of tandem repeat analysis (MLVA) and amplified fragment length polymorphism analysis (AFLP) are also typing methods with databases, but these methods are not widespread and also suffer from technical limitations (8-10).

The DiversiLab (DL) bacterial typing system (bioMérieux, Marcy l’Etoile, France) that allows to obtain results within a day may offer an alternative, although it is based on repetitive-sequence-based PCR (rep-PCR) which also suffers from poor reproducibility (11, 12). By standardisation of its procedures (PCR and analysis of the amplification products) and the use of a commercial microfluidics system DL improved its reproducibility and has the potential for multicenter comparisons of typing data, thereby possibly facilitating identification of international clones. The method can be easily introduced into routine settings and requires less hands-on time than PFGE. The ease of use is also facilitated by the use of the associated website that allows easy analysis and visualization of the data. However, comparisons between different centers have not yet been performed. The aim of this study was to evaluate the interlaboratory reproducibility of DL for *E. coli* and *Klebsiella* spp. in an
international, multicentre setting. Eleven centers in six countries typed 39 *E. coli* and 39 *Klebsiella* spp. isolates, which were previously characterized by PFGE and represent either outbreaks or unique isolates.
MATERIALS AND METHODS

Isolates and centers. In total, 39 *E. coli* isolates and 39 *Klebsiella* spp. (34 *K. pneumoniae* and 5 *K. oxytoca*) that had been typed previously by PFGE were selected from the collection of the Hospital Hygiene Department of the University Medical Center Utrecht and a study into the population distribution of β-lactamases conferring resistance to third-generation cephalosporins in human clinical isolates in the Netherlands (13). Clustering in PFGE was based on a cut-off of 80% and in agreement with epidemiological data. Repeated PFGE typing confirmed in both groups one cluster of six isolates, three clusters of three isolates each, three clusters of two isolates, and 18 isolates with unique profiles. Isolates were initially identified by standard microbiology methods and later confirmed by MALDI-TOF MS (Bruker Daltonics, Germany).

The isolates, from a single plate, were shipped on M40 Transystem Amies Agar Gel transport swabs (Copan Italia SpA, Brescia, Italy) to the 11 participating centers in 6 countries across Europe (Austria, England, Germany, Spain, The Netherlands) and Canada.

Typing. All centers used the same protocol. DNA was isolated using an UltraClean® Microbial DNA Isolation kit (Mo Bio Laboratories Inc., Carlsbad, USA) according to the manufacturer’s instructions with two changes for the *Klebsiella* spp. isolates. It was recommended to all centers to use a 10 µl loop of bacteria and 900 µl of the MD3 solution based on previous experience (8). Nanodrop™, a highly sensitive spectrophotometer, or equivalent was used to quantify the DNA. The minimal required concentration was 25 ng/µl. The DNA was required to have an OD 260/280 ratio >1.7 and OD 260/230 ratio >1.3. Each center performed the PCR with AmpliTaq (Invitrogen, Breda, The Netherlands) and the kits specified by the manufacturer for *E. coli* (kit no. 270613) and *Klebsiella* (kit no. 270615). PCR products were analyzed using standard chips (no. 270670). Each center uploaded the chip results to its own bioMérieux DiversiLab website for local analysis.
Data analysis and statistical methods. Analysis of the profiles was performed at two levels. First, at the level of the individual laboratory and secondly all profiles were examined by staff at the coordinating center. All centers received the same protocol for analysis of the data at the first level. DL results to be used for comparisons were recommended to lack automatic warnings and have peak intensities of at least 100 or above for at least one peak. Analysis was performed using Pearson correlation in the dedicated DL software of the manufacturer (version 3.4). Isolates with a similarity <95% were considered different and isolates with a similarity >98% were considered indistinguishable. All isolates with a similarity >95% and <98% were judged manually using the pattern overlay of the analysis tool in the software.

