MICROBIOLOGY INDONESIA
ISSN 1978-3477, eISSN 2087-8575
Vol 8, No 1, Maret 2014, p 9-15

Available online at
http://jurnal.permi.or.id/index.php/mionline
DOI: 10.5454/mi.8.1.2

Klebsiella pneumoniae from Indonesian Tempeh were Genetically Different from that of Pathogenic Isolates

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Tempeh is important traditional Indonesian fermented food made from soybeans employing *Rhizopus oligosporus* or *R. microsporus*. During the process of tempeh production, some bacteria from the environment and tempeh starter become an integral part of tempeh, and even have important roles in determining the final quality of tempeh it self. Several studies reported the presence of *Klebsiella pneumoniae* in tempeh as one of vitamin B12 producing bacteria in tempeh. However, *K. pneumoniae* also known as opportunistic pathogens causing pneumonia and liver abscess in human. In this study, Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction (ERIC-PCR) was employed to determine genetic diversity of *K. pneumoniae* isolated from tempeh and compared them with medical isolates. The result indicated that isolates from tempeh were genetically distinct from those of medical isolates.

Key words: ERIC-PCR, *Klebsiella pneumoniae*, tempeh

Tempe merupakan salah satu makanan utama tradisional Indonesia yang terbuat dari kacang kedelai dengan fermentasi menggunakan cendawan *Rhizopus oligosporus* atau *R. microsporus*. Selama proses pengolahan, bakteri yang berasal dari lingkungan dan inokulum awal menjadi bagian yang tidak terpisahkan dari tempe, bahkan memiliki peranan yang penting dalam menentukan kualitas akhir pada tempe. Beberapa penelitian telah melaporkan keberadaan *Klebsiella pneumoniae* pada tempe sebagai salah satu bakteri penghasil vitamin B12 pada tempe. Akan tetapi, *K. pneumoniae* juga dikenal sebagai patogen oportunis penyebab penyakit pneumonia dan abses hati pada manusia. Pada penelitian ini, metode Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction (ERIC-PCR) digunakan untuk membandingkan keragaman genetik *K. pneumoniae* pada tempe dibandingkan dengan isolat medis. Hasil penelitian ini menunjukkan bahwa secara genetik pneumoniae pada tempe berbeda dengan isolat medis.

Kata kunci: ERIC-PCR, *Klebsiella pneumoniae,* tempe

Tempeh is an indigenous Indonesian fermented food and has become an important part of the Indonesian diet for hundreds years. Tempeh is consumed in relatively large portion and can be found in a variety of types of cooking and processing methods. Excellent protein quality has made tempeh become a meat substitute and becomes popular among vegetarians (Liem *et al*.1977).

The process of tempeh making in Indonesia is still using conventional methods with uncontrolled condition (Barus *et al*. 2008). During the fermentation process, not only fungi involved but also bacteria play important roles in the formation of flavor and nutrition. Bacterial growth during tempeh production begins in the process of soybean soaking. During the fermentation process, the producers inadvertently adding bacteria so that the bacteria eventually become an inseparable part of tempeh, and even has an important role in determining the quality of tempeh (Barus *et al*. 2008; Seumahu e*t al*. 2013).

Several studies have reported the presence of bacteria in tempeh as *Klebsiella pneumoniae* and *Citrobacter freundii* (Keuth and Bisping 1994), and also bacteria of the phylum Proteobacteria and Firmicutes (Seumahu *et al.* 2012) which are reported as bacteria producing vitamin B_{12} in tempeh. Other contaminants such as *Brevibacterium epidermidis* and *Micrococcus luteus* known to play a role in the formation of antioxidants in tempeh (Klus and Barz 1995). Furthermore, bacteria in tempeh is also known as one of the factors that play a role in the formation of a bitter taste in tempeh (Barus *et al*. 2008). A diverse array of lactid acid bacteria and yeasts also play important role in Indonesian tempeh production (Efriwati *et al*. 2013).

