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Genetic Diversity of *Klebsiella* spp. Isolated from Tempe based on Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction (ERIC-PCR)

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Tempe is an Indonesian fermented food prepared by fermenting dehulled cooked soybeans with *Rhizopus oligosporus*. Many types of bacteria are also involved during tempe fermentation, and one of these is *Klebsiella* spp. Some isolates of *K. pneumoniae* produces vitamin B12 in tempe but it has also been classified as an opportunistic pathogen. For this reason *Klebsiella* spp. in tempe is important to be studied. The aim of this study was to investigate the genetic diversity of *Klebsiella* spp. from tempe employing ERIC-PCR method. Sixty-one isolates of *Klebsiella* have been isolated from sixteen tempe producers in Bogor, Jakarta, Malang, Tengerang, Bandung and Cianjur. 63F and 1387R primers were used to amplify 16S rDNA sequences, and 1R and 1F primers were used for ERIC analysis. The results of this research showed that sixty-one strains of *Klebsiella* were clustered into 17 groups. Based on ERIC-PCR analysis, isolates of *Klebsiella* could be grouped into different profiles which some of these groups consisted of isolates with identical ERIC-PCR profiles. Several identical ERIC-PCR profiles were found in tempe from the same producer. There was no correlation observed between genetic similarity among isolates with the origin of tempe.

Keywords: Tempe, *Klebsiella*, ERIC-PCR, vitamin B12

INTRODUCTION

Tempe is a traditional fermented soybean food from Indonesia, and it is an important protein source for Indonesian. Tempe is good for health because it contains essential compounds such as isoflavone (Ikehata *et al*. 1968) and vitamin B12 (Keuth & Bisping 1993; Wiesel *et al*. 1997). Liem *et al*. (1977) reported that Indonesian tempe, a protein-rich vegetarian food, is one of the world's first meat analogs. Therefore, tempe has been popular in Japan, USA, Australia, and Europe (Aderibigbe & Osegboun 2006).

Generally, vitamin B12 can only be found in foods derived from animals, therefore vitamin B12 in tempe is essential as a resource of vitamin B12 for certain people such as vegetarians. One type of bacteria that has an ability to synthesize vitamin B12 in tempe is *K. pneumoniae* (Keuth & Bisping 1993). It has been reported that *Klebsiella* sp. in tempe can achieve aproximately 108 CFU/g (Mulyowidarso *et al*. 1989; Mulyowidarso *et al*. 1990; Barus *et al*. 2008).

The presence of *K. pneumoniae* in tempe is advantageous because it produces vitamin B12. However, it has been reported that *K. pneumoniae* was classified as opportunistic pathogens (Podshun & Ullmann 1998). Since research on the characteristics of *Klebsiella* spp. in tempe is limited, an additional study needs to be conducted.

Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) (Wilson & Sharp 2006) is often used to analyze the diversity of enteric bacteria, such as *Klebsiella* spp. Characteristic of ERIC sequences are 126 bp, non-coding, and conserve (Versalovic *et al*. 1991). The position and number of ERIC sequences in bacteria vary so it can be used as genetic markers to study the diversity of bacterial isolates. Meanwhile, ERIC-PCR method has been successfully analyzed the diversity of different types of bacteria, such as *Mycobacterium tuberculosis* (Sechi *et al*. 1998) and *Vibrio parahaemolyticus* (Khan *et al*. 2002) because its method is rapid, sensitive, and consistent in analyzing bacterial diversity (Sechi *et al*. 1998; Huiyong *et al*. 2008). Therefore, to assess the genetic diversity of *Klebsiella* spp. from tempe in this study, ERIC-PCR method was used. It is expected that the

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results will be used as basis for further analysis of the *Klebsiella* spp. from tempe.

MATERIALS AND METHODS

Screening of *Klebsiella* Isolates from Tempe. Sixty-one isolates of *Klebsiella* have been isolated from tempe which were obtained direcly from sixteen tempe producers in Bogor, Jakarta, Malang, Tengerang, Bandung, and Cianjur. All isolates were tested by morphology (non-motile, rod-shaped, and Gram negative) and biochemical tests (positive on Voges Proskauer, citrate, urea, and lysine decarboxylase test; and negative on indole and methyl red test). Furthermore, the identification was done based on 16S rRNA gene sequences.

