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ORIGINAL ARTICLE

Antibiotyping, RAPD- and ERIC-PCR fingerprinting of *Klebsiella pneumoniae* clinical isolates at a tertiary reference hospital in Denpasar, Bali, Indonesia

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ABSTRACT

Background and Objectives: *Klebsiella pneumoniae* is a healthcare-associated infections agent and could be an extended spectrum β-lactamase (ESBL) producer. Understanding the transmission of this bacterium in a hospital setting needs accurate typing methods. An antibiogram is used to detect the resistance pattern of the isolates. Random Amplified Polymorphic DNA (RAPD) and Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR are rapid, technically simple, and easy-to-interpret DNA typing methods. This study aimed to evaluate the use of antibiotyping, RAPD-, and ERIC-PCR to investigate the heterogeneity of *K. pneumoniae* isolated from clinical specimens.

Materials and Methods: The antibiograms of 46 *K. pneumoniae* clinical isolates were determined by Vitek® 2 Compact. All isolates underwent RAPD-PCR using AP4 primer and ERIC-PCR using ERIC-2 primer. The dendrogram was generated using the GelJ software and analyzed to determine its similarity. The analysis of antibiogram and the molecular typing diversity index was calculated using the formula of the Simpson's diversity index.

Results: About 71.7% of the isolates were ESBL-producers, and more than 80% of isolates were susceptible to amikacin, ertapenem, and meropenem. The antibiotyping produced 32 diverse types with DI = 0.964. In addition, the RAPD-PCR produced 47 different types with DI = 1, while ERIC-PCR was 46 (DI=0.999).

Conclusion: Antibiotyping, RAPD- and ERIC-PCR showed powerful discrimination power among the isolates, supported the diversity of *K. pneumoniae* isolates in current study. These combination could be promising tools for clonal relationship determination, including in tracking the transmission of the outbreak's agent in hospital setting.

Keywords: *Klebsiella pneumonia*; Transmission; Antibiogram; Molecular typing; DNA fingerprinting

INTRODUCTION

Klebsiella pneumoniae is a Gram-negative, encapsulated, and easy to resistant bacterium (1, 2) that commonly establishes on human mucosal surfaces, such as the gastrointestinal (GI) tract and oropharynx (1). This bacterium causes a broad spectrum of infections (e.g. bacteremia, urinary tract infection (UTI), and pneumonia), making it an important human pathogen that is associated with healthcare-associated infections (HAIs) (3, 4). Prevalence of *K. pneumoniae* infections was up to 10% of HAIs globally (5).

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The prevalence of HAIs in Southeast Asia region was 21.6%, whereas Indonesia showed 30.4%, and the predominant bacteria that causes HAIs is *K. pneumoniae* (5). Furthermore, in the antibiogram at Prof. Dr. I.G.N.G Ngoerah Hospital, *K. pneumoniae* is one of the top three bacteria that were isolated from clinical specimens (unpublished data). Problems arise because this bacterium is easily resistant through mechanisms of producing extended spectrum β-lactamase (ESBL), carbapenemase, and AmpC enzyme, which destroy the wide classes of antibiotics. These resulted in high morbidity and mortality (6).

Understanding dissemination of specific strains is important for epidemiology and surveying the expansive spread of both their pathogenicity and multi-resistance (7). Overtime, many typing tools are utilized and documented to examine genetic diversity of bacteria (8). Antimicrobial susceptibility testing results, also known as antibiogram, is not only for helping the clinicians to choose antibiotics treatment, but also for early detection of bacterial transmission possibility and diversity (9). There are many methods known for genetic profiling of bacterial strains for genotypic typing. The molecular typing tool, pulsed-field gel electrophoresis (PFGE) has been widely accepted as the gold standard for bacterial typing, including *K. pneumoniae* (10). Since PFGE is labor-intensive and costly, there are other typing tools that can be the alternative, such as Random Amplified Polymorphic DNA (RAPD)- and Enterobacterial Repetitive Intergenic Consensus Sequence (ERIC)-PCR, which demonstrated substantial roles for genetic profiling of *K. pneumoniae* (8, 10-12). Both RAPD- and ERIC-PCR can be utilized to exhibit genetic differences between bacteria and the typing of organisms without previous knowledge of DNA sequences. The molecular typing, RAPD-PCR, amplifies segments of genomic DNA using primers of random sequence (13), while ERIC-PCR depends on the amplification of genomic DNA fragments utilizing sets of repetitive short nucleotide sequences (14). This repetitive intergenic consensus (ERIC) is an intergenic repetitive unit that is different from other bacterial repeats, which can be found in bacterial genomes, including Enterobacteriaceae. These discriminatory typing methods can be a guide in environmental sources identification and strain transmission between patients (11). It also presents whether strains emerging after antibiotic therapy are the original or newly acquired strain variants (7). Both RAPD- and ERIC-PCR can discriminate a wide variety of bacterial strains in a short time, suggesting that it will be a useful epidemiological tool. Therefore, this study aimed to analyze the antibiotyping, RAPD-PCR using AP4 primer, and ERIC-PCR using ERIC-2 primer to determine the heteregeneity of *K. pneumoniae* clinical isolates. This present study may be a guide for researchers to select the appropriate method for epidemiological tools.