K. pneumoniae which is included in family *Enterobacteriaceae* is also known both of causing

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pneumonia disease, an acute infection that attacks the alveoli (Gori *et al*. 1996) and liver abscess in human (Wang *et al.* 1998). Therefore, the study of genetic diversity is important for the identification and characterization of bacterial pathogenicity (Rademaker and de Bruijn 1997). One of the many molecular techniques for the study of genetic diversity is Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR. ERIC sequences are short sequences, which is 126 bp long, with sequences that are conserved as internal repeat and as non-coding sequences (Lupski *et al.* 1992). This technique is often used because it is simple, rapid, reproducible, and discriminative (Olive *et al.* 1999) and 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). ERIC-PCR has also been used to analyzed genetic diversity of *Klebsiella* spp. isolated from tempeh (Barus *et al*. 2013), as well as to study genetic heterogeneity of many types of *Vibrio cholerae* (Waturangi *et al*. 2012).

MATERIALS AND METHODS

This research was conducted in Research Laboratory, Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia from May to December 2013.

Medical isolates of *K. pneumoniae*. Four medical isolates ware used for comparison with *K. pneumoniae* from tempeh, i.e. *K. pneumoniae* ATCC BAA-2146, *K. pneumoniae* subsp. *pneumoniae* ATCC 10031, *K. pneumoniae* ATCC 35657, and one *K. pneumoniae* isolate originated from pneumonia patient, named as FK isolate, collection of Department of Microbiology, Faculty of Medicine, Atma Jaya Catholic University.

K. pneumoniae isolates from Tempeh. Tempeh EMP and WJB was produced in Bogor, West Java, Indonesia (Barus *et al*. 2008). A 10 g of fresh tempeh was placed into 90 mL of sterile physiological salt 0.85% (w/v) NaCl and homogenized in orbitar shaker (Yih Der) at a speed of 24 x g for one min. Dilution was made from 10^{-1} until 10^{-6} and a 100 µL from dilution of 10^{-4}, 10^{-5}, and 10^{-6} was spread on Eosin Methylene Blue (EMB) Agar (Oxoid) and incubated overnight at 37 °C. A single purple mucoid colony was typical character of *K. pneumoniae*. These colonies were further analyzed by cultivating them on Simmons' Citrate Agar (SCA) (Difco), and incubated at 37 °C for 24 h. Tempeh sampling were conducted twice, i.e. August and October 2013. *Klebsiella* sp. 135 isolated from tempeh was used for control (Maysella 2010).

Analysis of 16S rRNA Genes. Suspected blue colonies on Simmons' Citrate Agar were further verified using sequencing of genes encoding 16S rRNA. The whole cells from single colonies on plates were used directly in PCR reaction as described by Rademaker and de Bruijn in 1997. The 16S rRNA gene was amplified employing a PCR machine (Applied Biosystems, 2720 Thermal Cycler) using primer 63f (5'- CAGGCCTAACACATGCAAGTC-3') and 1387r (5'- GGGCGGWT GTACAAGGC -3') (Marchesi *et al*. 1998). Total PCR volume was 50 µl containing 2 µL DNA *template*, 25 µL Go*Taq* Green® Master Mix (Promega), 2 µL primer forward dan reverse (25 pmol μL^{-1}) dan 19 µL nuclease free water. The PCR protocol was as follows: initial denaturation at 94 °C for 5 min, denaturation at 92 °C for 30 s, annealing at 62 °C for 30 s, elongation at 72 °C for 30 s, and post extention at 72 °C for 7 min. The cycle was repeated for 30 times. A 5 µL of PCR amplification products were further verified by electrophoresis in 1% agarose (Bioline) in 1x TAE buffer for 60 min, 80 V. Sequencing of PCR products were performed in Macrogen Inc., Korea, and were analyzed employing a program SeqTrace. Sequencing results were compared to the database with the Basic Local Alignment Search Tool (BLAST) program which is provided by National Centre of Biotechnology Information (NCBI).