Genome Isolation. *Klebsiella* isolates were cultured in Luria-Broth medium (Oxoid, Hampshire, England) overnight at 37 °C. Genome isolation of each isolate was conducted using Genomic Purification Kit (Fermentas, Lithuania) based on the manufacturer's protocol with a slight modification. The modification was in the speed of centrifugation (13,000 x g), and the addition of 500 mL lysis solution. Obtained DNA genome was stored at -20 °C freezer for further usage.

Amplification of 16S rRNA Gene Sequences. Two specific primers were used to amplify 16S rDNA sequences, namely 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387R (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi *et al*. 1998). Amplification of 16S rRNA genes sequence of *Klebsiella* isolates was performed in GeneAmp® PCR System 2700 (Applied Biosystems, Carlsbad, CA, USA). PCR master mix (25 µL) contained 12.5 mL Go Taq (Promega, Madison, USA), 2 mL primer (25 pmol mL-1), 8.5 mL nuclease free water (Promega, Madison, USA), and 2 mL of DNA template. PCR conditions were as follows: pre-denaturation at 94 °C for 5 min, denaturation at 93 °C for 30 s, annealing at 62 °C for 30 seconds, extension at 72 °C for 30 min, and post-extension at 72 °C for 10 min. Denaturation, annealing, and extension were performed in 35 cycles. PCR products were observed using 1.8% electrophoresis agarose gel (Promega, Madison, USA) then stained with ethidium bromide (Sigma-Aldrich, USA) for 15 min and destained for 5 min before visualized on UV transilluminator using 1 kb ladder (Fermentas, Lithuania) as marker. PCR products were then partially sequenced in Macrogen Inc., Republic of Korea.

ERIC Sequence Amplification. Amplification of ERIC sequence of *Klebsiella* isolates was performed in GeneAmp PCR System 2700 (Applied Biosystems, Carlsbad, CA, USA) using the universal primers which comprised Primer 1R 5'-ATGTAACGT CCTG G GGATTCAC-3 ' and primer IF 5' AAGTAAGTGACTGGGGTGAGCG-3' (van der Zee *et al*. 2003; Huiyong *et al*. 2008). PCR master mix (25 µL) contained 12.5 mL GoTaq (Promega, USA), 9.5 mL Nuclease-Free Water (Promega, Madison, USA), 1 uL of each primer (25 pmol mL^{-1}), and 1 mL DNA template. PCR conditions were as follows: pre-denaturation 94 °C for 2.5 min, 94 °C denaturation for 30 s, annealing 47 °C for 1 min, extension 72 °C for 1 min, post-extension 72 °C for 4 minutes, and hold 4 °C. Denaturation, annealing, and extension were performed in 35 cycles. PCR products were observed using 1.8 % electrophoresis agarose gel (Promega, Madison, USA), stained with ethidium bromide (Sigma-Aldrich, USA) for 15 min, and then destained for 5 min. UV transilluminator was used to visualize DNA in gel electrophoresis using 1 kb ladder (Fermentas, Lithuania) as a marker. Based on binary data from profile of ERIC, phylogenetic tree was constructed using MEGA 5 software (Tamura *et al*. 2011).

RESULTS

Sixty-one isolates of *Klebsiella* spp. were randomly selected from tempe which was taken directly from many tempe producers (Table 1). The coloni isolates of *Klebsiella* were pink mucoid on media MCA, non-motile, rod-shaped, and Gram-negative. Based on biochemical tests, the isolates of *Klebsiella* were positive on Voges Proskauer, citrate, urea, and lysine decarboxylase test; and negative on indole and methyl red test.

Table 1. Sixty-one isolates of *Klebsiella* from tempe

Isolate code (Origin of tempe)
1, 2, 3, 4, 5a, 8, 9, 10a, 11a, 12, 13, 14, 15, 255, 339 (Jakarta)
5b, 7b, 10b, 11b, 17b, 165, 175, 194, 198 (Malang)
108, 110, 115, 120, 133, 135, 140, 145, 157, 210, 211, 215, 219, 221, 225, 226, 233, 240, 245 (Bogor)
16, 17a, 18, 19, 52, 59, 305 (Bandung)
20, 21, 22, 23, 24, 25, 248, 250, 49, 50, 51 (Cianjur)

A total of 15 isolates of *Klebsiella* spp. from Table 2 have been randomly selected for further identification based on 16S rDNA sequences. Sequencing of 16S rDNA was successfully performed. Based on BLASTN, partial sequence of 16S rRNA gene (about 800 to 940 nucleotides) show high similarity with *Klebsiella* spp. with maximum identities for each isolate ranging from 97 to 100% with E-value 0 (Table 2).