Statistical analysis (adjusted Rand’s and adjusted Wallace’s coefficients) was performed using the online tool of the Instituto de Medicina Molecular of the University of Lisbon (http://darwin.phyloviz.net/ComparingPartitions/index.php). The discriminatory power was estimated by Simpson’s index of diversity (14). Ninety-five percent confidence intervals (CI95) for discriminatory indices were calculated according to the method of Grundmann et al (15). Non-overlapping confidence intervals were regarded as representing statistically significant differences in discriminatory power (15).

Isolates designated as non-typeable, defined as failing to meet the minimum criteria of peak intensities, were included in the statistical analysis as unique values. All non-processed, non-viable, and isolates that could not be amplified, defined as failure to generate an amplification product in the PCR step, were removed from the statistical analysis. PFGE data were not communicated to the local centers.

Central analysis was performed by the chief investigators (GV, AF). The data were also transferred to a dedicated website created by bioMérieux with the same DL analysis software as used by the individual centers for the analysis of the combined data. The data
RESULTS

Typing data of 425 of 429 *E. coli* and 422 of 429 *Klebsiella* spp. were available for analysis. Three of the *E. coli* samples were not processed (<1%) and one showed no amplification in one of the centers (<1%). Similarly, seven of the *Klebsiella* spp. samples (1%) were not processed (<1%). The analyses performed by the individual centers were confirmed by the central laboratory.

The local analyses showed little consensus (Figure 1A), which is confirmed by the adjusted Rand’s and adjusted Wallace’s coefficients (Tables S1 and S2). Nevertheless, the local analyses of the *E. coli* DL data agreed in 96% of the cases for half of the unique isolates according to PFGE (Figure 1A; bottom nine isolates). A similar level of consensus was obtained for the cluster of isolates 31E and 32E and the cluster consisting of isolates 14E, 24E and 25E. However, there was no consensus in the assignment for the isolates in the three other clusters. The lack of consensus was confirmed by the adjusted Rand’s and adjusted Wallace’s coefficients (Tables S1 and S2) with mean values of 0.357 and 0.389, respectively. Both statistical analyses showed poor overall values (range 0.132-0.740 and 0.070-1.0, respectively). An exception for the adjusted Wallace’s coefficients was the comparison with center 3, which was due to the fact that analysis here resulted in one big cluster that encompassed the smaller clusters identified by other centers.

For *E. coli* DL analysis from local centers showed less discriminatory power compared to PFGE. Examples of this are the DL clusters with isolates 31E and 32E, 18E and 37E, and 14E, 24E, and 25E (Figure 1A).
The central analysis of *E. coli* DL also resulted in less discriminatory power in comparison to the PFGE analysis, which is reflected by lower Simpson’s index of diversity (Table S3). The Simpson’s index of diversity was 0.964 (95% CI 0.935-0.992) for PFGE and ranged from 0.709 (95% CI 0.550-.867) to 0.864 (95% CI 0.767-0.961). However, it showed improved consensus between the data from the different centers (Figure 1B and Tables S4 and S5) compared to local analysis. This was most apparent in the cluster consisting of 22E and 39E, the cluster of 18E and 37E, and the cluster of 34E up to and including 11E in Figure 1B, which improved significantly in concordance. This is reflected in the adjusted Rand’s and adjusted Wallace’s coefficients (Tables S4 and S5). The adjusted Rand’s coefficients have a mean value of 0.8 (range 0.473-1.0). The average is mainly lowered by results obtained by center 11. The directional adjusted Wallace’s coefficient also reflects the improved concordance with a mean value of 0.83 and for 6 of the 11 centers the mean was above 0.9 (range 0.290-1.0).

