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Original article

Non-sequencing based molecular bacterial typing as an affordable tool for outbreak investigation in low-income countries

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ABSTRACT

Background: Multidrug-resistant bacteria represent a potential threat to patients in healthcare facilities, with higher abundance and more profound consequences in the developing world. Controlling the spread of these bacteria requires timely identification of clonally related isolates that are cross transmitted from one patient to another within healthcare facilities. The relatively high cost of sequencing-based typing methods hinders their routine use in low-income countries. This highlights the need for an alternative easily applicable molecular typing method that can be routinely used in laboratories with basic equipment and low financial resources, for epidemiologic investigation of outbreaks. **Aim and methods:** This study aimed to evaluate molecular typing of 30 multidrug-resistant (MDR) *Klebsiella pneumoniae (K. pneumoniae)* isolates, that were isolated in a previous hospital outbreak and were of previously identified clonal relatedness, by 2 non-sequencing-based typing methods: Plasmid Profile Analysis and Random Amplified Polymorphic DNA (RAPD). The obtained banding patterns were converted into binary data and phylogenetically analyzed by DARwin 6.0.21 software. **Results:** There was a statistically significant fair agreement between the 2 methods in grouping of the isolates into distinct clusters (Kappa=.23, *p*=.025). Both methods proved to be of high typeability of near 100% and discriminatory power of more than 0.9. Both methods fulfilled the performance and convenience criteria evaluated. **Conclusion:** Any of the two methods may be applicable for routine investigation of outbreaks by MDR bacteria in the developing world, when more precise molecular typing methods are not applicable for shortage of resources.

Introduction

Outbreaks by MDR bacteria are a commonly encountered problem in the healthcare settings of developing countries, particularly in the intensive care units (ICUs). This renders ICUs deadly for most admitted patients [1]. Tracking the spread of bacteria causing an outbreak in a certain healthcare facility requires identification and differentiation of bacteria to beyond the species level, in what is called bacterial typing [2].

In developing countries, still, the basic phenotypic methods for bacterial typing such as

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antibiogram pattern comparison of isolates are the only routinely implemented typing method for outbreak investigation. This can be attributed to the higher cost of molecular typing methods (due to expensive equipment, such as automated DNA sequencers or scanners) and the lack of sufficient financial and technical resources. Molecular typing methods, however, allow better observation of the clonal relationships between bacterial isolates which is specifically important during outbreaks [3].

A good typing method should have excellent typeability, which means it should assign a type to all the tested isolates. Moreover, it should have discriminatory power to discriminate between two epidemiologically unrelated strains. It should also be rapid and highly reproducible. The ease of using the method, the financial and technical resources needed, and the suitability for computerized analysis, interpretation, and storage of results are also important criteria for the selection of a typing method [4]. With the availability of a variety of molecular typing techniques, each laboratory should make its own decision in the selection of the optimum technique that is applicable for use with the available resources and provides satisfactory results [4].

Despite being a highly attractive tool for epidemiological investigations, interpretation of results from Whole Genome Sequencing (WGS) represents a challenge to its routine implementation. Nevertheless, the high cost of WGS and other sequencing-based methods, such as multi-locus sequence type analysis (MLST) significantly hinders their routine use in low-income countries [5-7]. Microarray technology is also a perfect genotyping tool but with a high cost [8].

Other molecular typing techniques include PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) which is easy and of excellent reproducibility but with poor to moderate discriminatory power and low throughput [3].

Pulse field gel electrophoresis is another accurate method for short-term epidemiological studies, but it is time-consuming, labor-intensive, and requires complex computer-based analysis of banding patterns [9,10].

Random amplification of polymorphic DNA (RAPD) is a rapid and easy typing method with a relatively low cost. It relies on the use of short primers of random sequences for amplification of multiple regions of the bacterial genome at a low annealing temperature. The repeatability and reproducibility of this method, however, should be closely monitored, with the application of stringent standardization measures, due to the sensitivity to different reagents and machines[3,5].