ERIC sequences of 61 *Klebsiella* isolates have been successfully amplified using ERIC 1R and ERIC 1F primers (Figure 1 as representatif of ERIC-PCR). Visualization of the ERIC-PCR profiles shows that some of them have a diverse genetic

Table 2. BLASTN result of 16S rRNA partial gene sequences of some *Klebsiella* isolated from tempe

Isolate	Homology	Access. number	Max. Identity (%)
50	*Klebsiella pneumoniae* strain F3	FJ490057	98
52	*Klebsiella pneumoniae*	AF130981.1	98
194	*Klebsiella* sp. 2009I10	GU290327.1	98
198	*Klebsiella* sp. gx-118	FJ823040.1	97
135	*Klebsiella pneumoniae* strain 8	KC508812.1	99
59	*Klebsiella pneumoniae* strain MSO-32	KC710339.1	99
157	*Klebsiella pneumoniae* strain SZH12	GU384263.1	99
51	*Klebsiella pneumoniae* strain GN07-Cr.a	FJ447493.1	99
305	*Klebsiella pneumoniae* strain GN07-Cr.a	FJ447493.1	99
339	*Klebsiella pneumoniae* strain ECU-21	EU360793.1	100
219	*Klebsiella pneumoniae* strain fJ1	FJ947063.1	99
221	*Klebsiella pneumoniae* strain fJ1	FJ947063.1	98
49	*Klebsiella* sp. SZH11	GU384262.1	100
110	*Klebsiella* sp. SZH11	GU384262.1	100
233	*Klebsiella* sp. SZH11	GU384262.1	99