MATERIALS AND METHODS

Ethic statement. This study got approval from the Research Ethics Commission of the Faculty of Medicine, Udayana University, Bali, Indonesia (no. 1676/UN.14.2.2.VII.14/LT/2022).

Bacterial isolates. Forty-six glycerol stocks of *K. pneumoniae* clinical isolates isolated in 2021-2022 at the Clinical Microbiology Laboratory of Prof. Dr. I.G.N.G. Ngoerah Hospital Bali and *K. pneumoniae* ATCC 13883 (control isolate) were cultivated on MacConkey agar at 35 ± 2°C, for 24 hours. Colonies were subjected to DNA isolation.

Antibiotics susceptibility testing and detection of ESBL production. The isolates' antibiotics susceptibility pattern and ESBL production detection of the isolates were examined using Vitek® 2 Compact (bioMérieux, Marcy-l'Etoile, France). The test was conducted based on company standard procedures.

DNA isolation. *K. pneumoniae* ATCC 13883 and clinical isolate were resuspended in 200 µl of sterile phosphate buffered saline (PBS), and the genomic DNA was extracted using DNA isolation kit (High Pure PCR Template Preparation kit, Roche Diagnostics, Roche Diagnostics GmbH, Mannheim, Germany) according to company standard procedures. DNA concentration was measured (Implen Nanophotometer) before used for RAPD-PCR.

RAPD and ERIC-PCR. Extracted DNA were subjected to RAPD and ERIC-PCR. Primer AP4 (5'-TCACGATGCA -3') was used for randomly amplifying DNA fragments (15), while ERIC-2 primer (5'-AAG TAA GTG ACT GGG GTG AGC G-3') was for ERIC-PCR (12). PCR mixture (Go Taq® 2x Green master mix, Promega Corporation, Madison,

USA) with total volume of 12.5 µL was used for each reaction. About 1 µL (10 µM) each of AP4 or ERIC-2 primer was used in this study. The optimal amplification conditions were pre-denaturation at 94°C (4 min for RAPD and 2 min for ERIC); denaturation at 94°C for 1 minute, annealing for 1 minute (at 34°C for RAPD and 25°C for ERIC), and elongation at 72°C for 2 minutes (40 cycles), and final elongation at 72°C (10 min for RAPD and 5 min for ERIC).

The PCR products were run on 1% agarose gel with DNA staining (GelRed, Biotium). All RAPD and ERIC-PCR reactions were conducted in duplicate. The reproducibility of RAPD and ERIC-PCR protocol was assessed by repeating the experiment using different subculture of the same isolates.

Analysis of amplification results. Data was calculated and presented as tables and graphs using the Microsoft Excel spreadsheet for Windows 2019. The cluster analyses were analyzed using GelJ v.2.3 software, while the dendrogram and similarity index were performed using the unweighted pair group method with arithmetic mean (UPGMA) analysis (16). The discriminatory power of the antibiogram and molecular typing was calculated using the Simpson's index of diversity (17).

RESULTS

Sample characteristics. *Klebsiella pneumoniae* isolates (n = 46) used in this study were isolated from blood (11/46; 23.9%), sputum (10/46; 21.7%), urine (11/46; 23.9%), wound (9/46; 21.7%), and other type of specimens (4/46; 8.7%) (Table 1).

