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SCREENING OF RESTRICTION ENZYMES FOR THE DEVELOPMENT OF RESTRICTION FRAGMENT-BASED, GENOMIC BIOMARKER OF *ALCALIGENES JAVAENSIS*

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Abstract

Alcaligenes javaensis is a food-borne, Gram-negative bacterium, which has the closest relationship with Alcaligenes faecalis commonly causing contamination of clinical equipment and infection on humans. Hence, the development of a biomarker for members of genus Alcaligenes including A.javaensis is needed for early detection of this bacterium. This research aimed to screen the performance of 5 restriction enzymes consisting of EcoRV, Hind III, NotI, SalI, and BgIII as singles and in combinations to produce specific pattern of restricted, genomic DNA fragments of A.javaensis. Total DNA was first extracted from subcultured A.javaensis cells using DNA isolation kit and the obtained DNA isolate was then subjected to enzyme restrictions in vitro. Later, the restricted DNA fragments were evaluated using agarose gel electrophoresis method. Results showed that only the EcoRV-BgIII-HindIII combination within a restriction process of 18 h could produce smaller-sized DNA bands, while those from other combinations (5 enzymes as singles and their 2-combinations) could not do so. As conclucion, the combination of EcoRV-BgIII-HindIII is the most potential among the evaluated endonucleases to be used as a genomic biomarker for A.javaensis.

Keywords

Restriction Enzyme, Genomic Biomarker, *Alcaligenes Javaensis*, *Alcaligenes Faecalis*, Bacterial Detection

1. Introduction

Alcaligenes javaensis is a food-borne, obligate aerobic, Gram-negative isolated from Purwokerto region of Central Java, Indonesia (Ethica *et al*., 2013a; 2013b; Ethica & Raharjo, 2014; 2018). The bacterium has the closest relationship with *Alcaligenes faecalis*, a bacterium commonly reported as the cause of contamination of medical equipments and samples (Ethica *et al*., 2017; 2018; Kavuncuoglu *et al*., 2010; Bizet and Bizet, 1997). Having been isolated from various clinical materials, this opportunistic pathogen is responsible for many infections including pancreatic abscess and corneal ulcer. There are bare reports in the literature of the frequency of recovery of *A.faecalis* from human samples (Mordi *et al*., 2013; Kavuncuoglu *et al*., 2010; Berry, 1967; Aisenberg *et al*., 2004; Hwang *et al*., 2009).

There are morphological characteristics differing *A.faecalis* from other *pseudomonads* such as its motility with peritrichous flagella, and its non-pigmented, rod-shaped cells (Bizet and

Bizet, 1997). However, there are many similarities between members of genus *Alcaligenes* and other bacterial genus leading to high dynamics of species transfer from and to the genus *Alcaligenes* in taxonomy. Furthermore, several other *Alcaligenes* members such as *A.denitrificans*, *A.cupidus, A.pacificus, A.venustus, A.aestus*, and *A.aquamarinus* had been transferred to other genus causing changes of their names into *Achromobacter denitrificans, Deleya cupida, Deleya pacifica, Deleya venusta, Deleya aesta,* and *Halomonas aquamarinus*, respectively (Garrity, 2004).

1.1 Importance of Biomarker Development

Considering high dynamics in taxonomy of genus *Alcaligenes*, it is important to develop of a biomarker for members of this group of infectious bacteria including *A.javaensis* species. Restriction endonucleases and agarose gel electrophoresis have been widely used to indicate extensive nucleotide sequence diversity of various microorganisms (Lansman *et al*. 1981). Restriction enzymes *Hpa*II, *Ava*I, *Hha*I and *Hae*II had been found useful to distinguish *Xenopus laevis* somatic (erythrocyte) rDNA from amplified rDNA (Bird and Southern, 1978). It is possible to detect the organism in an environment using PCR and restriction enzyme analysis. For example, Johnston and Aust in 1994 could successfully detect fungi species, *Phanerochaete chrysosporium*, in soil by PCR and restriction enzyme analysis. Nevertheless, there are barely efforts to develop biomarkers to distinguish *A.javaensis* from other pathogens as part of its eradication steps.