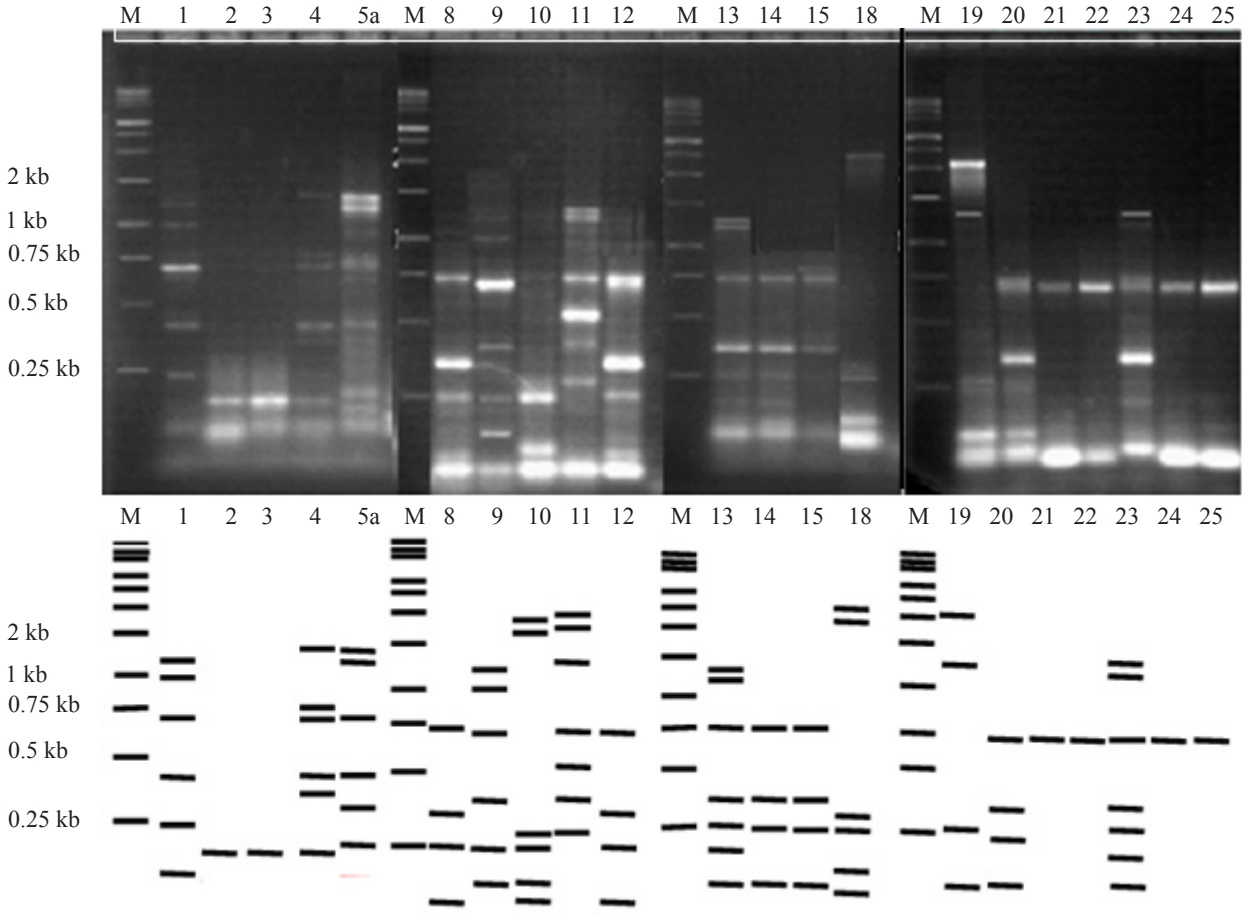

Figure 1. DNA fingerprints of several *Klebsiella* spp. isolated from tempe generated by ERIC- PCR. M, marker 1 kb ladders (Fermentas).

such as isolate 1, 2, and 5. This was indicated by the difference in the number and size of ERIC sequences found in each isolate. Otherwise, some of them are not diverse such as isolate 21, 22, 24, and 25. There were only two ERIC profile often found in each isolate (0.25 and 0.75 kb).

Phylogenetic tree as a representation of the genetic diversity of *Klebsiella* spp. was successfully constructed (Figure 2). Sixty-one isolates of *Klebsiella* sp. appear to form 16 clusters. Some of these isolates have a diverse genetic and some of them are not diverse. The position of the same

