TROPICAL AGRICULTURAL SCIENCE

Isolation and Identification of *Bacillus thuringiensis* from *Aedesaegypti* Larvae as Potential Source of Endotoxin to Control Dengue Vectors

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ABSTRACT

Dengue is an emergent disease transmitted by *Aedes aegypti* mosquitos prominent in tropical countries. Numerous methods have been used to prevent the spread of Dengue fever, such as fogging and treatment using anti-larvae chemicals, yet these methods are harmful. *Bacillus thuringiensis* found in *Aedes aegypti* larvae is capable of producing endotoxin that able to kill insects without any side effect on humans, thus it is able to control Dengue vectors without any adverse effects to the environment. *Aedes aegypti* larvae were crushed and mixed with saline solution to isolate the bacteria in the larvae. From all bacterial colonies extracted from the larvae, 13 colonies with appearance closest to *Bacillus* colonies were screened using gram staining, spore staining, and biochemical testing. From 13 colonies, 8 of them were further analysed using ARDRA and *cry1A* gene amplification. These analyses showed one of the colonies had *cry1A* gene, which indicated the colony was *Bacillus thuringiensis*. The isolated *Bacillus thuringiensis* was used for endotoxin production and efficacy assays.

Keywords: Aedes aegypti, *Bacillus thuringiensis*, Dengue vectors, endotoxin

INTRODUCTION

Aedes aegypti and *Aedes albopictus* mosquitoes are the main vector of several viruses such as Dengue virus, Yellow fever virus, Chikunguya virus (Kraemer et al., 2015) and Zika virus (World Health Organization [WHO], 2016). These viruses have become major public health concerns, with increasing disease incidence and

prevalence of dengue and other associated fevers. *Ae. aegypti* and *Ae. albopictus* distributions are predicted to occur in tropical and subtropical areas, with *Ae. albopictus* having wider area due to its higher tolerance to lower temperatures (Kraemer et al., 2015). In Indonesia, annual dengue haemorrhagic fever occurrence had increased from 0,05 per 100000 people in 1968 to 35-40 per 100000 people in 2013 (Karyanti et al., 2014).

Aedes aegypti and *Aedes albopictus* lifecycle is the following: Egg-larva-pupa-adult and require still water to live their non-adult stages of life. An adult female mosquito can produce 100-200 eggs per batch, and each egg can develop within 2 days in tropical climate. In larva stage, they undergo several changes in size called instars. Initially, a newly hatched larva has around 1.7 mm length, and can reach up to 7.2 mm at the 4th instar (Bar & Andrew, 2013). They feed on organic matters in the water, and if undisturbed, can be found floating in the water surface. After 4th instar, larvae will enter pupal stage. In this stage, they no longer eat and after a couple of days will emerge as an adult mosquito (Zettel & Kaufman, 2012).

To control its growth and population, several methods have been undertaken, such as treatment with DDT, Malathion or Temephos (Abate powder). However, some chemicals used to control mosquito growth have been found to be harmful for humans, especially DDT which was banned in several countries. Temephos are non-harmful to human, but several researches have reported increasing resistance of *Aedes aegypti* larvae against it (Diniz, Henriques, Leandro, Aguiar, & Beserra, 2014). *Bacillus thuringiensis* is capable of producing crystal protein consisting of δ-endotoxins, which possess insecticidal activity. The crystal proteins, or Bt toxins, are produced during sporulation stage of *B. thuringiensis* as a 130-140 kDa protoxins in bipyramidal shaped crystals. The crystal proteins are cleaved into an active toxin that can kill the insect (Regev et al., 1996). Cry toxin, one of the proteins, comprises crystal protein and has specific targets (Bravo, Gill, & Soberón, 2007). Thus, by finding a *B. thuringiensis* that is able to produce crystal proteins specific for *Aedes* larvae, we could make a specific larvicide for *Aedes* larvae.

Dengue fever is a major health concern 126.675 being infected with it resulting in 1,229 deaths in 2015 (Kementerian Kesehatan Republik Indonesia [Kemenkes], 2016). It is an endemic problem requiring an urgent solution. Proper pest controls to curb *Aedes aegypti* and *Aedes albopictus* is a must. Prevention is more economical than treating the disease. Using Temephos has been proven to completely eradicate the larvae, and there are indications the larvae has developed resistance to Temephos (Mulyatno, Yamanaka, Ngadino, & Konishi, 2012). This research attempts to isolate *Bacillus thuringiensis* from larvae with movement impairment and sign of sickness. The frailty and sickness may be caused by *B. thuringiensis* crystal protein, thus by isolating the bacteria, we can produce the crystal protein as anti-larvae. Production

of crystal protein from *B. thuringiensis* for this purpose has never been attempted in Indonesia.