Genetic Profiling of *K. pneumoniae* Isolates. 13 bacterial isolates were identified as *K. pneumoniae* from EMP tempeh and 10 isolates from WJB tempeh. A total PCR volume used was 25 µL containing 12.5 µL Go*Taq* Green® Master Mix (Promega), 1 µL of 25 pmol ERIC1R (5'-ATGTAAGCTCCTGGGGATTCA C-3'), 1 µL of 25 pmol ERIC2F (5'-AAGTAAGTGAC TGGGGTGAGCG-3')Give references for the primers (Versalovic *et al*. 1991), 9.5 µL nuclease free water, and 1 µL DNA template which was obtained directly from the isolates using a sterile toothpick. The PCR protocol was as follows: initial denaturation at 95 °C for 7 min, denaturation at 95 °C for 30 s, annealing at 49 °C for 1 min, elongation at 65 °C for 8 min, and post extention at 65 °C for 16 min (Applied Biosystems, 2720 Thermal Cycler). The PCR cycle was used 30 times. A 5 µL of PCR products was verified by electrophoresis for 90 min and 70 V, on 1% agarose in 1x TAE buffer. Formed band profiles were observed under the UV transilluminator. Formed band profiles were then compared as biner number and analyzed using *FreeTree* (Hampl *et al*. 2001) and TreeView to construct a phylogenetics tree (http://taxonomy.zoology.gla.ac.uk/

rod/treeview.html.

RESULTS

Isolation of *K. pneumoniae*. A total of 58 bacterial isolates (Table 1) were isolated from EMP and WJB tempeh. The bacterial colonies were purple in the center of colony, mucoid, and rounded shape on EMB medium. The colonies of these isolates changed from green to blue on Simmons'Citrate medium, which is specific character of *Klebsiella* sp.

Fig 1A and 1B showed hypermucoviscocity of *K. pneumoniae* ATCC35657 or FK colonies when touched with inoculating loop. However, colonies of *K. pneumoniae* from EMP (Fig 1C) and WJB tempeh (Fig 1D) did not show hypermucoviscosity. This character showed significant phenotypic difference between tempeh and those of medical isolates.

Analysis of 16S rRNA Genes. A total of 18 isolates from the EMP tempeh and 25 isolates from WJB tempeh were selected for further amplification of genes encoding 16S rRNA. Based on 16S rRNA gene sequence alignments with the NCBI database, *K. pneumoniae* were identified in 13 isolates obtained from EMP tempeh and 10 isolates from WJB tempeh (Table 1). The other microorganisms were found from EMP tempeh based on 16S alignment analysis were: *Klebsiella sp*., *Rhizobium sp*., and *Enterobacter sp*. while *Bacterium sp.*, *Klebsiella sp.*, and *Cronobacter sakazakii* were isolated from WJB tempeh.

Genetic Profiling of *K. pneumoniae* Isolates. A total 23 isolates which were identified as *K. pneumoniae* were further subjected to ERIC-PCR analysis in order to compare their genetic diversity from those of medical isolates. ERIC-PCR of 13 isolates of *K. pneumoniae* from EMP tempeh showed a similar pattern (Fig 2). The ERIC-PCR profiles of medical isolates showed a diverse patterns but distinctively different from tempeh isolates. This result indicated that *K. pneumoniae* presence in tempeh was not the same as pathogenic *K. pneumoniae*. Similar result were also found when we analysed isolates from WJB tempeh (Fig 3) which showed different ERIC-PCR profiles from the medical isolates. Although genetic profiles of *K. pneumoniae* isolates in WJB tempeh were more varied than EMP tempeh, we found noi dentical profiles when compared to those of medical isolates.

The resulting electrophoresis band profiles were further converted into binary data matrix. The results were used to construct a phylogenetic tree using FreeTree program and TreeView. Fig 4 showed the genetic relationship of *K. pneumoniae* isolated from EMP tempeh. Based on this analysis, the medical isolates clustered into a separate and distict group. Tempeh isolates formed two groups, the first one contains isolates which have similar ERIC profiles, i.e., I EMP16, I EMP13, I EMP9, I EMP8, I EMP5, I EMP4, and I EMP. The other group contains of I EMP1, II EMP5, II EMP2, II EMP4, II EMP1, and II EMP3. Isolates from WJB tempeh formed a separate group under one branch except one isolate, I WJB2 (Fig 5). Again, in this analysis, the medical isolates formed a separate group outside the branches which contained isolates from WJB.

