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Genetic diversity of mastitis cow's milk bacteria based on RAPD-PCR

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Abstract. *Mustopa AZ, Puspitasari IF, Fatimah, Triratna L, Kartina G. 2018. Genetic diversity of mastitis cow's milk bacteria based on RAPD-PCR. Biodiversitas 19: 1714-1721.* Mastitis in cow is caused by several pathogenic bacteria, including antibiotic-resistant bacteria. Identification of the pathogenic bacteria's diversity that is contaminating cow's milk needs to be done. The aim of this research was to conduct molecular identification in mastitis cow's milk bacteria through RAPD-PCR (Random Amplified Polymorphism DNA-Polymerase Chain Reaction) analysis. Bacteria from mastitis cow's milk were enumerated using selective media. Based on the result of media selection, there were 72 isolates of bacteria from mastitis cows in Ciguha, Guranteng, Cikarenceng Village (Pagerageung, Tasikmalaya), and Warnasari Village (Pangalengan, Bandung), West Java, Indonesia. The genomes from these isolates were extracted and then subjected to RAPD-PCR analysis. The results of RAPD-PCR analysis showed 8 clusters of dendrogram which 4 dominant clusters were selected. Identification of 4 dominant clusters, which contained representative strains, using 16s rRNA showed the isolates BPA-12, MHA-6, L-4, and XLDA-8 were identified as *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, and *Enterobacter bugandensis.*

Keywords: 16S rRNA analysis, mastitis cow's milk bacteria, RAPD-PCR analysis

INTRODUCTION

Mastitis in dairy cow is an important problem in Indonesia's dairy industries. Mastitis subclinical gives about 75-83% of cases until 2006 (Sudarwanto et al. 2006). Rahayu (2009) reported that the prevalence of subclinical mastitis increased by 85% and caused reduction in milk production by up to 15%. Ditjennak (2006) also reported that 80% of lactation cows in Indonesia are mastitis subclinical. It becomes the main problem for animal farmer because of milk production decline to 20%. In East Java, the prevalence of subclinical mastitis case is low, only 51.8% (Winarso 2008), compared to the same case in small dairy farm in Java Island that reached 85% in term of 2008-2010 (Nurhayati and Martindah 2015).

Ditjennak (2006) stated Indonesia's dairy population reach to 368,470 cows with milk production of 6.5-8.5 L/cow/day. While, ideal dairy production in Indonesia is 14-16 L/cow/day. Low milk production is analogous with 60-90% cows affected by mastitis in Indonesia, which most of cases caused by subclinical mastitis. Almost all cattle ranchers are unaware about subclinical mastitis because there is no physical change in milk and udder (Nurdin 2007). Economically, milk contamination gives disadvantage to producer and consumer because of low milk production and low quality. Milk quality is the most important factor for consumer and can determine the quality of production during processing (Collins et al. 2010). Normally, fresh cow's milk can last for 4 hours. Subclinical and clinical mastitis can contaminate milk and accelerate damage in milk.

However, treatments for this contamination in Indonesia is still not effective. They usually use broad-spectrum antibiotics without analyzing the specific causal agent. Improper mastitis treatment and using large-scale antibiotics could rise antibiotic resistance in certain bacteria (Sandholm and Pyorala 1995). It is necessary to test the mastitis-causing agent and consult with veterinarian in determining the type of drug and antibiotic to be used, so the treatment will be more effective (Waldner 2007).

There are 137 pathogens causing mastitis. The most common types of pathogens that infect large animal groups, are *Staphylococcus aureus*, *Streptococcus agalactiae*, other *Streptococcus* species and coliforms. Mastitis is often associated with other organisms such as *Actinomyces pyogenes*, *Pseudomonas aeruginosa*, *Nocardia asteroides*, *Clostridium perfringens*, *Mycobacterium*, *Mycoplasma*, *Pasteurella*, and *Prototheca*. Cases that occur are mostly caused by *Staphylococcus*, *Streptococcus*, coliforms, and *A. pyogenes* (Herlina et al. 2015).

Random Amplified Polymorphism DNA-Polymerase Chain Reaction (RAPD-PCR) technique is a type of molecular marker widely used in molecular biology research and diagnostic. The analysis results can be stored computerized and allowing rapid identification of unknown isolates. In addition, this technique is simpler, easier in its preparation, gives faster results, and produces unlimited characters that can help to analyze bacterial genetic

diversity. RAPD is an inexpensive yet powerful method to study diversity (Mustopa and Fatimah 2014; MacGowan et al. 1993; Williams et al. 1990).