MATERIALS AND METHODS

Materials

Aedesaegypti larvae were obtained from water containers within the vicinity of Universitas Surabaya, Surabaya, Indonesia. Restriction enzymes were purchased from Thermo Fischer.

Collecting *Aedesaegypti* Larvae

Aedesaegypti larvae were taken from water containers and still water from unused tires in Universitas Surabaya. Collected larvae were put in glass jars, and each species was confirmed by observing larva's comb under microscope, and later stored in a small plastic container for bacterial isolation.

Bacterial Isolation from Larvae

Aedes aegypti larvae were selected for bacterial isolation. Larvae with impaired movement and slow response were transferred into sterile test tubes. Each test tube was filled with 4-5 larvae before they were crushed. 4 ml of 0.85% NaCl was added and incubated at room temperature for 5 minutes. A serial dilution was prepared up to 10^{-2} using 0.85% NaCl sterile saline solution, and then each dilution was incubated in 70°C for 15 minutes. After incubation, each diluted sample was plated into Nutrient agar with 0.3% w/v Yeast extract. Each petri dish was incubated at 37°C for 16-24 hours.

Bacterial Selection

Colonies were screened for *Bacillus*-like characteristics, such as white to yellowish colonies, entire or undulate arborescent colony, motile and smooth (for yellowish colonies) or dull (for white colonies). Every colony with similar characteristics were inoculated at pH 6.8 LB (Luria Bertani)-Acetate broth selective medium. LB-Acetate medium was made based on Travers, Martin, & Reichelderfer (1987). LB-Acetate medium was incubated at 37°C, 150 rpm for 24 hours, and then transferred to water bath at 80°C for 5 minutes. 100 µL of each LB-Acetate medium was inoculated in LB agar medium (pH 7,2) and incubated at 37°C for 16-24 hours.

Biochemical Assay and Staining

Colonies grown in LB agar pH 7.2 were screened for biochemical tests and staining. Each colony was tested in the following: starch hydrolysis, motility, and nitrate. A starch agar with addition of 2% amylum was prepared for starch hydrolysis analysis of samples. Each suspected colony was inoculated into the agar and incubated at 37°C for 16 hours. Then iodine solution was added into the plates and spread evenly. Positive results can be inferred from the formation of white area surrounding the colonies (modified from Clarke & Cowan, 1952). For motility assay, a bacterial colony was taken using a toothpick, and then inoculated into an agar medium by stabbing. The medium then was incubated at 37°C for 16-24 hours (modified from Bergey, 2005).

Nitrate reduction test was performed based on Zobell (1932) with modifications. Agar medium was prepared by adding 1 gram of KNO_3 per litre of nutrient agar medium. Each colony was then inoculated into the medium and incubated at 37°C for 24-48 hours. A drop of α-naphthylamine and sulfanilic acid was added into the medium and the colour change was observed. A toothpick of zinc powder was added into the medium after observing the change in colour. Citrate utilisation was analysed using Simmons Citrate Agar. The SCA slant medium was prepared, and bacterial sample was inoculated into the medium. After 16-24 hours of incubation at 37°C, the alteration in the colour of the medium would indicate the result (modified from Hemraj, Diksha, & Avneet, 2013).

Gram staining was done based on a method modified from Gram (1884), and spore staining (modified from Schaeffer & Fulton, 1933) of colonies were used to analyse cell characteristics of each colony. Gram positive and spore producing colonies were used for further molecular tests.

DNA Extraction from Bacterial Colonies

Bacterial colonies were transferred using tip of toothpicks to PCR tubes filled with 100 µL ddH20 and mixed thoroughly. They were incubated at -80°C for 20 minutes and then immersed in boiling water for 10 minutes to lyse the cells. Later, they were centrifuged at 10,000×g for 10 seconds and the supernatant collected and checked for DNA concentration using Nanodrop (modified from Bravo, 1998).

16S rRNA Amplification

Primers used for PCR amplification of 16S rRNA were 27F primer (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R primer (5'-TACGGYTACCTTGTTACGACTT-3'). The PCR was carried out in 50 µL tubes. 1 µL DNA template was mixed with 10 µL Master Mix 2x concentration and 0.5 µL 10 pmol primers, then ddH$_2$O was added until total volume is 20 µL. PCR cycles are the following: 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 3 min. PCR results were visualised in a 1% agarose gel electrophoresis in 100V for 30 minutes.