Plasmid profile analysis is one of the early used molecular typing methods targeting extrachromosomal DNA. The limitation of this method is linked to the plasmid where it can be gained or lost through conjugation, although still, small plasmids tend to be more stable [11]. The advantages of this method include its relatively low cost and simplicity. Also, being a DNA non-amplification method saves the time required for PCR [12].

The aim of this study was to evaluate molecular typing of 30 MDR *K. pneumoniae* isolates by 2 non-sequencing-based typing methods; Plasmid Profile Analysis and Random Amplified Polymorphic DNA (RAPD), to evaluate the possibility of their routine epidemiologic use in low-income countries. The evaluation was based on the typeability, discriminatory power, cost and equipment needed, interpretation procedure, typing system concordance, and the possibility of simultaneous typing of all isolates under investigation in a one-time session.

Materials and methods

Bacterial isolates

This study included 30 MDR *K. pneumoniae* isolates obtained from different types of clinical samples from patients in the ICU of a pediatric University Hospital in Alexandria. Identification of the isolates was done by conventional biochemical reactions, and antimicrobial susceptibility testing was done by VITEK 2 system (BioMerieux, Durham, NC). Identification was further confirmed by PCR amplification of 16S–23S internal transcribed spacer (ITS) region using specific primers [13]. The isolates under study were previously genotyped by multiple locus variable number tandem repeat analysis (MLVA) and *wzi* gene sequencing in a previous study, and many of them were found to be clonally related [14]. The isolates' previous identification numbers, their equivalent code number in the current study, and the clonal groups to which the isolates were assigned are illustrated in **table (S1),** supplementary material. The isolates were stored at -80 °C for subsequent work. Fresh subcultures were prepared from the stored isolates for subsequent molecular tests, which

were all performed with blinded testing. All isolates were tested only once, to evaluate the typeability of the method in case of lack of enough time and/or additional resources for repeated testing.

Plasmid profile analysis

Each test isolate was inoculated in 20 ml LB broth medium, with incubation for 16 hours at 37°C with vigorous shaking (at approximately 300 rpm). Plasmid DNA was extracted from 5ml of the culture by GeneJET™ Plasmid Miniprep Kit (Thermo Scientific, USA), following the manufacturers' instructions. Agarose gel electrophoresis of the extracted plasmid DNA preparations was done using 1% (w/v) agarose gel in TAE buffer containing 1 mg/ml ethidium bromide. Each 10 isolates were loaded in a separate gel to facilitate comparability of images. Electrophoresis was always performed at 100 volts for 40 minutes. Visualization and photography of the separated plasmid DNA was done on an UV transilluminator. The apparent bands of the plasmids were compared to ThermoscientificTM O'GeneRuler 1 kb Plus DNA Ladder. Photographs of the separated plasmid DNA from the 30 isolates were aligned. Visual assignment of band levels was performed by each member of the research team individually, and the results were compared to ensure the repeatability of interpretation.

RAPD

DNA was extracted from the bacterial isolates by boiling method [15]. The primer 640 (CGTGGGGCCT) [16], purchased from Thermofisher, was used for RAPD PCR analysis. The 25 µL reaction mixture contained 12.5 µL of 2x my Taq HS mix, master mix (Bioline, UK), 2 µL of the primer (20 pmol), and 3 µL of the template DNA. The thermal profile was 94°C for 3 minutes, 36 cycles of denaturation at 94°C for 1 minute, annealing at 36°C for 1 min, and extension at 72°C for 2 minutes, followed by final extension at 72°C for 9 minutes. Each 10 isolates were loaded in a separate gel to facilitate comparability of images. The amplicons were separated by agarose gel electrophoresis at 100 Volts using 1% (w/v) agarose in TAE buffer, stained with 1 mg/ml ethidium bromide. The DNA ladder was run with isolates. Time of electrophoresis was always set at 40 minutes to obtain comparable images. Banding patterns in the gel were visualized and photographed on an UV transilluminator. Photographs were aligned and visual assignment of band levels was performed individually by the research team members, with comparison of results.