This study aimed to analyze genetic diversity of mastitis cow's milk bacteria in West Java, Indonesia by using RAPD-PCR technique. The results of this study are expected to provide information of polymorphism pattern and obtained phylogenetic tree from pathogenic bacteria in mastitis cow's milk in Indonesia.

MATERIALS AND METHODS

Study area

Samples were collected at cattle farms in Ciguha, Guranteng, Cikarenceng Villages (Pagerageung, Tasikmalaya) and Warnasari Village (Pangalengan, Bandung), West Java, Indonesia. The research was conducted in Laboratory for Applied Genetic Engineering and Protein Design, Research Center for Biotechnology, Indonesian Institute of Sciences, Bogor, West Java, Indonesia. The research was begun from March to September 2017.

Procedures

Sample preparation

About 10 to 15 ml of milk samples were milked from cows infected with mastitis in 4 different villages. The milk sample was tested by Mastitis Test, homogenized sample for negative mastitis and slimy or dense sample for positive mastitis. Then, fresh milk samples were diluted and enumerated by total plate count (TPC) method (Yunita et al. 2015). As much as 100 µL of bacterial suspension were removed from each dilution into petri dishes containing media selection of Baird Parker Agar (BPA) (Baird-Parker 1962), Mueller Hinton Agar (MHA) (Mueller and Hinton 1941), Xylose Lysine Deoxycholate Agar (XLDA) (Zajc-Satler and Gragas 1977), and Listeria Oxford Agar Base (LOAB) (Lee and McClain 1986). Suspension was spread into agar medium in petri dish. The petri dish was incubated at 37°C for 24-48 hours. The growing colony is transferred into Nutrient Broth (NB) medium, incubated at 37°C overnight and stored at 4°C.

Genomic DNA extraction

The DNA genome from the growing colony (bacteria isolate) was extracted by the method developed by