ARDRA Analysis

PCR amplicons were treated with restriction enzymes PstI, HindIII, EcoRI and HaeIII. 5 µL of PCR results were mixed with 1 µL 10x Restriction buffer, 0.5 µL Restriction enzyme and 8.5 µL ddH$_2$O then incubated at 37°C for 15 minutes (modified from Thermo Fisher protocol). The result was visualised in 1.5% agarose gel electrophoresis in 100V for 45 minutes.

Cry1 Gene Amplification

DNA sample was amplified by Lep1A (5'-CCGGTGCTGGATTTGTGTTA-3') and Lep1B (5'-AATCCCGTAT TGTACCAGCG-3') primers to detect *Cry1* gene on *Bacillus thuringiensis*. For 10 µL total PCR reaction, 1 µL DNA template, 5 µL Master Mix and 0.5 µL for each primer was mixed in a PCR tube and mixed thoroughly. PCR reaction was performed at

94ºC initial denaturation for 2 minutes, then 35 cycles of 94ºC for 30 second, 50ºC for 30 second, and 72ºC for 2 minutes then 72ºC for 10 minutes final elongation (modified from Rijzaani & Bahagiawati, 2003).

RESULTS AND DISCUSSION

Bacterial Isolation from *Aedes aegypti* Larvae Collection and Selective Medium Treatment

Aedes aegypti larvae were collected from water containers in Universitas Surabaya's greenhouse and static water contained in unused tires around the vicinity of the campus. Larvae were identified using their comb spine, observed under microscope (Figure 1). *Aedes aegypti* has comb spines arranged in a row, with each comb spines pointed and curved alongside with smaller denticles (Bar & Andrew, 2013). Larvae was collected twice, and bacteria extracted from the first larvae collection was numbered I, and the second larvae collection were numbered II.

Preliminary examinations of morphology showed numerous types of bacterial colonies, and mostly growing close to each other. Colonies were transferred into a new medium to separate each of them. Each colony was inoculated in LB-Acetate selective medium. Spore-forming *Bacillus thuringiensis* can be distinguished from other bacteria by inoculating in medium with sodium acetate. *Bacillus thuringiensis* spores were unable to germinate in presence of sodium acetate, and heat applied after incubation destroys other unwanted vegetative bacteria still alive in the medium

(Travers et al., 1987). This treatment should eliminate most unwanted bacteria. Colonies that were able to grow after LB-Acetate medium treatment were screened using biochemical tests and staining.

Figure 1. Aedes aegypti larvae (A) and larvae comb (B with 40× magnification). The comb spine of *Aedes aegypti* is circled.

Biochemical Assay and Staining

Gram staining of samples (Figure 2) showed various bacterial types; however, only rod-shaped gram-positive bacteria were used for further experiment. Some bacteria have endospores, which can be seen gram stain as hollow point in the cells. Spore staining
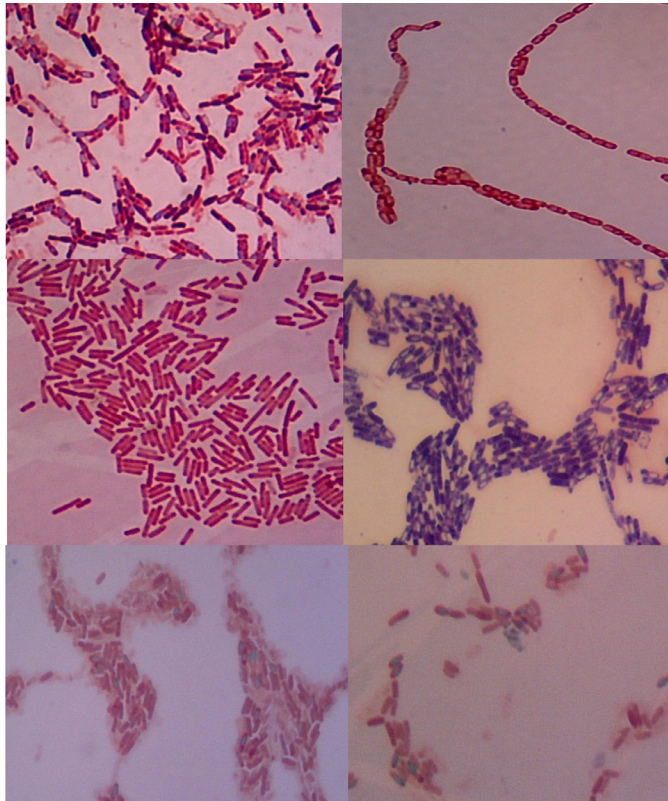
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Figure 2. Several bacterial staining results. Top left and middle right are gram positive, spore-producing *Bacillus*. Bottom pictures show spore staining result, with both having greenish spore in red cells.