As shown in Table 1, most isolates were ESBL-producers (71.7%), and collected from wards other than ICU (87.0%).

Antibiotics susceptibility testing results. The antimicrobial susceptibility testing resulted more than 80% of isolates were susceptible to ertapenem, meropenem, and amikacin, which were 91.3%, 93.5%, and 89.1%, respectively, and 76.1% of isolates were susceptible to tigecycline. In this study, less than 60% of isolates were susceptible to the rest of the antibiotics tested, and all isolates were resistant to ampicillin, as shown in Fig. 1.

As described in Table 2, there were 32 antibiotypes produced from *K. pneumoniae* antibiogram.

Table 1. Characteristics of *Klebsiella pneumoniae* isolates based on specimen type, ESBL producers phenotype, and ward of specimen collection

Isolates Characteristics	N (%)
Specimen type	
Urine	11 (23.9)
Sputum	10 (21.7)
Blood	11 (23.9)
Wound	10 (21.7)
Others	4 (8.7)
Phenotype Extended Spectrum Beta Lactamase (ESBL) producer	
Yes	33 (71.7)
No	13 (28.3)
Ward of Collection	
Intensive Care Unit (ICU)	6 (13.0)
Non-ICU	40 (87.0)

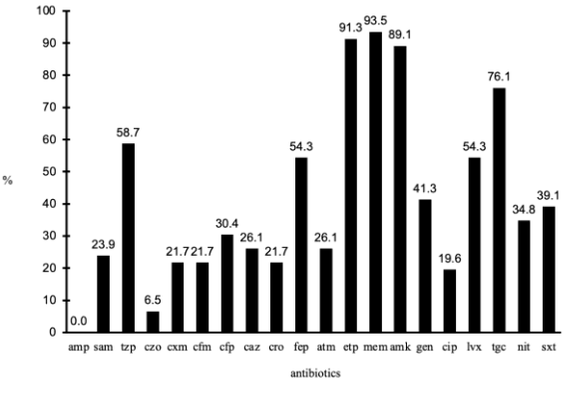

Fig. 1. Antibiogram of 46-non replicative *Klebsiella pneumoniae* clinical isolates used in this stud. (antibiotics abbreviation: amp=ampicillin; sam=ampicillin sulbactam; tzp=piperacillin tazobactam; czo=cefazolin; cxm=cefuroxime; cfm=cefixime; cfp=cefoperazon; caz=ceftazidime; cro=ceftriaxone; fep=cefepime; atm=aztreonam; etp=ertapenem; mem=meropenem; amk=amikacin; gen= gentamicin; cip=ciprofloxacin; lvx=levofloxacin; tgc=tigecycline; nit=nitrofurantoin; sxt=trimethoprim-sulfamethoxazole)

After analysis, DI of antibiogram typing was 0.964 (Table 3).

RAPD and ERIC-PCR. In the first stage, the RAPD using AP4 primer and ERIC-PCR using ERIC-2 primer protocols were optimized. The optimized results of both primers are shown in Fig. 2.

Since the duplicate experiment shown in Fig. 2 generated similar band pattern in both AP4 and ERIC-2

Table 2. Antibiotypes of *K. pneumoniae* clinical isolates based on antibiogram pattern