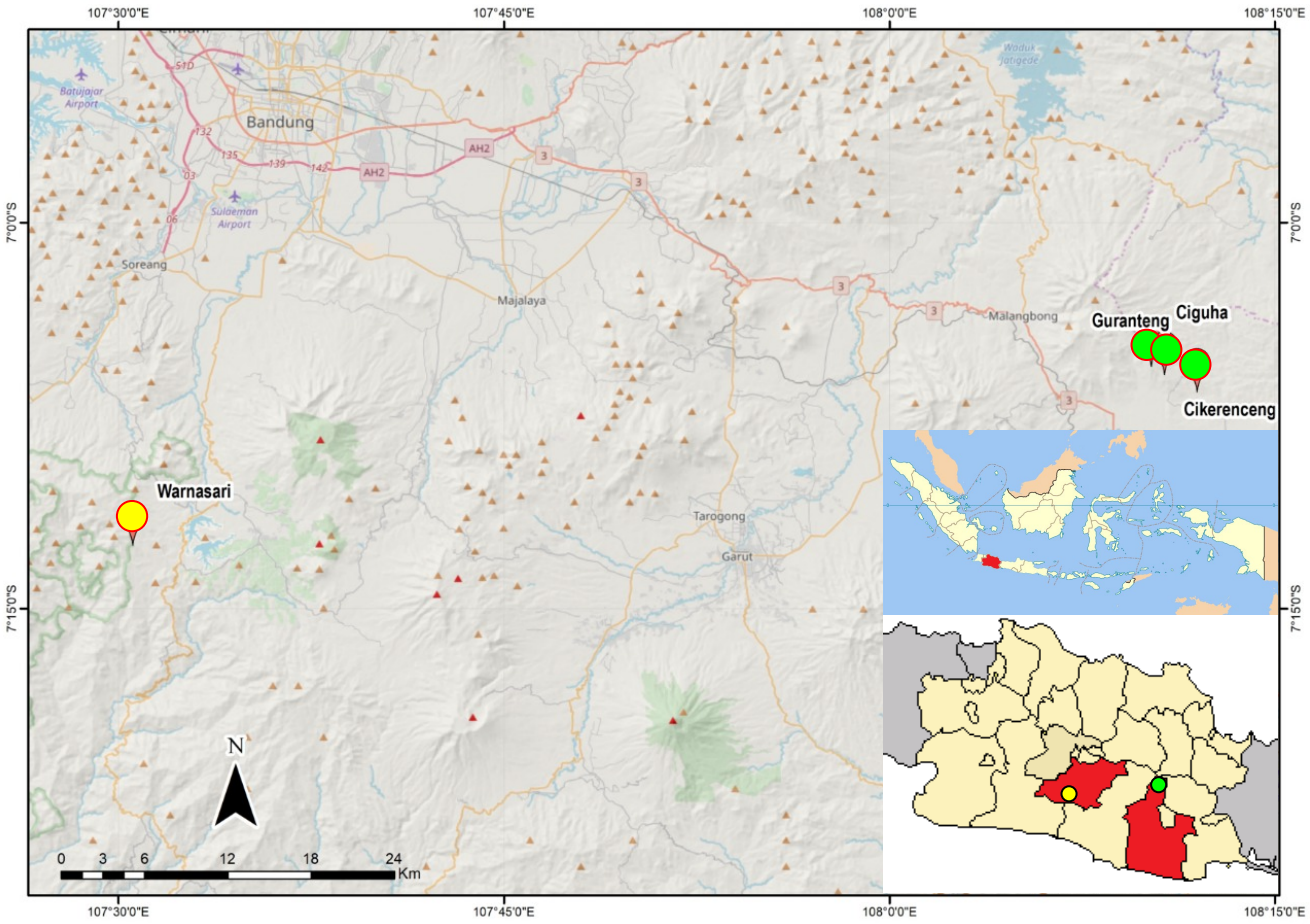

Figure 1. Location of sample collection in Pagerageung Sub-district (●) of Tasikmalaya and Pangalengan Sub-district (●) of Bandung, West Java, Indonesia

Mustopa and Fatimah (2014). The isolates were transferred into 1.5 mL sterile tube and centrifuged at 11,000 x g for 10 minutes, at 4°C. The cell pellets were suspended with 540 µL of Tris-EDTA buffer and 10 µL lysozyme, then homogenized and incubated at 37°C for 60 minutes. The solution was added with 200 µL SDS 10%, 100 µL NaCl 5 M, 80 µL 10% CTAB and incubated at 68°C for 30 minutes. Then, chloroform was added 1:1 (v/v), and centrifuged at 23000 x g for 10 minutes. The top phase solution was transferred to a new microtube, then isopropanol was added with a volume ratio of 1:1 and centrifuged. The DNA pellets were added by 1 mL 70% cold ethanol and inverted. Then, centrifuged at 10000 x g for 2 minutes at 4°C. The DNA pellets were aired overnight. Dry DNA was solubilized in 27 µL of sterile water (ddH_2O) and 3 µL RNAse. The DNA solution incubated at 37°C for 30 minutes, then stored at 4°C.

RAPD-PCR

The composition of RAPD-PCR reaction of mastitis cow's milk bacteria can be seen in Table 1. The PCR was performed by pre-denaturation at 94°C, 3 mins; followed by 44 cycles of denaturation at 94°C, 1.5 mins; annealing at 36°C, 1 min; extension at 72°C, 2 mins; and final extension at 72°C, 5 mins. The PCR product further analyzed by electrophoresis on 1.5 % (w/v) agarose gel in 0.5x TBE buffer and stained with Ethidium Bromide (EtBr). Marker Universal Ladder was used as standard DNA size (Chao et al. 2008). The results of RAPD-PCR can be visualized in electrophoregram.

Identification of mastitis cow's milk bacteria

16S rRNA gene sequencing for bacterial identification was using PCR with two primers. The primers were 8F primer (5'-AGAGTTTGATCATGGCTCAG-3') and 16R primer (5'-AAGGAGGTGATCCAACCGCA-3'). The PCR mix comprised 16 µL distilled water, 1 µL DNA template, 0.2 µL 8F primer, 0.2 µL 16R primer, and 0.2 µL *Taq* (Dream). The PCR was performed using pre-denaturation at 94°C, 3 mins, followed by 30 cycles of denaturation at 94°C, 1 min; annealing at 50°C, 1 min; extension at 72°C, 2 mins; and final extension at 72°C, 5 mins. The PCR product was analyzed by electrophoresis on 1% (w/v) agarose gel in 0.5x TBE buffer and stained with EtBr. Marker 1 kb Ladder was used as standard DNA size. The 16S rRNA gene is then direct sequenced. The sequencing results were analyzed using Basic Local Alignment Search Tool (BLAST) in the NCBI Program. The phylogenetic tree is further prepared using MEGA 6.06 application (Chao et al. 2008).

Data analysis

Evaluation of the DNA bands was based on specific molecular weight. The resulting amplification fragment is the dominant DNA locus. The RAPD marker was scored manually. Scoring criteria based on the presence of locus, 1 for presence and 0 for absence. Electrophoregram was manually analyzed to be a binary number of 1-0 in excel format. The binary data further processed into dendrogram or called phylogenetic tree using NTSYS 2.02 program (Rohlf 1993).

RESULTS AND DISCUSSION

Bacterial isolation of mastitis cow's milk samples in four different regions were selected by four different selective media, which were BPA, MHA, XLDA, and LOAB. The bacterial isolation showed 72 isolates as shown in Table 2.