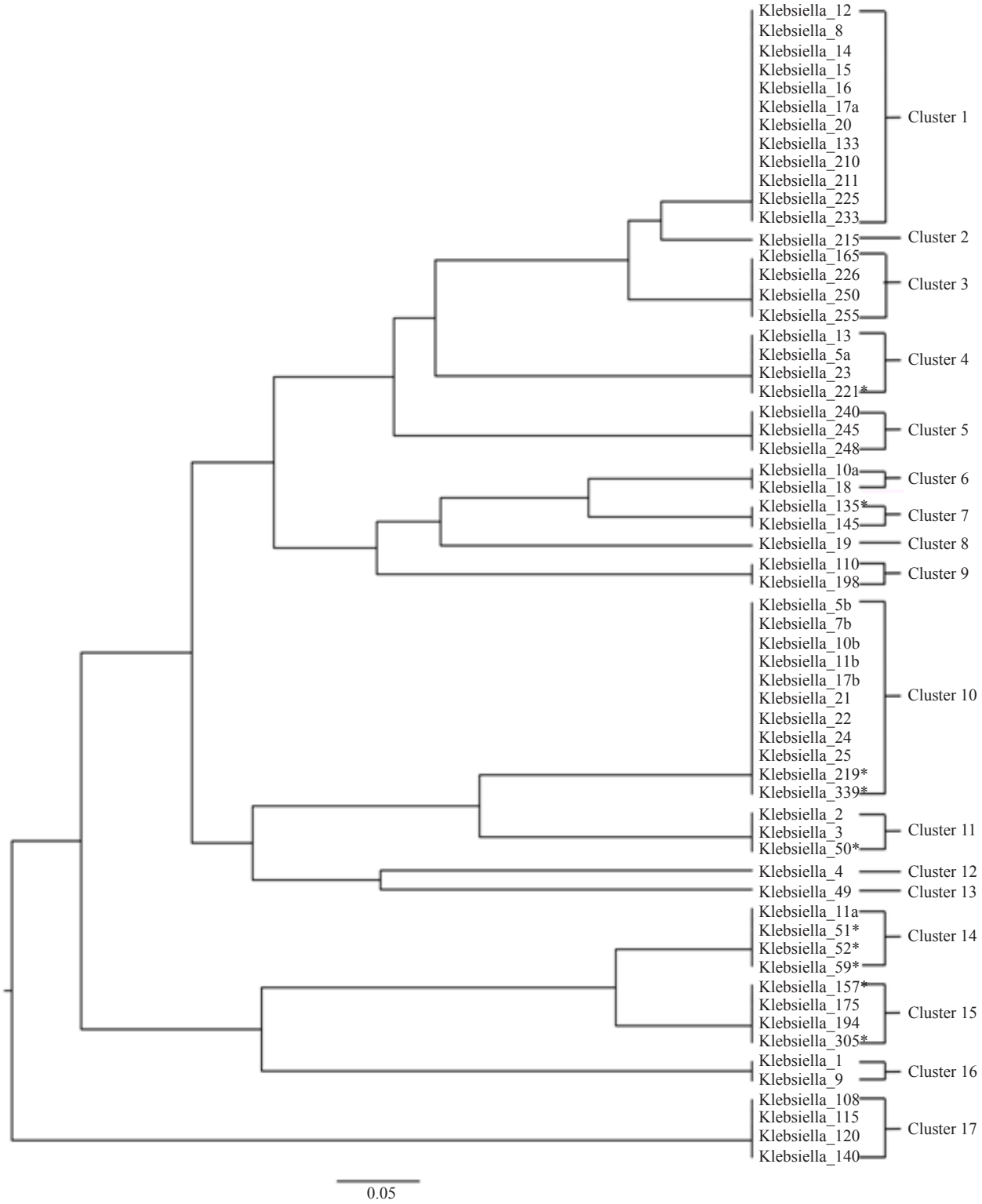

Figure 2. Dendrogram resulting from analysis of ERIC-PCR performed on sixty-one *Klebsiella* strains obtained from tempe. The Neighbour joining (NJ) tree was constructed using Mega 5 with 100 bootstrap analysis of replication. *Isolates was similar to *Klebsiella pneumoniae* based on 16S rDNA sequences (Table 2).

isolates have been shown in the same cluster (Figure 2). Ten isolates in Table 2 were similar to *K. pneumoniae* based on 16S rDNA sequences. ERIC-PCR profiles of ten *K. pneumoniae* were also diverse. In Figure 2, these isolates were found spread on six different clusters, namely: clusters 4 (221 isolate), cluster 7 (135 isolate), cluster 9 (isolates 339, 219), cluster 10 (50 isolate), cluster 13 (isolates 59, 52, 51), and cluster 14 (isolates 305, 157).

Tempe successfully made in sterile conditions using *Klebsiella* sp. and *Rhizopus* sp. *Klebsiella* sp. made the pH of the soybean soaking water drop from seven to about four after 24 hours of soaking. At these conditions *Rhizopus* sp. grow well so that the resulted tempe is good. Nevertheless, pH of the soybean soaking water which did not involve *Klebsiella* sp. was about six after 24 hours of soaking. In this condition, *Rhizopus* sp. did not grow and caused soybeans spoil (Figure not shown).

DISCUSSION

Genome of each isolate of *Klebsiella* was isolated using Genomic DNA Purification Kit (Fermentas, Lithuania) according to the procedure but with a slight modification. The modification included a change of sentrifugation's speed from 10,000 × g to 13,000 × g for 10 min and the addition of lysis solution from 400 mL to 500 mL. This modification could increase the quality of lysis and separation of Exopolysaccharide (EPS) isolates of *Klebsiella*. *Klebsiella* produce EPS containing acid kolanat, clavan, and fukogel (Domenico *et al*. 1994) thereby inhibiting genomic isolation. However, with this modification, *Klebsiella* genomes were successfully isolated.