Antibiotype	Antibiogram pattern	No. of isolates (%)
A1	amp	1 (2.17)
A2	amp-czo	4 (8.70)
A3	amp-czo-nit	2 (4.35)
A4	amp-czo-gen-cip-nit	1 (2.17)
A5	amp-sam-czo-tgc-nit	1 (2.17)
A6	amp-sam-gen-cip-lvx-tgc-sxt	1 (2.17)
A7	amp-czo-cxm-cfm-cfp-caz-cro-atm	1 (2.17)
A8	amp-sam-czo-cxm-cfm-cfp-caz-cro-atm-cip-sxt	2 (4.35)
A9	amp-sam-czo-cxm-cfm-cfp-caz-cro-gen-cip-nit	1 (2.17)
A10	amp-czo-cxm-cfm-cfp-caz-cro-atm-cip-nit-sxt	2 (4.35)
A11	amp-sam-czo-cxm-cfm-cfp-caz-cro-atm-gen-cip	1 (2.17)
A12	amp-sam-czo-cxm-cfm-cfp-caz-cro-fep-atm-cip-sxt	2 (4.35)
A13	amp-sam-czo-cxm-cfm-cfp-caz-cro-atm-cip-nit-sxt	1 (2.17)
A14	amp-sam-czo-cxm-cfm-cfp-cro-atm-gen-cip-lvx-sxt	1 (2.17)
A15	amp-sam-czo-cxm-cfm-cfp-caz-cro-atm-gen-cip-nit-sxt	1 (2.17)
A16	amp-sam-czo-cxm-cfm-cfp-caz-cro-atm-gen-cip-lvx-sxt	2 (4.35)
A17	amp-sam-czo-cxm-cfm-cfp-caz-cro-fep-atm-cip-nit-sxt	1 (2.17)
A18	amp-sam-tzp-czo-cxm-cfm-cfp-caz-cro-atm-cip-lvx-sxt	1 (2.17)
A19	amp-sam-czo-cxm-cfm-cfp-caz-cro-atm-gen-cip-lvx-nit-sxt	2 (4.35)
A20	amp-sam-czo-cxm-cfm-cfp-caz-cro-fep-atm-gen-cip-nit-sxt	1 (2.17)
A21	amp-sam-tzp-czo-cxm-cfm-cfp-caz-cro-fep-atm-gen-cip-lvx-nit	2 (4.35)
A22	amp-sam-tzp-czo-cxm-cfm-cfp-caz-cro-fep-atm-gen-cip-tgc-nit	1 (2.17)
A23	amp-sam-tzp-czo-cxm-cfm-cfp-caz-cro-fep-atm-gen-cip-nit-sxt	1 (2.17)
A24	amp-sam-tzp-czo-cxm-cfm-cfp-caz-cro-atm-gen-cip-lvx-tgc-nit-sxt	2 (4.35)
A25	amp-sam-tzp-czo-cxm-cfm-caz-cro-fep-atm-etp-gen-cip-lvx-nit-sxt	1 (2.17)
A26	amp-sam-tzp-czo-cxm-cfm-cfp-caz-cro-fep-atm-gen-cip-lvx-nit-sxt	1 (2.17)
A27	amp-sam-tzp-czo-cxm-cfm-cfp-caz-cro-fep-atm-gen-cip-lvx-tgc-nit	3 (6.52)
A28	amp-sam-tzp-czo-cxm-cfm-cfp-caz-cro-fep-atm-gen-cip-lvx-tgc-nit-sxt	1 (2.17)
A29	amp-sam-tzp-czo-cxm-cfm-cfp-caz-cro-fep-atm-amk-gen-cip-lvx-tgc-nit	2 (4.35)
A30	amp-sam-tzp-czo-cxm-cfm-caz-cro-fep-atm-etp-mem-amk-gen-cip-nit-sxt	1 (2.17)
A31	amp-sam-tzp-czo-cxm-cfm-cro-fep-atm-etp-mem-amk-gen-cip-lvx-tgc-nit-sxt	1 (2.17)
A32	amp-sam-tzp-czo-cxm-cfm-caz-cro-fep-atm-etp-mem-amk-gen-cip-lvx-tgc-nit-sxt	1 (2.17)

primers, the intra-laboratory variation from these primers was minimal.

The RAPD-PCR using AP4 primer generated 9 bands on average within range 100-3000 bp. On the other hand, ERIC-PCR produced 6 different bands on average within the similar range of bp to that of RAPD. All clinical isolates and *K. pneumoniae* ATCC 13883 were fingerprinted and successfully typable by RAPD and ERIC-PCR.

When the similarity level used is 85%, it showed 47 diverse types using AP4 primers (Fig. 3).

On the other hand, ERIC-PCR method produced 46 different types (Fig. 4). RAPD- and ERIC-PCR discriminatory power was calculated using the Simpson's index of diversity (17). For the similarity level of 85%, the DI of RAPD-PCR and ERIC-PCR were 1 and 0.999, respectively (Table 3). These results suggested that both of these methods could detect the isolates' heterogeneity.

As shown in Table 3, the diversity index of antibiogram typing was relatively good (DI=0.964), however, both RAPD- and ERIC-PCR showed a higher discriminatory power than that of antibiogram typing.