Table 1. RAPD amplification reaction of mastitis cow's milk bacteria

Substance	Final concentration	1X (*) (µL)
10X PCR buffer	1X	2
20 µM RAPD primer	0.3 µM	3
5 µM dNTPs	0.3 µM	2
Taq Polymerase		0.2
ddH_2O		10.7
DNA samples		2
Volume total		20

Note: * Total of samples prepared; (#) sample volume 2 µL per reaction

Table 2. Isolate code and the origin of the used isolates

Isolate code	Origin	No	Isolate code	Origin
BPA-1	Ciguha	37	L-13	Warnasari
BPA-2	Ciguha	38	L-14	Warnasari
BPA-3	Ciguha	39	L-15	Warnasari
BPA-4	Ciguha	40	XLDA-1	Guranteng
BPA-5	Ciguha	41	XLDA-2	Guranteng
BPA-6	Guranteng	42	XLDA-3	Guranteng
BPA-7	Guranteng	43	XLDA-4	Guranteng
BPA-8	Guranteng	44	XLDA-5	Guranteng
BPA-9	Guranteng	45	XLDA-6	Cikarenceng
BPA-10	Guranteng	46	XLDA-7	Cikarenceng
BPA-11	Cikarenceng	47	XLDA-8	Cikarenceng
BPA-12	Cikarenceng	48	XLDA-9	Cikarenceng
BPA-13	Cikarenceng	49	XLDA-10	Cikarenceng
BPA-14	Cikarenceng	50	XLDA-11	Warnasari
BPA-15	Cikarenceng	51	XLDA-12	Warnasari
BPA-16	Cikarenceng	52	XLDA-13	Warnasari
BPA-17	Cikarenceng	53	XLDA-14	Warnasari
BPA-18	Cikarenceng	54	XLDA-15	Warnasari
BPA-19	Cikarenceng	55	MHA-1	Ciguha
BPA-20	Cikarenceng	56	MHA-2	Ciguha
BPA-21	Warnasari	57	MHA-3	Ciguha
BPA-22	Warnasari	58	MHA-4	Ciguha
BPA-23	Warnasari	59	MHA-5	Ciguha
BPA-24	Warnasari	60	MHA-6	Guranteng
L-1	Warnasari	61	MHA-7	Guranteng
L-2	Warnasari	62	MHA-8	Guranteng
L-3	Warnasari	63	MHA-9	Guranteng
L-4	Warnasari	64	MHA-10	Guranteng
L-5	Warnasari	65	MHA-11	Cikarenceng
L-6	Warnasari	66	MHA-12	Cikarenceng
L-7	Warnasari	67	MHA-13	Cikarenceng
L-8	Warnasari	68	MHA-14	Cikarenceng
L-9	Warnasari	69	MHA-15	Cikarenceng
L-10	Warnasari	70	MHA-16	Warnasari
L-11	Warnasari	71	MHA-17	Warnasari
L-12	Warnasari	72	MHA-18	Warnasari