The *Klebsiella* spp. were not separated into different species as most of them were *K. pneumoniae* (n=34) and only a few *K. oxytoca* (n=5) were included and these were unique isolates based on PFGE. The assignments of *Klebsiella* spp. agreed in 97% of the cases for two thirds of the unique isolates according to PFGE (Figure 2A; last 12 isolates). Concordance of isolates belonging to a cluster was larger than found for *E. coli*, though more outliers were present. This is reflected in overall higher and more consistent values of the adjusted Rand’s and adjusted Wallace’s coefficients (Table S6 and S7), although both statistical analyses show poor overall values (range 0.091-0.864 and 0.056-1.0, respectively). An exception to this finding for the adjusted Wallace’s coefficients is center 9, where analysis identified one big cluster, which encompassed the smaller clusters that other centers identified.
In comparison to the PFGE analysis for Klebsiella spp. DL did not show a significant difference in discriminatory power in the local analysis (Figure 2A and Tables S6 and S7). Examples of this are the isolates 03K and the isolates 35K and 37K.

A marked improvement in the degree of concordance between the centers was obtained by central analysis and the discriminatory power remained acceptable (Figure 2B and Table S3). Most notably, the formation of the clusters containing the isolates 23K, 09K, and 36K, the cluster of 02K and 33K, the cluster of 26K and 31K, the cluster of 37K and 35K and the cluster consisting of 21K and 38K improved in concordance significantly. This is reflected in the statistical analysis (Table S8 and S9); the adjusted Rand’s coefficients have a mean value of 0.71 (range 0.513-0.965). The values of the adjusted Wallace’s coefficient also increased in comparison to the local analyses to a mean value of 0.73 (range 0.425-1.0). It has to be noted that although different clusters could be assigned to the isolates 02K, 05K, 06K, 11K, 26K, 28K, 31K, and 33K, the patterns of these clusters were closely related and that these clusters and unique isolates may be considered a clonal complex (Figure 3).

DISCUSSION

We performed an international multicenter evaluation of the DiversiLab bacterial typing system for *E. coli* and Klebsiella spp. In order to assess whether DL is suitable to compare isolates analyzed at different centers. Some local studies (8, 16-18) showed that DL performed well for several species, e.g., Klebsiella spp., and to a lesser extent, for some others, e.g., *E. coli*. Moreover, it was shown to identify some of circulating high risk clones such as ST131 *E. coli* harbouring CTX-M-15 extended spectrum β-lactamasae and clonal complex 147 from *K. pneumoniae* expressing carbapenemases (19-20).

In our study, 11 centers from six different countries typed 39 *E. coli* and 39 Klebsiella spp. isolates that were selected based on PFGE results. The main findings of the study were:
1) DL had a lower discriminatory power for the E. coli isolates than PFGE; 2) the clustering obtained by the different centers was only partly concordant; 3) central analysis improved the clustering to an acceptable level. In comparison to PFGE typing, DL had less discriminatory power, creating larger clusters and clustering of isolates that using PFGE are considered unique (Figure 1-2). This has also been demonstrated by other studies (21-23). Isolates considered to be different by DL are also considered to be different by using PFGE.

A number of factors might have contributed to the less than optimal concordance between the centers. The main factors are incorrect clustering by Pearson correlation and misclassification of isolates due to variation in the amplification products between isolates. In some cases the amplification signals were faint or even completely absent causing incorrect clustering by Pearson correlation. In the central analysis, which completely relied on interpretation of the overlays, a more consistent clustering was obtained (see Figures 1B and 2B). This was further aided by the fact that, for each isolate, at least 10 replicates were available. This allowed for assessing and mitigation of continuous minor changes in the patterns of the isolates when examining individual clusters. Sometimes, replicates for the same isolates did not cluster next to each other but across the whole cluster (Figure 3). These data indicate that the reproducibility of one or more of the steps before analysis is insufficient.

Several issues can be responsible for this lack of reproducibility: 1) variation between persons performing the assays; 2) amplification may be inconsistent; 3) inconsistent quality and/or amount of DNA obtained during its isolation. However, this also indicates the importance of a central database to compare isolates. Moreover, person-to-person variation, variation in thermocycler performance and the quality and amount of DNA isolated are known from the experience at the central laboratory to lead to variation in the results (unpublished data). This study lacked an experimental design to measure person-to-person variation within a center.
Regardless, it is difficult to control for person-to-person variation, particularly within a multicenter setting.