Previous studies have reported that *Klebsiella* was one type of bacteria involved in the fermentation (Keuth & Bisping 1993; Barus *et al*. 2008) and production of vitamin B12 and B6 (Keuth & Bisping 1993) in tempe. Results of this study have shown that *Klebsiella* also has a role in the process of acidification during soaking of soybean in tempe fermentation. *Klebsiella* can decrease the pH to about 4. Barus *et al*. (2008) reported that during soaking, the soybean soaking water will drastically decrease the pH from 7 to 4. This condition is an important factor to support the growth of *Rhizopus*. Acidification in the making of Indonesian tempe takes place naturally, ie. by bacteria, while in production of tempe in other countries, such as Holland and the United States, the acidification is carried out with the addition of organic acids.

Although it is known that *Klebsiella* has a role in producing vitamin B12 and acidification, this still needs further characterizations. For example, there is a need to assess the difference between non-pathogenic *Klebsiella* and *Klebsiella* that are pathogenic for humans. There should also be an assessment on the potential of intra-species of *Klebsiella* to produce vitamin B12 in tempe. This information is vital not only for the security of food but also for the improvement of tempe quality.

This research has identified *Klebsiella* based on morphological, biochemical, and molecular traits. The morphological and biochemical identification showed the same results with molecular identification based on 16S rRNA gene sequences. Furthermore, the genetic diversity of *Klebsiella* has been successfully analyzed by ERIC-PCR.

Visualization of the ERIC-PCR (Figure 1) has demonstrated the existence of differences in the number and size of ERIC-PCR profiles. Meacham (2003) reported that the difference in the number and size of the ERIC-PCR profiles describe the genetic diversity among bacterial isolates. Based on ERIC-PCR profiles, a phylogenetic tree that describes the genetic diversity of *Klebsiella* has been successfully constructed. Figure 2 shows that 61 isolates of *Klebsiella* spp. formed 16 clusters. Each cluster has a different ERIC profile describing the genetic diversity of *Klebsiella* spp. The results of this research also indicate the existence of genetic diversity within intraspesies of *K. pneumoniae*. This was demonstrated by isolates 50, 52, 135, 59, 157, 51, 305, 339, 219, 221 which were similar to *K. pneumoniae* (Table 2). However, in Figure 2 these isolates were found to be spread on six different clusters.

There was no positive correlation between the origin of isolates with genetic diversity. For example, cluster 1 consists of *Klebsiella* isolates originating from Jakarta (8, 12, 14, 15 isolates), Bogor (133, 210, 211, 215, 225, 233 isolates), Bandung (16, 17a isolates), and Cianjur (20 isolates). The results of this study indicate that *Klebsiella* strains from one location spread to multiple clusters (Figure 2). For example *Klebsiella* isolates from Jakarta can be found in cluster 2, 4, 6, 9, 10, 11, and 15. Thus, it can be concluded that strains of *Klebsiella* in tempe are genetically diverse. We are currently identifying particular isolates that would play significant role(s) in vitamin B12 production.

The 16S rRNA gene of ten isolates has been sequenced (Figure 1). Genetic diversity of 10 isolates of *Klebsiella* has been compared based on

16S rRNA sequences with ERIC sequences (data not showed). The result showed that ERIC-PCR sequences were more discriminative than 16S rRNA sequences. Therefore, ERIC-PCR profile has been used to study genetic heterogeneity of many types of bacteria (Dalla-Costa *et al*. 1998; Waturangi *et al*. 2012).

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REFERENCES

Aderibigbe EY, Osegboun AO. 2006. Acceptability of tempe among health workers in Ado-Ekiti, Nigeria. *Pakis J Nutr* 5:122-124. http://dx.doi.org/10.3923/pjn.2006.122.124

Barus T, Suwanto A, Wahyudi A, Wijaya H. 2008. Role of bacteria in tempe bitter taste formation: microbiological and molecular biological analysis based on 16S rRNA gene. *Microbiol Indones* 2:17-21. http://dx.doi.org/10.5454/mi.2.1.4

Dalla-costa LM, Irino K, Rodrigues J, Rivera ING, Trabulsi. 1998. Characterisation of diarrhoeagenic *Escherichia coli* clones by ribotyping and ERIC-PCR. *J Med Microbiol* 47:227-234. http://dx.doi.org/10.1099/00222615-47-3-227

Domenico P, Salo RJ, Cross AS, Cunha BA. 1994. Polysaccharide capsule-mediated resistance to opsonophagocytosis in *Klebsiella pneumoniae*. *Infect Immun* 62:4495-4499.