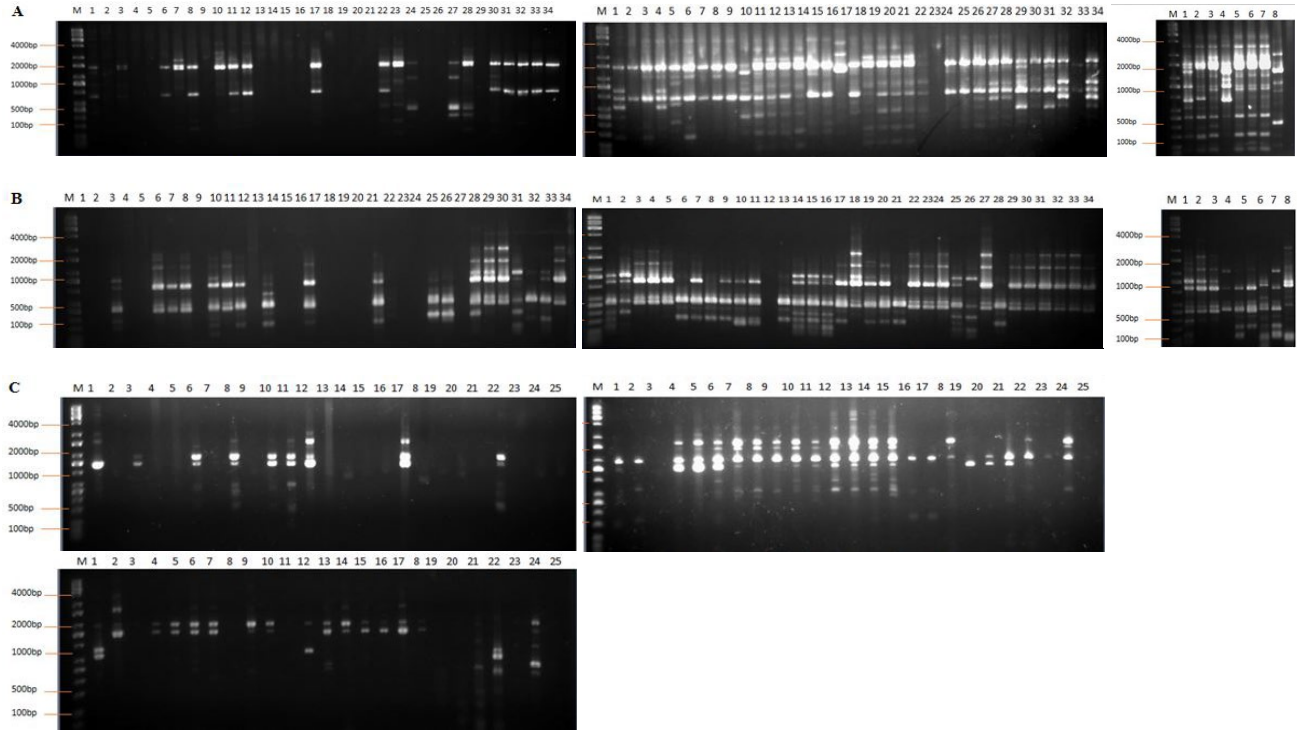

Figure 2. DNA profiling of mastitis cow's milk bacteria using RAPD PCR. A. Primer A, B. Primer B, C. Primer C

Genomic DNA extraction results

Qualitatively, the genomic DNA extraction result from 72 bacterial isolates were visualized in electrophoresis gel. The results show a band size greater than 10 kb, even though there are 4 samples that do not show DNA band (XLDA-15, L-9, L-12, and L-15). Quantitative analysis of genomic DNA showed different concentration each isolates as shown on Table 3. The purity of DNA from protein were analysed by 260/280 ratio and show great purity in 23 bacterial isolates. While the purity of DNA from polysaccharide were analysed by 260/230 and show great purity in 9 bacterial isolates.

RAPD-PCR identification and analysis of genetic relationship of mastitis cow's milk bacteria

Polymorphism identification was done with RAPD-PCR method. This PCR used 3 random primers (primer A, primer B, and primer C). The results were visualized in electrophoregram (Figure 2). The band pattern similarities on the electrophoregram showed the bacterial diversity. Electrophoregram was analyzed manually and changed into binary number 1 and 0. Then, the binary number data were analyzed in NTSYS 2.02 program to show the genetic relationship of mastitis cow's milk bacteria in dendrogram (Figure 3). The result of dendrogram was divided in 8 clusters with 4 dominant clusters (red circle).

16S rRNA analysis of mastitis cow's milk bacteria

PCR results using primer 8F and 16R primers for 16S rRNA identification can be seen in Figure 4. Positive results were indicated by the appearance of DNA bands of 1500 bp. The positive PCR results were sequenced to determine the base sequence of the 16S gene from each isolate. The gene sequence was then analyzed with Basic Local Alignment Search Tool (BLAST) using MEGA program and obtained phylogenetic tree for BPA-12 isolate (Figure 5), isolate MHA-6 (Figure 6), isolate L-4 (Figure 7), and XLDA-8 isolates (Figure 8). The identification of 16S rRNA showed that BPA-12 is *S. aureus* bacteria, L-4 is *Listeria monocytogenes* bacteria, MHA-6 is *Escherichia coli* bacteria, and XLDA-8 is *Enterobacter bugandensis* bacteria.

Discussion

Pathogenic bacteria diversity from mastitis cow's milk was evaluated. Seventy-two of pathogenic bacteria were isolated using 4 different media selections. Then, bacteria DNA was extracted and analyzed by 2 parameters, which are qualitative analysis and quantitative analysis. Qualitatively, the genomic DNA extraction results were visualized in electrophoresis gel. All samples show a band size greater than 10 kb, except XLDA-15, L-9, L-12, and L-15 sample. It might happen because its low DNA concentration, therefore, it is slightly invisible on the electrophoregram. Damerdesh et al. (2012) stated the chromosomal DNA from bacteria has a size more than 10 kb which is about 21-23 kb. Spectrophotometric analysis was used to quantitate DNA genome from the isolates by using a UV-VIS spectrophotometer that measured absorbance at 230 nm, 260 nm, and 280 nm. All ratio A260/280 results were in the range from 1,086 to 2,431. Good quality DNA will have an A260/280 ratio of 1.8–2.0 A (Sambrook et al. 1989). Lower A260/280 values may indicate protein contamination. Then, the ratio A260/230