Analysis of banding patterns

For images of agarose gel electrophoresis from both methods, band levels were assigned binary values of 1 or 0 when present or absent, respectively. The assigned binary data were phylogenetically analyzed using DARwin 6.0.21 software. The trees were constructed by hierarchical clustering based on unweighted neighbor joining algorithm and Dice correlation coefficient.

Results

Plasmid profile analysis

All the 30 *K. pneumoniae* isolates were typeable by plasmid profile analysis as every isolate gave a discrete banding pattern on the agarose gel **(Figure 1)**. The binary data from each isolate is illustrated in **figure (S1),** supplementary material.

According to the dendrogram **(Figure 2)**, based on 100% identity, the 30 *K. pneumoniae* isolates under study displayed 18 distinct plasmid profile patterns. The 30 isolates grouped into 3 main clusters: cluster A, comprising 10 isolates with 2 distinct patterns, cluster C, comprising 11 isolates with 9 distinct patterns, and cluster B, with 9 isolates of 7 distinct patterns**.** Based on Simpson's diversity index, the calculated discriminatory power of plasmid profile analysis was 0.94, indicating that the method is highly discriminatory.

RAPD

Agarose gel electrophoresis of the amplicons from RAPD PCR reaction of the 30 *K. pneumoniae* isolates revealed unique banding patterns for 29 isolates **(Figure 3)**, with one isolate showing no bands, probably due to PCR inhibition. Accordingly, the typeability of RAPD for the 30 isolates under study, considering a one-session testing, was 96.7%. The binary data from each isolate is illustrated in **figure (S2),** supplementary material.

The dendrogram showed that the 29 typed isolates displayed 23 distinct patterns with 100% identity. The isolates grouped into 3 main clusters, Cluster A, comprising 20 isolates, Cluster B, with 8 isolates having 4 distinct patterns, and cluster C comprising 1 isolate. The 20 isolates in cluster A were further divided into 2 subclusters, A1, including 14 isolates of 13 distinct patterns, and cluster A2, including 6 isolates of 5 distinct patterns **(Figure 4)**. The calculated discriminatory power of RAPD was 0.97,

based on Simpson's diversity index, indicating that the method is highly discriminatory.

Typing system concordance

Among the 29 isolates that were typed by the 2 methods; 8 isolates were assigned into the same cluster (cluster A) by plasmid profile analysis as well as by RAPD. In addition, 5 isolates were assigned into the same cluster (cluster B) by both methods. On the other hand, 9 isolates were assigned together in cluster C by plasmid profile analysis, whilst RAPD typing resulted in their clustering together in cluster A. Data was analyzed using the Statistical Package for Social Sciences (SPSS ver.20 Chicago, IL, USA). Evaluation of the concordance between the two methods was performed by using Kappa statistic. The test revealed that there was a statistically significant fair agreement between the 2 methods in grouping of the isolates into distinct clusters (Kappa=.23, *p*=.025), **(Table 1)**.

Table 1. Evaluation of the concordance between molecular clustering of the 30 *K. pneumoniae* isolates by plasmid profile analysis versus RAPD

			RAPD			**Total**
			A	B	C	
Plasmid	A	Count	8	2	0	10
		% Within RAPD	40.0%	25.0%	0.0%	34.5%
	B	Count	3	5	0	8
		% Within RAPD	15.0%	62.5%	0.0%	27.6%
	C	Count	9	1	1	11
		% within RAPD	45.0%	12.5%	100.0%	37.9%
Total		Count	20	8	1	29
		% within RAPD	100.0%	100.0%	100.0%	100.0%

		Value	**Approximate Significance**
Measure of agreement*	Kappa	.231	.025
N of valid cases		29	

*Data were analyzed using the Statistical Package for Social Sciences (SPSS ver.20 Chicago, IL, USA).