DISCUSSION

K. pneumoniae presence in both of tempeh samples (EMP and WJB) and consistantly exist from the first

Table 1 *Klebsiela pneumoniae* from fresh tempeh

Tempeh sample	Number of sampling	EMB agar	SCA	Number of Isolates Sequencing of 16S rRNA	DNA Sequence Alignment *(K. pneumoniae)*
EMP	I	18	18	10	8
	II	8	8	8	5
Total of EMP		26	26	18	13
WJB	I	17	10	10	7
	II	15	15	15	3
Total of WJB		32	25	25	10
Grand Total		58	51	43	23

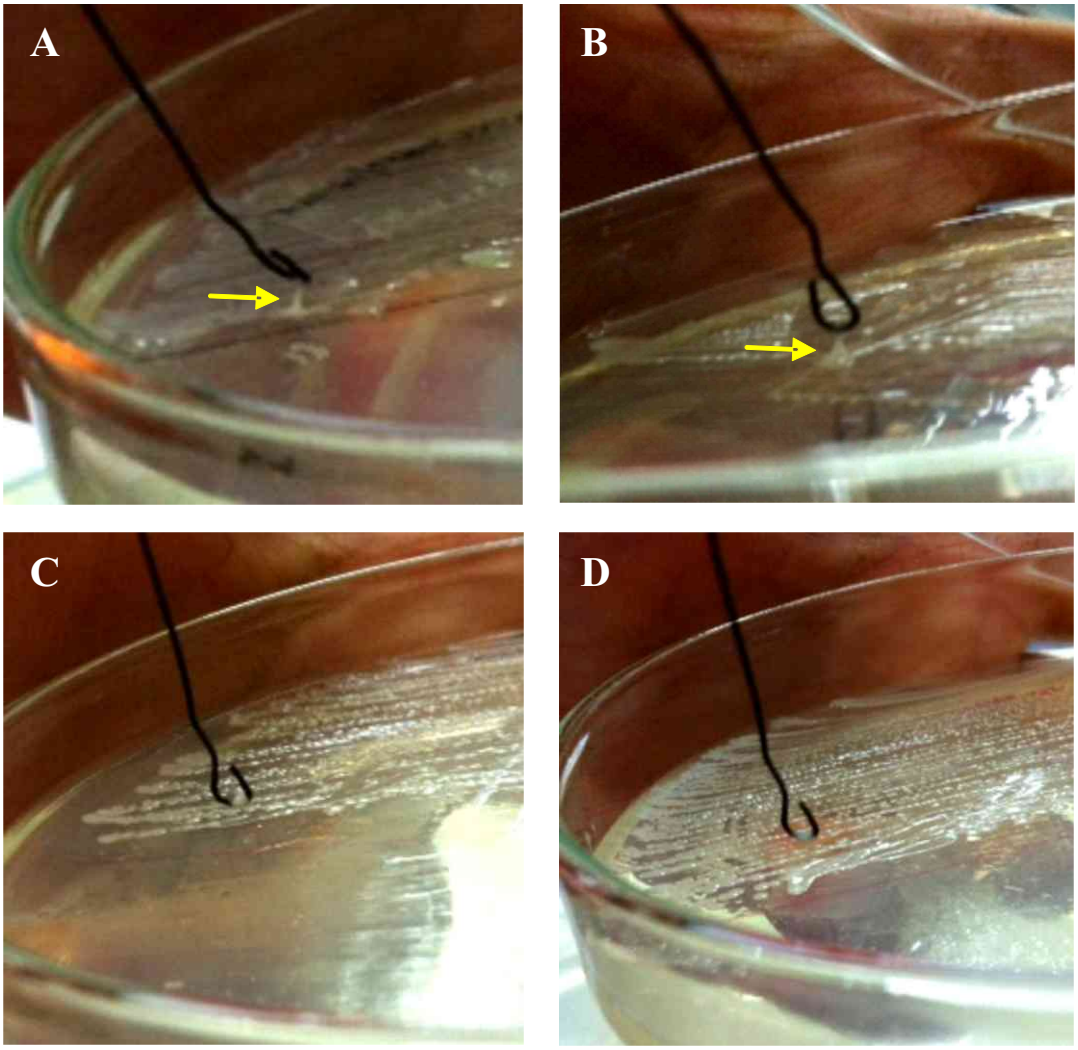

Fig 1 The phenotype of *K. pneumoniae* ATCC 35657 (A), FK isolate (B), *K. pneumoniae* from EMP (C), and WJB tempeh (D).