from these isolates were in the range of 1.124-2.309. A260/230 values greater than 1.8 are typically suitable for analysis. Lower A260/230 values indicate contamination with salts or some solvents (e.g., phenol). These results showed that 22 isolates have a good level of purity (not contaminated by protein) and 8 isolates are not contaminated by polysaccharide, salt, or some solvents. It means that the majority of genomic DNA extracted from 72 isolates having un-pure DNA. According to Weishing et al. (2005), this is caused by endonuclease activity that destroys DNA, the high content of polysaccharides that increases the viscosity of the isolated product, and the inhibitor components (e.g. polyphenols and other secondary metabolites) contamination. DNA concentration was determined by measuring absorbance at 260 nm (Sambrook et al. 1989). The DNA concentrations of 72 isolates were varied from 20 ng/µL to 1607 ng/µL. BPA-2 isolate has the highest concentration (1607 ng/µL) although its purity level is low (low than 1.8). While, the lowest DNA concentration is L-4 and it can be detected by electrophoresis. XLDA-15 DNA that was not detected by electrophoresis because it has a low concentration (24 ng/µL). While L-9, L-12, and L-15, which are also not detected by electrophoresis, have concentrations of 107, 226, and 64 ng/µL, respectively.

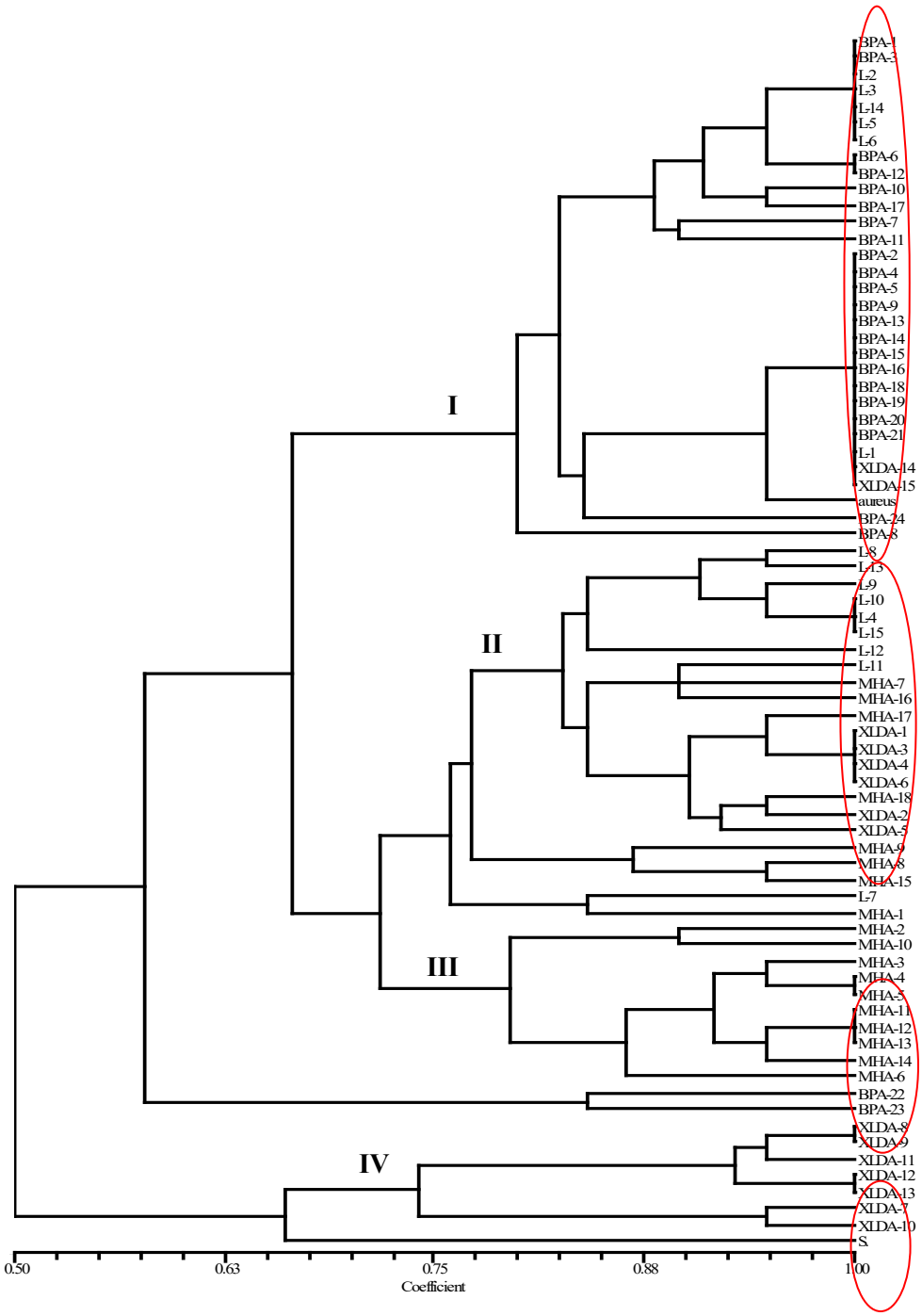

Figure 3. Dendrogram of RAPD-PCR analysis using NTSYS 2.02 program