Huiyong D, Tongjie C, Yumei C, zhaobing Z, Meiling Y, Xingxiao Z. 2008. Transmission identification of *Escherichia coli* aerosol in chicken houses to their environments using ERIC-PCR. *Sci China: Life Sci* 51:164-173.

Ikehata H, Wakaizumi M, Murata K. 1968. Antioxidant and antihemolytic activity of a new isoflavone, "factor 2" isolated from Tempe. *Agr Biol Chem* 32:740-746. http://dx.doi.org/10.1271/bbb1961.32.740

Keuth S, Bisping B. 1993. Formation of vitamins by pure cultures of tempe moulds and bacteria during the tempe solid substrate fermentation. *J Appl Bacteriol* 75:427-434. http://dx.doi.org/10.1111/j.1365-2672.1993.tb02798.x

Khan AA, McCarthy S, Wang RF, Cerniglia CE. 2002. Characterization of united States outbreak isolates of *Vibrio parahaemolyticus* using enterobacterial repetitive intergenic consensus (ERIC) PCR and development of a rapid PCR method for detection of O3:K6 isolates. *FEMS Microbiology* 206:209-214. http://dx.doi.org/10.1111/j.1574-6968.2002.tb11011.x

Liem ITH, Steinkraus KH, Cronktt TC. 1977. Production of vitamin B-12 in Tempe, a fermented soybean food. *Appl Environ Microbiol* 34:773-776.

Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, Wade WG. 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl Environ Microbiol* 64:795-799.

Meacham KJ, Zhang L, Foxman B, Richard Bauer RJ, Marrs CF. 2003. Evaluation of genotyping large numbers of *Escherichia coli* isolates by enterobacterial repetitive intergenic consensus-PCR. *J Clin Microbiolog* 41:5224-5226. http://dx.doi.org/10.1128/JCM.41.11.5224-5226.2003

Mulyowidarso RK, Fleet GH, Buckle KA. 1989. The microbial ecology of soybean soaking for tempe production. *Int J Food Microbiol* 8:35-46. http://dx.doi.org/10.1016/0168-1605(89)90078-0

Mulyowidarso RK, Fleet GH, Buckle KA. 1990. Association of bacteria with the fungal fermentation of soybean tempe. *J Appl Bacteriol* 68:43-47. http://dx.doi.org/10.1111/j.1365-2672.1990.tb02546.x

Podschun R, Ullmann U. 1998. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin Microbiol Rev* 11:589-603.

Sechi LA, Zanetti S, Dupré I, Delogu G, Fadda G. 1998. Enterobacterial repetitive intergenic consensus sequences as molecular targets for typing of *Mycobacterium tuberculosis* strains. *J Clin Microbiol* 36: 128-132.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731-2739. http://dx.doi.org/10.1093/molbev/msr121

van der Zee A, Steer N, Thijssen E, Nelson J, van't Veen A, Buiting A. 2003. Use of multienzyme multiplex PCR amplified fragment length polymorphism typing in analysis of outbreaks of multiresistant *Klebsiella* pneumoniae in an intensive care unit. *J Clin Microbiol* 41:798-802. http://dx.doi.org/10.1128/JCM.41.2.798-802.2003

Versalovic J, Koeuth T, Lupski JR. 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucl Acids Res* 19:6823-6831. http://dx.doi.org/10.1093/nar/19.24.6823

Waturangi DE, Joanito I, Yogi Y, Thomas S. 2012. Use of REP- and ERIC-PCR to reveal genetic heterogeneity of *Vibrio cholerae* from edible ice in Jakarta, Indonesia. *Gut Pathog*. 4:2. http://dx.doi.org/10.1186/1757-4749-4-2

Wiesel I, Rehm HJ, Bisping B. 1997. Improvement of tempe fermentations by applicationof mixed cultures consisting of *Rhizopus* sp. and bacterial strains. *Appl Microbiol Biotechnol* 47:218-225. http://dx.doi.org/10.1007/s002530050916

Wilson LA, Sharp PM. 2006. Enterobacterial repetitive intergenic consensus (ERIC) sequences in *Escherichia coli*: evolution and implications for ERIC-PCR. *Mol Biol Evol* 23:1156-1168. http://dx.doi.org/10.1093/molbev/msj125