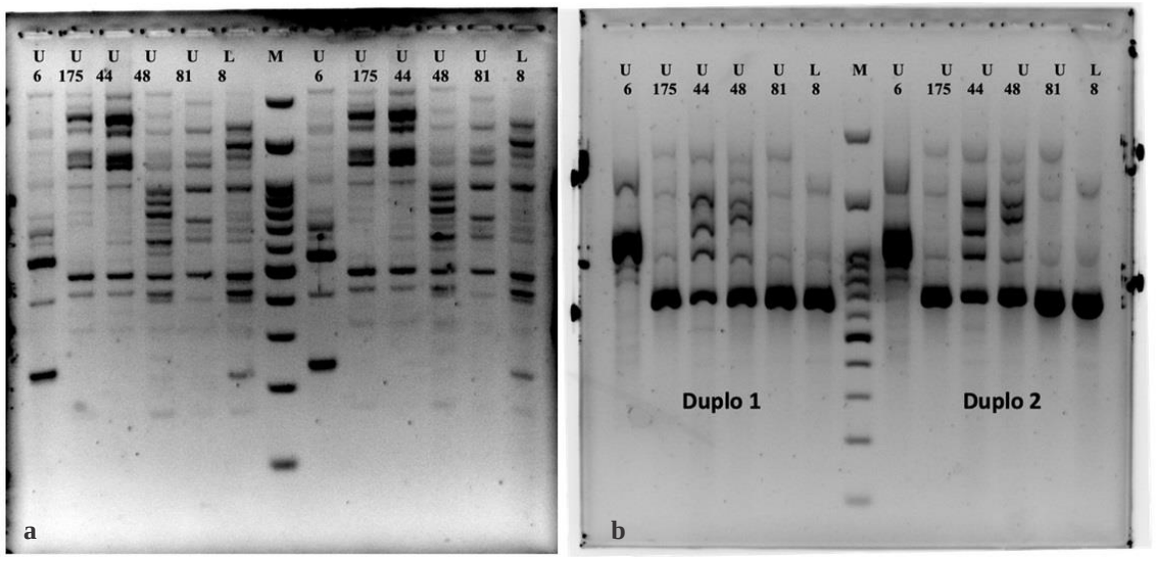

Fig. 2. The optimized RAPD-PCR profiles of *K. pneumoniae* clinical isolates amplified by using AP4 primer (a) and ERIC-PCR using ERIC-2 primer (b). The RAPD- and ERIC-PCR products were run on 1% gel agarose. (U = urine specimens; L = other specimen; M = 100 bp DNA Ladder, Geneaid Biotech. LTD., Taiwan). The RAPD- and ERIC-PCR were conducted in duplicate. The results showed that both fingerprinting methods revealed a consistent results.

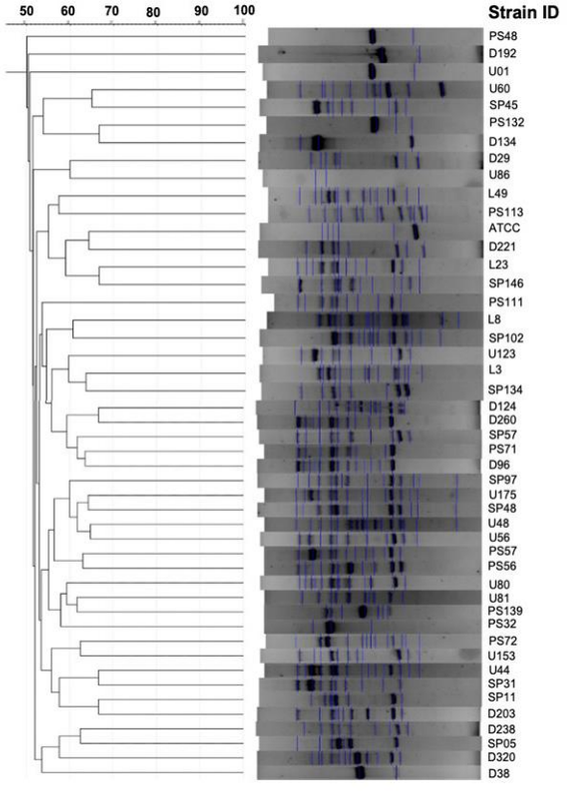

Fig. 3. Dendrogram cluster analysis of RAPD-PCR typing. The dendrogram and Similarity was generated from GelJ analysis. (D = blood; U = urine; SP = sputum; PS = wound; L = others; ATCC = *K. pneumoniae* ATCC 13883)