Table S1. Isolates' ID in the previously published study (14), their code equivalents in the current study, and the clonal groups assigned to the isolates by all 4 applied molecular typing methods

Isolate ID	Isolates' current code equivalent	Clonal group assigned by Plasmid profile analysis	Clonal group assigned by RAPD
1	1[2a]	P-B	R-B
4	2[2a]	P-B	R-B
5	3[2]	P-B	R-B
6	4[2]	P-B	R-B
7	5	P-B	R-B
10	6[a]	P-A	R-B
11	7	P-B	R-A2
12	8[a]	P-A	R-B
13	9[2]	P-B	R-A1
14	10[2]	P-C	R-B
15	11[1c]	P-C	R-A1
16	12[4]	P-C	R-A1
18	13	P-A	R-A1
19	14[2]	P-C	R-A1
20	15[b]	P-C	R-A2
21	16[2a]	P-C	R-A1
22	17	P-C	R-C
24	18[b]	P-C	R-A1
25	19[3]	P-C	R-A1
27	20[3c]	P-A	R-A1
28	21[4]	P-A	R-A1
30	22[1c]	P-A	R-A1
31	23[4]	P-A	R-A1
32	24	P-A	R-A1
33	25[4]	P-A	R-A1
34	26[2]	P-B	R-A2
38	27	P-B	R-A2
40	28	P-A	R-A2
41	29	P-C	R-A2
43	30[b]	P-C	NT

[1,2,3,4]: Clonal groups by MLVA (100% identity) (14); [a,b,c]: Clonal groups by *wzi*-gene sequencing (14); P-A, P-B, P-C: Clonal groups by Plasmid profile analysis; R-A1, R-A2, R-B, R-C: Clonal groups by RAPD, NT: not typed.

Figure 1. Agarose gel electrophoresis showing banding patterns of the 30 *K. pneumoniae* isolates by plasmid profile analysis typing method in comparison with the bands of a DNA ladder (ThermoscientificTM O'GeneRuler 1 kb Plus). Lane numbers indicate the code of the tested isolates.