This research aimed to screen a group of restriction enzymes consisting of EcoRV, Hind III, NotI, SalI BgIII as singles as well as combinations of EcoRV-BgIII, NotI-SalI, EcoRV-BgIII-HindIII, and EcoRV-BgIII-HindIII based on restriction ability on genomic DNA of *Alcaligenes javaensis* to assess their potential to be used as a restriction fragment-based biomarker of the infectious bacterium.

2. Methods

2.1 Materials

Samples used in this research were overnight-cultured of *A.javaensis* cells in LB medium, isopropanol, ethanol and 70%, RNA-se free water. A DNA isolation kit A1123 for Gram-negative bacteria (Promega). Materials needed for restriction process and DNA band evaluation were 5 common restriction enzymes: EcoRV (Biosystem), NotI (Promega), BglII (Nippon), Hind

III (Promega) dan SalI (Promega). Reagents for restriction process included ddH_2O, Acetylated BSA, buffer 10x, special buffer (E, D and H, as suggested by enzyme manufacturer) agarose (Merck), buffer, DNA marker, and ethidium bromide. Product after restriction was extracted using phenol-chloroform method (Sambrook *et al*., 1989). Instruments needed in this study were centrifugation machine (Thermo), vortex machine, electrophoresis, and UV lamp. Equipment used for all works included 1.5-ml micro centrifuge tubes, water-bath, ice block, micropipette (Gilson), pipette tips, and absorbent paper.

2.2 Bactrial Genomic DNA Isolation

The main steps of method applied in this research were isolation of bacterial genomic DNA (Ethica *et al*. 2013a; 2013b), process of enzyme restriction, extraction of restriction products and analysis of DNA products. First, genomic DNA isolation process was carried out by following instructions of the manufacturer (Promega) for Gram-negative bacteria omitting the use of cell lysis solution.

2.3 Enzyme Restriction Process

Enzyme restriction process on genomic DNA was carried out based on manufacturer's manual (Biosystem, Promega and Nippon), where minimal amount of DNA was used to correspond with 10 unit of enzyme in a total 20-ul reaction volume for each sample. Restriction duration was set 4 min as the shortest duration suggested by the manufacturers, and 18 min as part of investigation on critical time for the restriction process. Table 1 described the components of each of 9 samples representing 5 enzymes of EcoRV, Hind III, NotI, SalI, and BglII as singles, 2-combinations and 3-combinations. Each of all samples with all its set components including DNA, but without the enzyme was placed in a micro-centrifuge, and then mixed by pipetting. Next, enzyme was added followed by microcentrifugation for few seconds. The obtained DNA-restriction enzyme mixture was incubated for 18 hours.

Extraction of DNA products resulted from enzyme restriction on bacterial genomic DNA was carried out using phenol-chloroform method (Ethica *et al*. 2013a). Evaluation of extracted DNA products after the restriction process was conducted using agarose gel electrophoresis after previously checked by spectrophotometer for its OD_{600} (Raharjo *et al*., 2012). The EtBr-stained gel with the loaded sample was run under 50 V for 60 min for each sample.

3. Results and Discussion

The necessity to identify a group of restriction enzymes generating unique DNA band patterns distinguishable from all other bacteria for diagnostic purposes have been widely reported. Such information is required to design and developed a PCR-RFLP (Polymerase Chain Reaction—Restriction Fragment Length Polymorphism) assay leading to a more specific, sensible and faster detection of bacterium (Mandakovic *et al*., 2016).

In this study, the performance of 5 restriction enzymes and their combinations in providing specific pattern of DNA cuts when subjected to bacterial genomic DNA of *Alcaligenes javaensis* was tested and evaluated. Such evaluation is required to determine the potential of a restriction enzyme to be used for biomarking *A.javaensis* species, giving this infectious organism genetic specificity among other pathogens sharing high phenotypic character similarities.