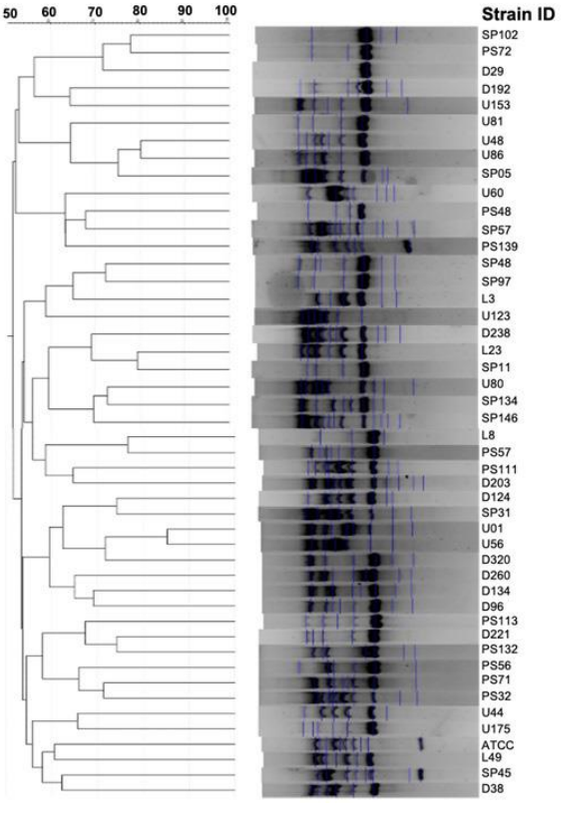

Fig. 4. Dendrogram cluster analysis of ERIC-PCR. The dendrogram and Similarity Index was generated from GelJ analysis. (D = blood; U = urine; SP = sputum; PS = wound; L = others; ATCC = *K. pneumoniae* ATCC 13883)

Table 3. Comparison of index of diversity calculation between antibiotyping and molecular typing methods

Typing Method	Number of diverse type	Simpson's index diversity
Antibiogram typing	32	0.964
RAPD-PCR	47	1
ERIC-PCR	46	0.999

DISCUSSION

Antibiotyping and two discriminatory molecular typing methods, RAPD- and ERIC-PCR, were used to analyze the clonal relationship among *K. pneumoniae* clinical isolates. *Klebsiella pneumoniae* is an opportunistic pathogen that can lead someone to severe conditions with a broad spectrum of diseases (18). This bacterium is one of the most concerning multidrug-resistant (MDR) pathogens and is a significant source of healthcare-associated infections that affect humans (4). In this study, most isolates were ESBL-producers (71.3%). A study that reviewed the prevalence of ESBL-producer *K. pneumoniae* demonstrated that the pooled prevalence was 27% (19). In comparison, another review of ESBL prevalence in Iran showed a pooled prevalence of 43.5% (20). In our hospital, the prevalence of ESBL-producer *K. pneumoniae* is high, partly due to the high use of third-generation cephalosporins, especially ceftriaxone. Furthermore, in the present study, the antibiogram of *K. pneumoniae* isolates showed that majority of them were less susceptible to many tested antibiotics. However, over 80% of isolates were still susceptible to amikacin, ertapenem, and meropenem. Furthermore, all isolates (100%) were resistant to ampicillin due to the intrinsic resistant trait of *K. pneumoniae* (21, 22). The current study also analyzed typing based on antibiogram pattern and found relatively good discriminatory power. Although it was not as powerful as the molecular typing methods in this study, the antibiotyping could be used as an early screening method for strain-relatedness detection.