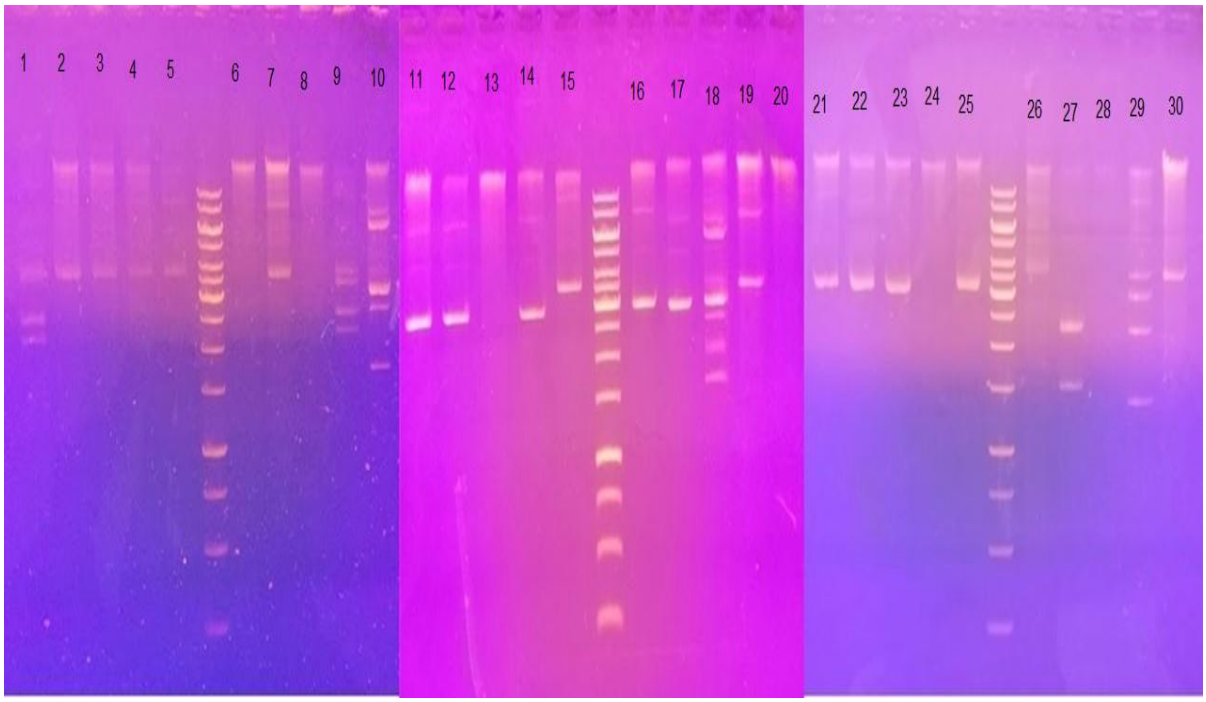

Figure 2. Dendrogram generated by hierarchical clustering method based on unweighted neighbor-joining algorithm and Dice correlation coefficient, showing the genetic similarity among *K. pneumoniae* isolates by Plasmid Profile Analysis typing method.

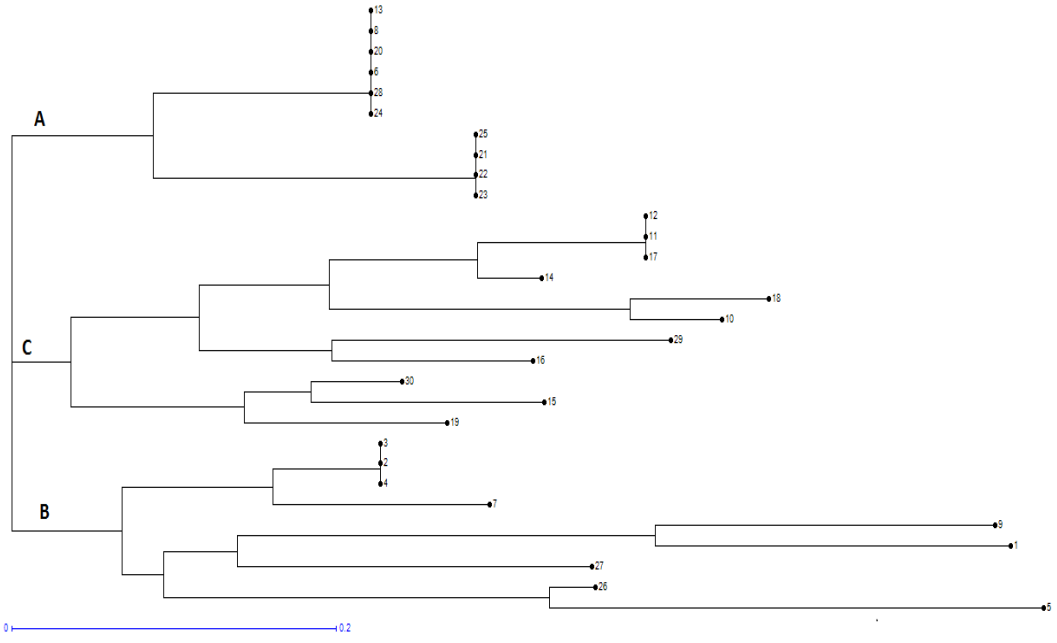

Figure 3. Agarose gel electrophoresis showing banding patterns of the 30 *K. pneumoniae* isolates by Random Amplified Polymorphic DNA (RAPD) typing method in comparison with the bands of a DNA ladder (ThermoscientificTM O'GeneRuler 1 kb Plus). Lane numbers indicate the code of the tested isolates.