3.1 Genomic DNA Isolation

Genomic DNA isolation of freshly cultured *A.javaensis* was carried out to obtain DNA sample as subject of enzyme restriction process by several enzymes and their combinations set in this study. As the first screening effort to determine restriction enzymes potential to be used as a genomic biomarker of *A.javaensis*, 5 restriction enzymes such as EcoRV, Hind III, NotI, SalI, and BgIII because these enzymes are commonly used and widely available in the market. Bacterial genomic DNA was successfully obtained from the isolation step, which appeared as transparent white residue at the bottom of the micro-centrifuge tube after the last step of isolation process.

3.2 Enzyme Restriction

Restriction process was carried out in 18-h durations. The 4-h restriction duration was suggested by most manufacturers of enzyme used in this study. However, the 18-h one was set in this study to make sure that the restriction process on bacterial genomic DNA could complete also considering that the cut DNA band did not show any signs of degradation after 18-h restriction process. For each test, an Eppendorf tube was prepared, and then filled with mixture as described in Table 1, and then coded accordingly. Total of 16 tubes represented 15 different samples including a control sample containing similar ingredients but without any restriction enzyme added.

Table 1: *Composition of Mixture in each Sample and Control used in Restriction Process*

Component	Sample Code and Content					
	C	E	H	N	S	B
DNA isolate (µl)	4	1.5	1.5	1.5	1.5	1.5
Acetylated BSA (µl)	-	0.2	0.2	0.2	0.2	0.2
Buffer 10x (µl)	-	2.0 (buffer H)	2.0 (buffer E)	2.0 (buffer D)	2.0 (buffer D)	2.0 (buffer H)
Enzyme (unit)	-	10	10	10	10	10
ddH_2O (µl)	16	15.3	15.3	15.3	15.3	15.3
Total volume (µl)	20	20.0	20.0	20.0	20.0	20.0
Incubation temp.(°C)	N/A	37	37	37	37	37
T. Termination (°C)	N/A	80	65	65	65	65

Note: C = control, E: EcoRV, N: NotI, B: BglII, H: HIndII. S: SalI

Results of the restriction performance test of each of 5 enzymes as singles, 2- and 3-combinations could be seen in Figure 1 and 2, respectively. As seen in Figure 1, each of 5 enzymes within durations of 18 h could not produce restriction products since the size of each genomic DNA appeared in the same length showing no difference with genomic DNA. Clearly there were no DNA cuts from the restriction enzymes when used as singles, although some shadings were observed on bands other than that from NotI.

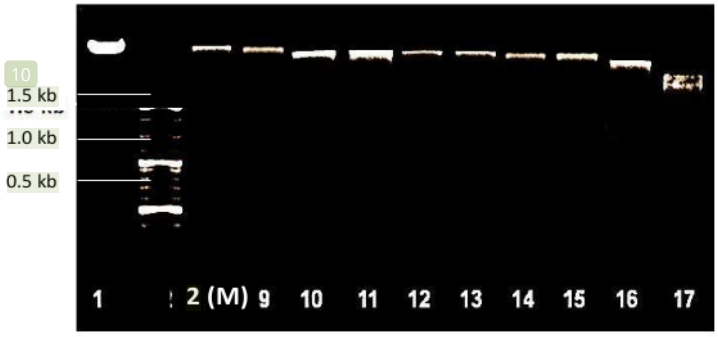

Figure 1: *Restriction Enzyme Performance on Genomic DNA of Alcaligenes Javaensis. Lane 1. Bacterial genomic DNA (uncut). Lane 2 (M) = 1,5 kb DNA ladder (Marker). Lane 3 = EcoRV. Lane 4 = HindlII. Lane 5 = NotI. Lane 6 = SalI. Lane 7 = BglII.*

Further test using combination of 2 and 3 enzymes from total of 4 enzymes (EcoRV, HindlII, SalI, and BglII) was conducted. This time NotI enzyme was omitted because per

previous result, DNA band from restriction treatment using single NotI did not show any shading, which means clearly NotI could not give any restriction effect to *Alcaligenes* genomic DNA. Results of cutting using 2 and 3-enzyme combinations are displayed in Figure 2.