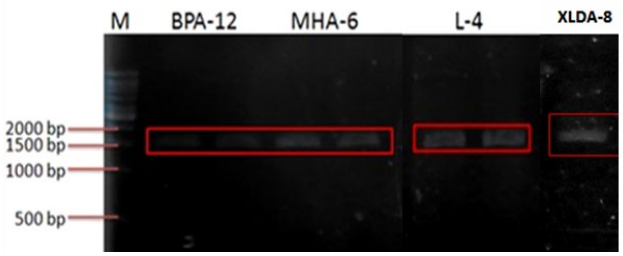

Figure 4. Electrophoregram of 16S rRNA identification result

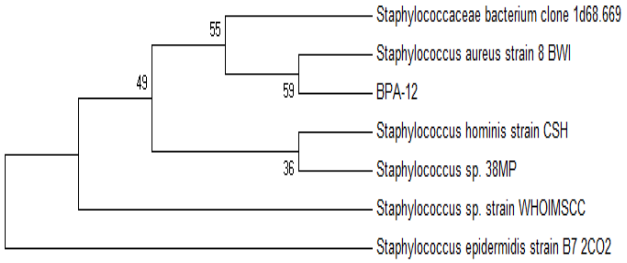

Figure 5. Phylogenetic tree of BPA-12 isolate

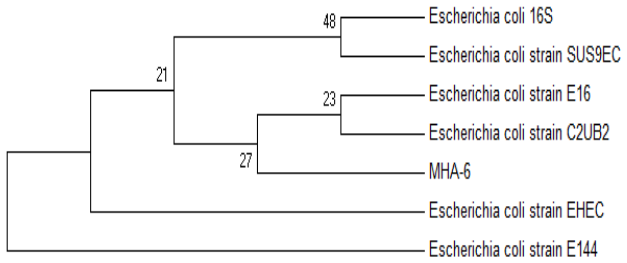

Figure 6. Phylogenetic tree of MHA-6 isolate

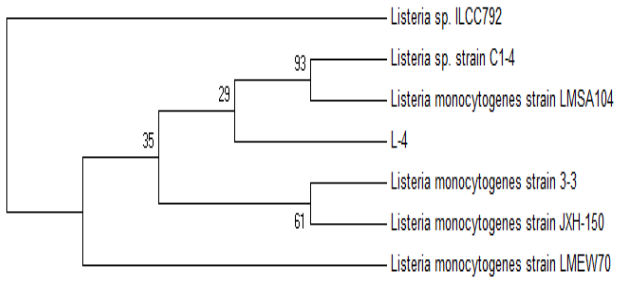

Figure 7. Phylogenetic tree of L-4 isolate

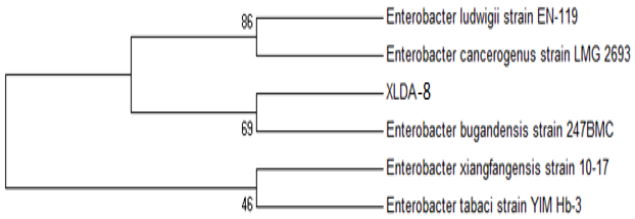

Figure 8. Phylogenetic tree of XLDA-8 isolate

Then polymorphism identification was done by RAPD-PCR method. DNA amplification is exponentially carried out with the aid of an enzyme and in vitro (Yuwono 2006). This PCR uses 3 random primers (primer A, primer B, and primer C). RAPD's excessive technique is tolerant to the level of DNA purity so it does not require high DNA purity (Prana and Hartati 2003). This technique is suitable for 72 isolates in this study which have low purity levels. Fingerprint analysis results showed the high level of polymorphisms. The polymorphism level reached 100%. This result indicates that 72 isolates from the 5 different regions are not really identical at the molecular level. Electrophoregram results showed that there is no characteristic of monomorphism, only one band appears at the same size in each isolate. Although this technique is tolerant to DNA purity level, but the intensity of the band of amplification product is influenced by the purity and concentration of DNA (Beishir 1991).