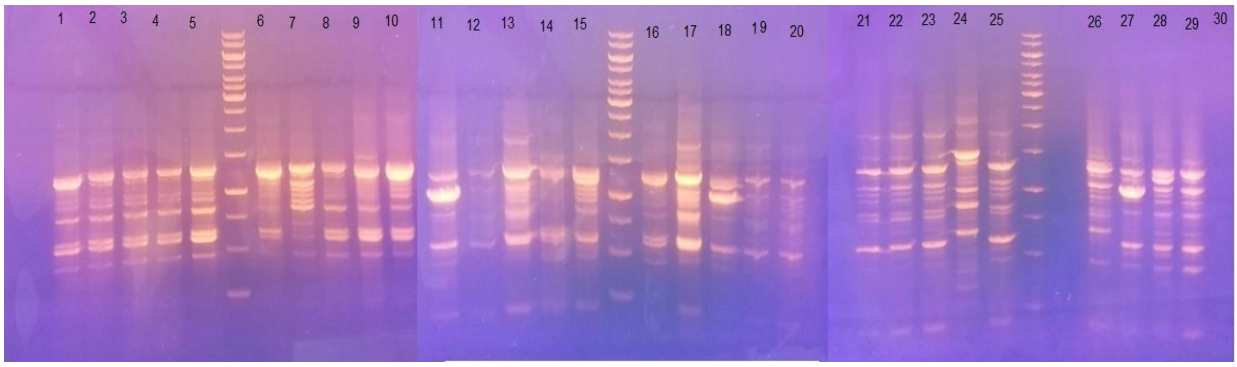

Figure 4. Dendrogram generated by hierarchical clustering method based on unweighted neighbor joining algorithm and Dice correlation coefficient, showing the genetic similarity among *K. pneumoniae* isolates by Random amplification of polymorphic DNA(RAPD) typing method.

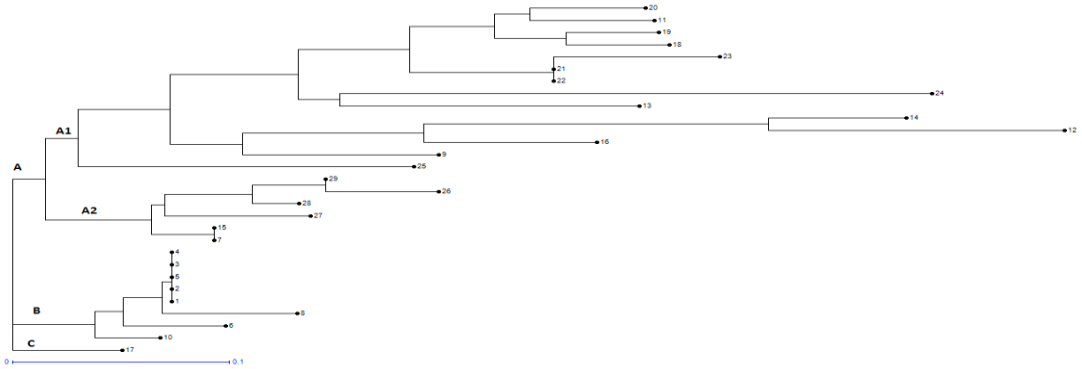

Figure S5. Binary data generated from visual analysis of banding pattern of Plasmid profile analysis of the 30 isolates (P1-P30). Columns: A: >10000, B: 10000bp, C: 9000bp, D: 8000bp, E: 7000bp, F: 6000bp, G: 4500bp, H: 3750bp, I: 3500bp, J: 3250bp, K: 3000bp. L: 2500bp. M: 2100bp, N: 2000bp, O: 1750bp, P: 1500bp, Q: 1400bp

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
p1	0	0	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0
p2	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
p3	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
p4	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
p5	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
p6	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
p7	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
p8	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
p9	0	0	0	0	0	0	0	0	1	1	0	1	1	0	0	0	0
p10	1	0	0	0	1	1	0	0	0	0	1	1	0	0	1	0	0
p11	1	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0
p12	1	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0
p13	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
p14	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0
p15	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
p16	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0
p17	1	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0
p18	1	0	0	0	1	1	0	0	1	0	1	1	0	1	1	0	0
p19	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0
p20	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
p21	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
p22	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
p23	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
p24	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
p25	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
p26	1	0	1	0	0	1	0	1	0	0	0	0	0	0	0	0	0
p27	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0
p28	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
p29	1	0	0	1	0	0	0	0	1	0	1	0	1	0	0	0	1
p30	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0