There are many DNA banding pattern-based fingerprinting methods that can be used. Some methods are Amplified Fragment Length Polymorphic (AFLP), Pulsed Field Gel Electrophoresis (PFGE), Whole-Genome Sequencing (WGS), Random Amplified Polymorphic DNA PCR (RAPD-PCR) and Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) to differentiate *K. pneumoniae* clinical isolates clonally (23). Some aspects must be evaluated to determine the efficiency of a typing method such as typeability, reproducibility and discriminatory power. Other additional aspects that must be considered are time, expenses, and complexity of performance and interpretation (10). PFGE was established as the definitive molecular typing method, but it is laborious and expensive. With their reduced time requirements, technical demands, and enhanced typeability, PCR-based methods are promising for typing *K. pneumoniae* isolates (10). These methods require only a small amount of DNA and eliminate the need for blotting and hybridization steps, DNA probes, or specific primers information (24).

RAPD is a method involving a single short primer (8-12 nucleotides) that randomly amplifying segments of DNA (25). In our study, we investigated the performance of RAPD-PCR with AP4 primer. The result found AP4 had high discriminatory index (DI = 1). The AP4 primer generated, on average, 9 different bands and produced bands within the 100-3000 bp range. This data is similar to a study in Iran that showed AP4 performed better than other primers, such as RAPD-7, OPAR3, OPAR8, Primer640, and HLWL74. AP4 was the most discriminatory primer according to some factors such as the number of PCR products, the intensity of bands, smear formation and size range. Despite that our results had a lower number of bands (15 vs. 32 bands) and range (100-3000 vs. 200-5000 bp), from the discriminatory index and repeatability aspects, our results were almost the same: 1 vs. 0.982 in discriminatory index and both of the data had high repeatability which also showed heterogeneity among the *K. pneumoniae* isolates (Similarity coefficient 100%) (15). Another study using the same primer AP4 differentiate the result of two methods, PFGE and RAPD-PCR. Data showed that PFGE generated 30 bands with a range of 15-700 kb, 42 bands in similarity level 85%, 0.987 DI, and high repeatability (Similarity coefficient 100%) (10). Overall, the results showed that both PFGE and RAPD-PCR are equally valuable and it is better to combine the two methods. RAPD-PCR can be the preliminary method for quick investigation, and PFGE is a confirmatory method (10). In using RAPD-PCR, some problems or disadvantages in reproducibility could be seen. Some factors that can influence the outcome of RAPD (altered the amplified fragments) such as

purity of DNA, DNA and primer concentration, and annealing and extension time (26).

ERIC-PCR is a method which amplification focused on the region between the ERIC sequences and differentiates variations the bacterial strain in the location of ERIC sequences (27). The current study investigated the performance of ERIC with ERIC-2 primer. The result showed ERIC-PCR with ERIC-2 primer generated 6 bands (100-3,000 bp) and with DI of 0.999. This finding is directly proportional to a study of ERIC-PCR using ERIC-2 primer to determine the clonal relatedness among *K. pneumoniae* isolates, which showed that ERIC-PCR produced DI of 0.8704, with 17 bands in range of 100-1500 bp. Furthermore, the study suggested that ERIC-PCR better than matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) in discriminatory index, reproducibility, specificity of clustering isolated; therefore, it is easier to determine the strain's relatedness in an epidemiological outbreak (28).

Another study had investigated RAPD- and ERIC-PCR of *Klebsiella pneumoniae* isolates. The dendrograms analyses revealed 21 distinct patterns with ERIC- and 18 patterns with RAPD-PCR of *K. pneumoniae* isolates with similarity >80%. In discriminatory index, ERIC got higher index than RAPD, which showed DI of 0.969 vs. 0.955. Both of methods significantly correlated with resistance patterns (29). In addition, in one study but using another species, the discriminatory index RAPD is better than ERIC (0.878 vs. 0.8591) (30).

The low number of isolates was one of this study limitations. Examining more clinical isolates and using gold standard and more powerful discriminating typing methods such as PFGE or Whole Genome Sequencing (WGS) may improve the quality of the current study results. Additionally, the study was limited to a specific bacterial strain of *K. pneumoniae*, and it would be beneficial to explore the performance of RAPD- and ERIC-PCR in other bacterial species to validate its generalizability.

CONCLUSION

Due to their typeability and discriminatory power, RAPD and ERIC-PCR were more successful in discriminating *K. pneumoniae* isolates than antibiotyping. However, since the antibiogram type showed relatively good discrimination index, the combination of the antibiotyping and the molecular typing could provide useful tools for epidemiology study of *K. pneumoniae*, including in an outbreak investigation.

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