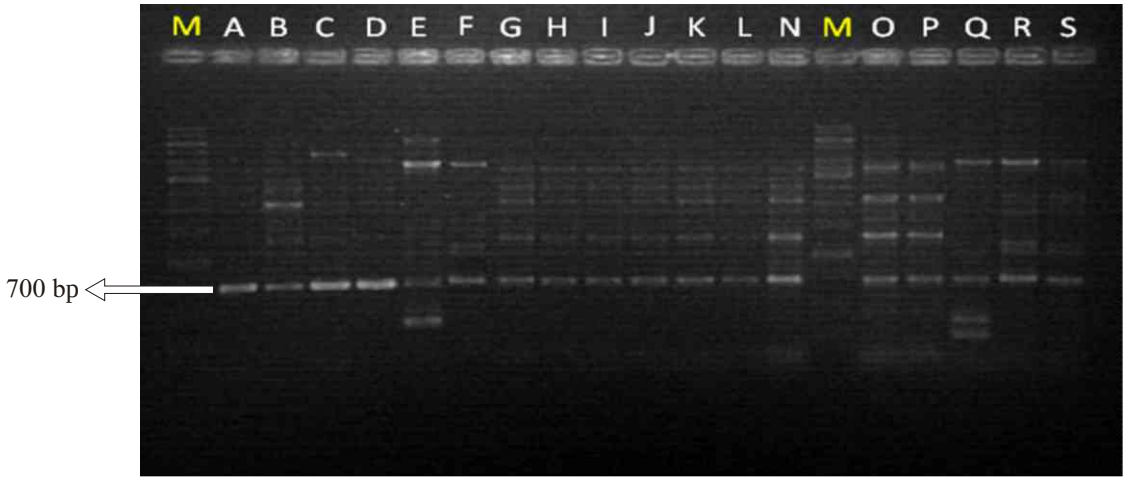

Fig 2 ERIC-PCR from *K. pneumoniae* from EMP tempeh, ie. (M) Molecular markers, (A) ATCC 10031, (B) ATCC 2014, (C) ATCC 35657, (D) FK, (E) *Klebsiella* sp. 135, (F) I EMP1, (G) I EMP3, (H) I EMP4, (I) I EMP5, (J) I EMP8, (K) I EMP9, (L) I EMP13, (N) I EMP16, (O) II EMP1, (P) II EMP2, (Q) II EMP3, (R) II EMP4, and (S) II EMP5.

and second sampling of each of these tempeh samples. The presence of *K. pneumoniae* in tempeh have been reported before (Keuth *et al.* 1994, Barus *et al.* 2008). Growth of *K. pneumoniae* on EMB medium showed distinctive colony morphology, which were dark purple in the center, mucoid, and rounded shape.