Inconsistent DNA amplification is most likely not due to the PCR kits used as these are quality checked for consistent performance and the same batches were used in all centers included in this study. However, the thermocycler used may be a source of variation, especially since during the amplification process in a rep-PCR based system and sometimes less than optimal interactions between primers and target occur and slight differences in initial conditions such as small variations in primer concentrations can have a major impact on the results. Although it is possible at a local level to assign a single machine to the DL assays, this is not feasible in a multicenter setting. One of the most important parameters in amplification is the quality and amount of DNA added. The study protocol required a minimum amount and quality of the DNA (25 ng/µl and an OD 260/280 ratio >1.7 and OD 260/230 ratio >1.3). This, however, still allows for variations between centers. In addition, the possible use of different quantification platforms may contribute to variation as different platforms may yield different results (unpublished observations). Moreover, only 2 µl of DNA solution is added and small absolute variations lead to a large relative variation in volume. Furthermore, amplification products of different lengths are generated with one amplification protocol leading to competition for amplification. The variation in DNA quality and amount may be addressed by a more robust protocol. The use of automatic DNA extraction can provide a useful contribution in better controlling DNA amount and quality. It should be noted though, that the use of different types of automatic extraction machines still might contribute to problems with reproducibility of amplification.

The final factors that contributed to the differences in the assignment of isolates to different clusters are errors in administration and/or exchange of isolates and/or results and failure to adhere to protocol. These factors were most notable with isolates included for
analysis that generated poor signals or warning signals, even if a good result of a retest was present.

Despite the ability of the amplification protocol and the DL system to type every isolate in most centers, some centers reported isolates that were non-typeable according to the given criteria. Some centers retested isolates because the quality of the initial data was unacceptable. All centers performed at least a small number of retests, whereas a few required a considerable number of retests, though the number of retests varied greatly, e.g., one center retested three isolates once and another center retested 90% (70 of 78) of the isolates and repeated one isolate seven times. This also indicates that adequate training is required. No pattern could be discerned with particular isolates being retested more often than others among the centers.

We conclude that DiversiLab may have the potential to indicate the occurrence of outbreaks in an international setting, at least for *E. coli* and *Klebsiella* spp., although with lower discriminatory power than PFGE. However, this will require more reproducible DNA amplification and isolation methods, strict adherence to protocols and an international database to allow comparison of isolates. In addition, reference isolates should be used with every chip to inform on the quality of each amplification.
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REFERENCES


FIG 1  Panel A. Comparison of the local clustering of *E. coli* and the clustering of the isolates using PFGE. Isolates belonging to one cluster according to local analysis or PFGE are indicated by the same color. Panel B. Comparison of the central clustering of *E. coli* and the clustering according to PFGE. Isolates belonging to one cluster according to central analysis or PFGE are indicated by the same color. Isolates left blank were considered unique isolates according to central analysis. NT: Non-typeable. NP: Not processed by the center. NA = No amplification.

FIG 2  Panel A. Comparison of the local clustering of *Klebsiella* spp. and the clustering of the isolates using PFGE. Isolates belonging to one cluster according to local analysis or PFGE are indicated by the same color. Panel B. Comparison of the central clustering of *Klebsiella* spp. and the clustering according to PFGE. Isolates belonging to one cluster according to central analysis or PFGE are indicated by the same color. Isolates left blank were considered unique isolates according to central analysis. NT: Non-typeable. NP: Not processed by the center. NA = No amplification.

FIG 3  Clonal complex of isolates 02K, 05K, 06K, 11K, 26K, 28K, 31K, and 33K according to the central analysis. Three clusters were detected: cluster. The clusters are indicated by colors. The clusters correspond to the clusters and unique isolates in Figure 2B. Red is equivalent to light green in Figure 2B; green is equivalent to middle green; blue is equivalent to dark green; light blue is equivalent to light blue in Figure 2B, the other isolates have unique types. Isolates indicated by a, b, and c are 11K isolates who do not group with the other 11K isolates (key 79–86). The pattern for isolate d, is most likely due to a poor DNA sample.
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**Diagram A**

**Diagram B**