Figure S6. Binary data generated from visual analysis of banding patterns of the *29* isolates by RAPD (R1-R29). Columns A: 2000 bp, B: 1750 bp, C: 1500 bp, D: 1300 bp, E: 1200 bp, F: 1100 bp, G: 1000 bp, H: 900 bp, I: 800 bp, J: 650 bp, K: 600 bp, L: 550 bp, M: 400 bp, N: 350 bp, O:300 bp.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
R1	0	0	0	1	1	0	1	1	1	0	1	1	1	0	0
R2	0	0	0	1	1	0	1	1	1	0	1	1	1	0	0
R3	0	0	0	1	1	0	1	1	1	0	1	1	1	0	0
R4	0	0	0	1	1	0	1	1	1	0	1	1	1	0	0
R5	0	0	0	1	1	0	1	1	1	0	1	1	1	0	0
R6	0	0	0	1	1	0	1	1	1	0	1	1	0	0	0
R7	0	0	0	1	1	1	1	1	1	0	0	1	1	0	0
R8	0	0	0	1	1	0	0	1	1	0	1	1	1	0	0
R9	0	0	1	1	0	0	0	1	1	0	1	1	1	0	0
R10	0	0	0	1	1	1	1	1	1	0	1	1	1	0	0
R11	0	0	0	1	0	0	1	0	1	0	0	1	0	0	0
R12	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
R13	0	1	0	1	0	1	0	1	1	0	0	1	0	0	0
R14	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0
R15	0	0	0	1	1	1	1	1	1	0	0	1	1	0	0
R16	0	0	0	1	0	0	0	0	1	0	1	1	1	0	0
R17	0	1	1	1	1	0	1	1	1	0	1	1	1	0	0
R18	0	1	0	1	0	0	1	0	1	0	0	1	0	0	0
R19	0	1	1	1	0	0	1	0	1	0	0	1	0	0	0
R20	0	0	1	1	0	0	1	0	1	0	0	1	0	0	0
R21	0	1	1	1	0	0	1	1	1	0	0	1	0	0	0
R22	0	1	1	1	0	0	1	1	1	0	0	1	0	0	0
R23	0	1	1	1	0	0	1	1	1	0	0	0	0	0	0
R24	1	1	1	1	0	1	1	1	0	1	0	1	0	1	1
R25	0	1	0	1	0	1	1	1	1	0	1	0	1	0	0
R27	0	0	1	1	1	1	1	1	1	1	0	0	1	0	0
R28	0	0	1	1	1	1	1	0	1	0	0	1	1	0	0
R29	0	0	0	1	1	1	1	1	1	1	0	1	1	0	0
R30	0	0	1	1	1	1	1	1	1	1	0	1	1	0	0

Discussion

In this study we assessed plasmid profile analysis and RAPD, for use as rapid molecular typing tools for routine epidemiologic investigation in developing countries. The assessment was based on processing of all tested isolates simultaneously under the same conditions, and within the same time frame, to evaluate the typeability of each method in case of an emergency outbreak when no time is available for repeated testing, and/or where no excess reagents are available for repeated testing due to limited resources, which are the problems very likely to be encountered in low-income countries.

The assessment relied on evaluating the two typing methods included in the study with regards to performance related criteria, such as the typeability, the discriminatory power, the ease of data interpretation, and the typing system concordance. In addition, the assessment also included convenience related criteria, which were mainly; the ease of use, the financial and technical resources needed, and the time required for the obtaining the typing results [5].

The typeability of both methods was satisfactory, with slightly higher typeability for plasmid profile analysis (100%), compared to 96.7% for RAPD. This could be attributed to the fact that plasmid profile analysis does not include a PCR reaction, which could be subject to inhibition by different PCR inhibitors necessitating test repetition. Alternatively, the use of silica-based genomic DNA extraction kit can improve the quality of the extracted DNA, however, this will increase the cost per test.