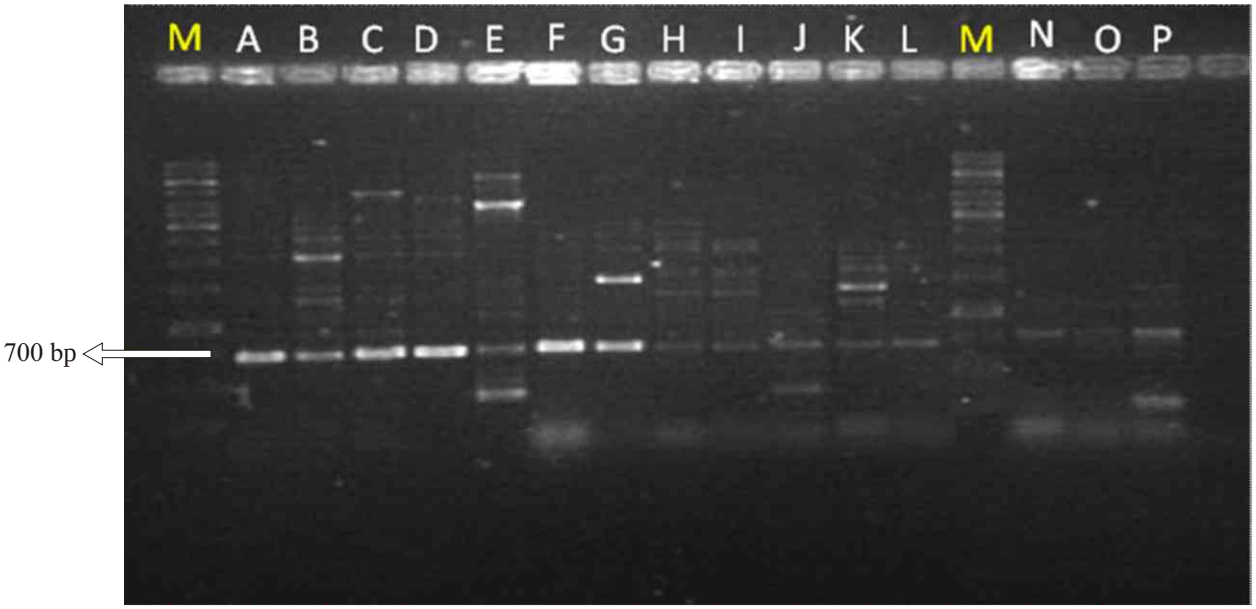

Fig 3 ERIC-PCR profiles of *K. pneumoniae* from WJB tempeh, ie. (M) Molecular markers, (A) ATCC 10031, (B) ATCC 2014, (C) ATCC 35657, (D) FK, (E) *Klebsiella* sp. 135, (F) I WJB1, (G) I WJB2, (H) I WJB3, (I) I WJB4, (J) I WJB5, (K) I WJB6, (L) I WJB16, (N) II WJB3, (O) II WJB5, and (P) II WJB8.

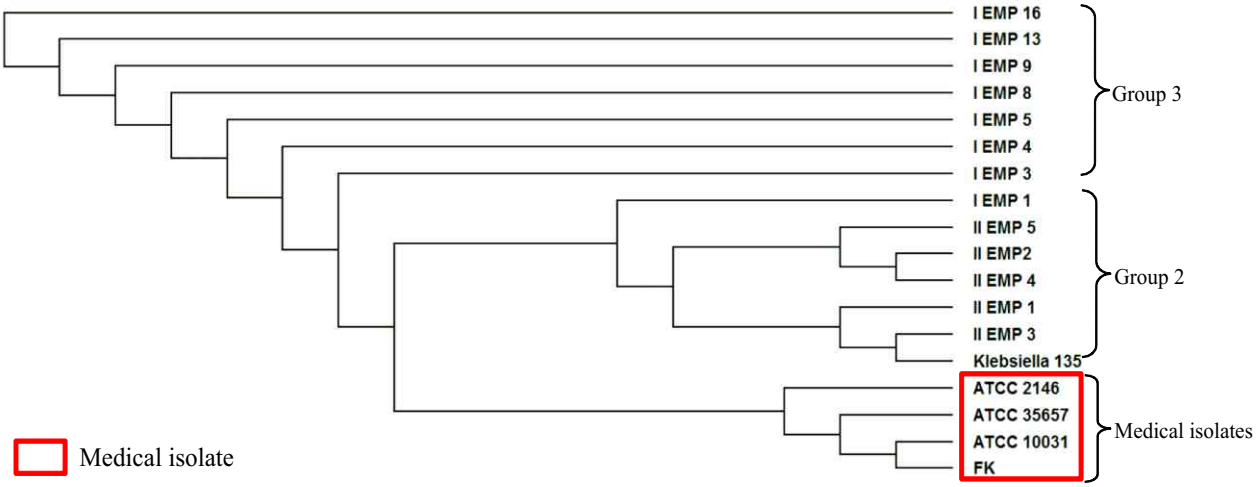

Fig 4 Philogenetic tree generated from ERIC-PCR profiles of *K. pneumoniae* isolated from EMP tempeh.