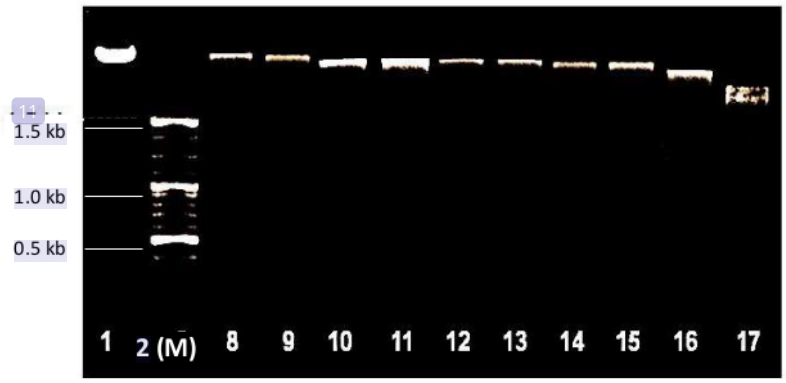

Figure 2: *Restriction Enzyme Performance on Genomic DNA of A.javaensis. Lane 1. Bacterial genomic DNA (uncut). Lane 2 (M) = 1,5 kb DNA ladder (Marker). Lane 8 = EcoRV – HindIII. Lane 9 = EcoRV – SalI. Lane 10 = EcoRV – BglII. Lane 11 = HindII – SalI. Lane 12 = HindII – BglII. Lane 13 = SalI – BglII. Lane 14 = EcoRV – SalI – BglII. Lane 15 = EcoRV – HindIII – BglII. Lane 16 = EcoRV – HindIII – SalI. Lane 17 = HindIII – SalI – BglII.*

Scientists have used biomarkers to distinguish the microorganisms causing the infection, or to assess the survival chances of patients infected by them. Bacterial genes, which are part of bacterial genomes, quantify the state of acute bacterial sepsis, so they could be regarded as biomarkers. (Pankla *et al*., 2009; Panchot *et al*., 2006). A whole-genome approach could be used to identify biomarkers, one of which is using restriction enzymes. In fact, restriction enzyme is the basic in DNA analysis and DNA fingerprinting for cancer studies (Samuelsson *et al*., 2010).

Restriction endonucleases are known as enzymes capable of identifying specific DNA through cutting up process, which could produce double-stranded cut in a nucleotide sequence. Abalaka (2011) stated that inside a bacterial host, the restriction enzymes selectively cut up foreign DNA in a process called restriction; host DNA is methylated by a modification enzyme (a methylase) to protect it from the restriction enzyme's activity. To cut the DNA, a restriction enzyme makes two incisions, once through each sugar-phosphate backbone or double helix. Results of this study demonstrated how the combination of EcoRV-BglII-HindIII endonucleases within restriction duration of 18 h could produce distinct restriction products from *A.javaensis*

genomic DNA. By far, this result is the first report about a group of restriction enzymes tested for their ability to detect this species.

In many cases combinations of several restrictions are required to cut out genomic DNA of bacteria (Murray *et al*., 1990; Mise and Miyahara, 1993). However, in the case of *P. Salmonis* it is possible that a single restriction enzyme *Pma*CI could generate several informative and easily recognizable bands in the 16S rDNA gene of the organism (Mandakovic *et al*., 2016). Although the use of single endonuclease seems to be less complicated for diagnostic purpose of infectious bacterium, it is evidence that combination of EcoRV-BglII-HindIII could produce distinct cut on genomic DNA of *A.javaensis* showing its potential in the development of restriction fragment-based biomarker of the bacterium.

4. Conclusion

Results of this experiment showed that the ability of 5 enzymes, EcoRV, HindIII, NotI, SalI, and BglII, when used as singles within restriction durations 18 hours on genomic DNA of *A.javaensis* species could not be detected. Yet, a 2-combination of EcoRV-BglII as well as a 3-combination of EcoRV-BglII-HindIII within the same duration of 18 h could show restriction ability proven by the presence of 2 DNA bands which sizes were different (smaller) than that of bacterial genomic DNA on electrophoresis gel. It means that among all endonucleases tested in this study, the combination of EcoRV-BgIII-HindIII is the most potential to be developed as restriction fragment-based biomarker for the infectious bacterium *A.javaensis*.

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