The result of the analysis of the NTSYS 2.02 program with the UPGMA method is a dendrogram that can express the proximity of each isolate (Figure 3). Dendrogram divided 72 isolates into 8 clusters with 4 main clusters (red circle). This grouping is based on the formation of the same band pattern produced by the same type of bacteria (Aqmarina 2014). Genetic relationship between bacteria was analyzed by genetic distance (Nei 1978). Cluster I consisted of 31 bacterial isolates, namely BPA-1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 24; L-1, 2, 3, 5, 6, 14; XLDA-14, and 15. The dominant isolates of cluster I was BPA isolates with 22 of 31 isolates. Cluster II consisted of 18 isolates, namely L-4, 8, 9, 10, 11, 12, 13, 15; MHA-7, 16, 17, 18; XLDA-1, 2, 3, 4, 5, and 6. Cluster II was dominated by isolate L with 8 of 18 isolates. Cluster III consisted of 10 isolates of bacteria, namely MHA-2, 3, 4, 5, 6, 10, 11, 12, 13, and 14. All isolates in cluster III are MHA isolates. Cluster IV consisted of 7 isolates of bacteria, namely XLDA-7, 8, 9, 10, 11, 12, and 13. Isolates in cluster IV were all XLDA isolates. Isolates of BPA that dominate cluster I consisted of isolates from Tasikmalaya (Ciguha, Guranteng, Cikarenceng) and Bandung (Warnasari). Isolate L which dominates cluster II came from Bandung (Warnasari). Isolate MHA that dominates cluster III came from Tasikmalaya (Ciguha, Guranteng, and Cikarenceng). Isolates XLDA that dominate cluster IV came from Tasikmalaya (Cikarenceng) and Bandung (Warnasari). Clustering is based on bacterial diversity with 80% coefficient. Isolates dominating each cluster were used as a reference in selecting bacterial isolates to be identified by the 16S rRNA technique. Four isolates were selected and had been identified namely as BPA-12, L-4, MHA-6, and XLDA-8.

PCR results using primer 8F and 16R primers for identification of 16S rRNA can be seen in Figure 4. Positive results are indicated by the appearance of DNA bands of 1500 base pair (bp). The results of 16S rRNA can be seen as phylogenetic tree for isolate BPA-12 (Figure 5), isolate MHA-6 (Figure 6), isolate L-4 (Figure 7), and XLDA-8 isolates (Figure 8). The identification of 16S rRNA showed that BPA-12 isolate is included as *S. aureus* bacteria. L-4 isolate is included as *L. monocytogenes*

bacteria. MHA-6 isolate is included as *E. coli* bacteria, and XLDA-8 isolate is included as *E. bugandensis* bacteria. While, the similarity level of each isolate with gene bank data is 97%, 94%, 98%, and 94%. Herlina et al. (2015) showed that the dominant bacteria present in mastitis cattle were *Staphylococcus* sp., *Pseudomonas aeruginosa*, *L. monocytogenes*, and *E. coli*. Epidemiological studies in Egypt found the main agents of subclinical mastitis isolated from positive CMT were *S. aureus*, *S. agalactiae*, and *E. coli*. with prevalence are 52.5%, 31.25%, and 16.25%, respectively. *S. aureus* is bacteria that causes the most subclinical mastitis. It can move quartile during the process of milking so that the transmission occurs. While the incidence of mastitis in dairy cows caused by *Pseudomonas* bacteria is very rare and sporadic (Supar and Ariyanti 2008). *E. bugandensis* is a highly pathogenic species of the genus *Enterobacter*. It is a nosocomial pathogen that can cause life-threatening infections in neonates and immunocompromised patients (Pati et al. 2018).

In conclusion, mastitis cow's milk bacteria in Ciguha, Guranteng, Cikarenceng, and Warnasari Village have 100% polymorphism value. The results of RAPD-PCR analysis showed 8 clusters from 72 bacterial isolates, with 4 dominant clusters. Coefficient of diversity and polymorphism value show bacterial isolates from 4 areas of milk sampling have a high bacterial similarity. Dominant bacterial isolates are BPA-12. MHA-6, L-4, and XLDA-8. BPA-12 are identified as *S. aureus* bacteria, MHA-6 are identified as *E. coli* bacteria, L-4 are identified as *L. monocytogenes* bacteria, and XLDA-8 are identified as *E. bugandensis* bacteria.

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