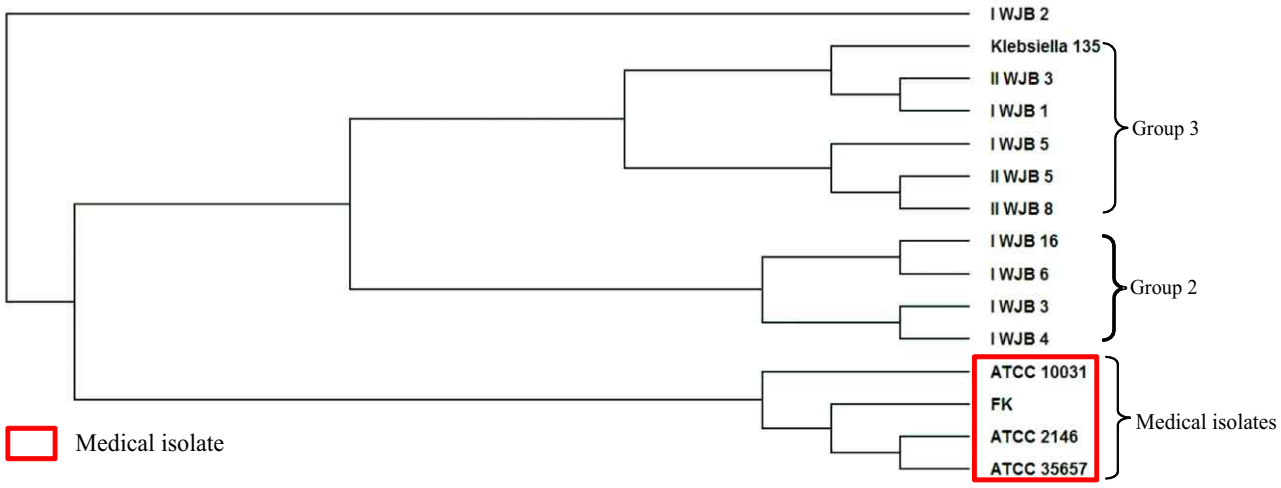

Fig 5 Philogenetic tree generated from ERIC-PCR profiles of *K. pneumoniae* isolated from WJB tempeh.

Fig1showed the phenotype difference tha the colonies of medical isolates showed more sticky on solid media than those isolated from tempeh. Mutation in *mag*A is one of the factors that mutants lost the hypermucoviscosity phenotype and became susceptible tophagocytes and a virulent to mice (Fang *et al*. 2004).

16S rRNA gene sequences are the most common house keeping genetic marker used for identifying bacteria in the laboratory (Janda *et al*. 2007). Although 16S rRNA gene (approximately 1 500 bp) is large enough for bioinformatics purposes, but usually it is not reliable for intra-strain differentiation. For example, *Escherichia coli* O104: H4 that is known pathogenic as well as isolate in recent out breaking Germany (Mellmann *et al*. 2011) were confirmed only as *E. coli* based on 16S DNA sequence analysis.

We confirmed colonies obtained from tempeh samples as *K. penumoniae* based on 16S DNA sequence analysis before subjected them for ERIC-PCR analysis to reveal intra-species genetic diversity (Barus *et al*. 2013).

In this study, ERIC-PCR method has been successfully employed to differentiate genetic profiles of *K. pneumoniae* isolated from tempeh and those of medical isolates. The ERIC-PCR profiles of *K. pneumoniae* from EMP tempeh (Fig2) and WJB tempeh (Fig3) were different from those of medical isolates. Keuth and Bispingin 1994 reported that *K. pneumoniae* isolated from Indonesian tempeh were negative for three known enterotoxins, i.e. Shiga-like toxin SLTIIA, heat-labil enterotoxin LTIh, and heat-stable enterotoxin STIh. Therefore, intraspecies genetic diversity within K. pneumoniae might reflect different phenotypes which could make the pathogenic or non-pathogenic.

The phylogenetic analysis showed two separate clusters representing K. pneumoniae from tempeh and the medical isolates. The phylogenetic trees generated from EMP tempeh showed unambiguously that tempeh isolates were genetically different from pathogenic *K. pneumoniae* (Fig4). That figure showed three main groups were formed from tempeh isolates, while the medical isolates were clearly separated in a different group. Similar result was also obtained from isolates derived fromWJB tempeh (Fig 5)

Our results suggested that *K. pneumoniae* isolates inIndonesian tempeh could be a distinctive non-pathogenic group of this species. This is also in line with the facts that tempeh production and consumption have been practiced in Indonesia for centuries and, to our knowledge, there is no single report on infectious disease associated with *K. pneumoniae* in tempeh. On the other hand, their presence in tempeh might be beneficial due to their ability to synthesize vitamin B12 and their immunomodulatory effects in human.

To conlude tempeh produced in Indonesia naturally harbors *K. pneumoniae* with unique genomic profiles. *K. pneumoniae* from tempeh samples in this study showed that they were genetically different from isolates known to be pathogenic to human.

ACKNOWLEDGMENT

This work was supported by DANA DIPA IPB (code : 2013.089.521219). We also acknowlegde the Department of Microbiology, Faculty of Medicine, Universitas Katolik Atma Jaya for the FK isolate.

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