Unlike our findings, **Lopes et al.** reported 90% typeability for Plasmid Profile Analysis compared to 100% typeability in RAPD [17]. On the other hand, 100% typeability for RAPD typing method was also reported by the study of **Wasfi et al.** who compared this typing method with ERIC PCR typing as well as typing by resistance pattern [18]. Several other studies also reported that typeability for RAPD was 100%, though none of these aforementioned studies stated that they depended on a one-time analysis for evaluating the parameter of typeability, which was the case in our study [19-21].

The calculated discriminatory power according to Simpson's diversity index revealed that RAPD was slightly more discriminatory (0.97) than plasmid profile analysis (0.94). This was in

agreement with the findings of **Lopes et al.** who also concluded that RAPD was more discriminatory than plasmid profile analysis [17]. A similarly high discriminatory power for RAPD typing method was also reported by **Wasfi et al.** [18], **Purighalla et al.** [15] and **Ripabelli et al.** [20] who stated that Simpson's diversity index for this typing method was 0.95, 0.96 and 0.98, in their studies, respectively.

From the prospect of typing system concordance, statistical analysis of the phylogenetic data obtained from typing of the 30 isolates in this study revealed that there was a statistically significant fair agreement between clustering of the isolates by the two methods (Kappa=.23, p=.025).

Typing by both methods was of relatively low cost in comparison with sequencing-based typing methods, especially with the paucity of sequencing facilities in low-income countries, which necessitates distant shipment of samples for sequencing, and the consequent delay in obtaining sequencing data, which may be further compromised by obtaining data of low quality that would hinder obtaining any result for a number of samples under investigation. On the other hand, typing by RAPD requires no more than the purchase of random primers, and the availability of a thermal cycler, aside from the availability of agarose gel electrophoresis equipment and reagents. Meanwhile, even in the absence of a thermal cycler, plasmid profile analysis can still be employed for typing. The use of free software for phylogenetic analysis of the data obtained represents an added advantage from the prospect of the overall cost of the molecular typing method.

Concerning the ease of interpretation of data obtained from typing of the isolates by both methods; visual comparison of banding patterns was facilitated by the referral to the 1 KB ladder used during gel electrophoresis. The fixed duration of migration in gel enabled comparable separation of the ladder bands, facilitating comparison of banding patterns of isolates with minimal bias. Alignment of the gel images enabled overview of the banding patterns from all isolates simultaneously, which further facilitated the interpretation of results.

It is mandatory for each laboratory, however, to optimize and standardize the procedures for testing and results interpretation to maintain repeatability and consistency of the results. This should include establishment of a detailed standard operating procedure with standardization of all variable parameters such as the type of reagents (also the brand, if possible), and the amount of input DNA from each sample. In addition, a dedicated team of at least two members should be assigned to the task of interpretation of banding patterns, ensuring that the results are repeatedly interpreted in the same way by each one of the team members.

Conclusion

Both plasmid profile analysis and RAPD were of high typeability, discrimination power, and relatively low cost. In addition, both methods do not require high technical skills for test performance and results interpretation, nor the presence of expensive lab equipment. The two methods can provide satisfactory results within a reasonable time frame, which makes them an excellent option for genotypic molecular investigation of outbreaks caused by bacteria in developing countries where the expenses of more precise typing methods are unaffordable.

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Competing interests

The authors have no potential conflict of interest to declare.

Authors' contribution

Both authors have equally participated in the conception of the study, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content, and final approval of the version to be submitted.

Ethics approval

The research design has been approved in 2018 by the ethical committee of the Medical Research Institute, Alexandria University. IORG 0008812. The research was performed on bacterial isolates collected from clinical samples that were already cultured as part of the routine work in the microbiology laboratory of the pediatric hospital of Alexandria University. No human participants, their data nor biological material from them was utilized in the research.

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