using malachite green was also performed to visualise the spores. *B. thuringiensis* from the previous unpublished research was used as positive control.

Non-spore forming, gram positive bacteria were eliminated, while the *B. thuringiensis* candidate cultures were analysed further. All strains were stained to observe their cell morphology after LB Acetate treatment. There were 13 bacteria samples, and 10 were Gram positive while 3 of them were Gram negative. The observed cell shape were mostly bacilli, with 2 strains streptobacilli, and 7 out of 13 samples have endospore (Table 1). Samples taken

from first bacterial extraction (I) were all gram-positive bacteria, and from the second bacterial extraction, there were 3-gram negative bacteria among 8 samples observed.

These colonies were inoculated in biochemical assay medium. Colonies were tested on several biochemical characteristics displayed in Bergey's Manual of Systematic Biology (De Vos et al., 2009). Nitrate reduction test, starch hydrolysis, citrate fermentation and bacterial motility were analysed using respective medium for each assay. Based on Bergey's manual, *B. thuringiensis* would display positive result

Table 1
Gram staining, cell shape and *endospore* of *bacterial sample isolated from* A. aegypti *larvae*

Sample	Gram	Cell shape	Endospore
Positive Control	Gram positive (+)	Bacilli	Yes
I-2B	Gram positive (+)	Bacilli	Yes
I-4B1	Gram positive (+)	Bacilli	-
I-4B2	Gram positive (+)	Bacilli	Yes
I-4D1	Gram positive (+)	Streptobacilli	Yes
I-4D2	Gram positive (+)	Bacilli	Yes
II-1A16B	Gram negative (-)	Bacilli	-
II-1A2	Gram positive (+)	Streptobacilli	-
II-1C	Gram positive (+)	Bacilli	Yes
II-224	Gram negative (-)	Bacilli	-
II-1F1	Gram positive (+)	Bacilli	-
II-2C	Gram negative (-)	Bacilli	-
II-1F2	Gram positive (+)	Bacilli	Yes
II-212	Gram positive (+)	Bacilli	Yes

in starch hydrolysis, nitrate reduction, citrate fermenting and motile. Citrate fermentation on *B. thuringiensi* positive control should give positive result, yet it showed negative result along with all citrate tests. This may be due to the fact citrate media (Simmons Citrate Agar) pH indicator was already degraded. Biochemical assay result (Table 2) showed that all samples had diverse assay results, with only 2 showed similarities

Table 2
Biochemical assay results of bacteria isolated from A. aegypti *larvae*

Sample	Starch hydrolysis	Nitrate reduction	Motility	Citrate
Positive Control	+ (Positive)	+ (Positive)	+(Positive)	-(Negative)
I-2B	+(Positive)	+(Positive)	- (Negative)	- (Negative)
I-4B1	- (Negative)	- (Negative)	- (Negative)	- (Negative)
I-4B2	+(Positive)	- (Negative)	- (Negative)	- (Negative)
I-4D1	+(Positive)	+ (Positive)	- (Negative)	- (Negative)
I-4D2	+(Positive)	+(Positive)	+ (Positive)	- (Negative)
II-1A16B	- (Negative)	- (Negative)	- (Negative)	- (Negative)
II-1A2	- (Negative)	+(Positive)	- (Negative)	- (Negative)
II-1C	- (Negative)	+(Positive)	- (Negative)	- (Negative)
II-224	- (Negative)	+(Positive)	- (Negative)	- (Negative)
II-1F1	- (Negative)	+(Positive)	- (Negative)	- (Negative)
II-2C	- (Negative)	-(Negative)	- (Negative)	- (Negative)
II-1F2	- (Negative)	+(Positive)	- (Negative)	- (Negative)
II-212	+(Positive)	+(Positive)	+ (Positive)	- (Negative)

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with positive control, sample I-4D2 and II-212.The diverse biochemical assays results obtained might indicate that samples different from positive control were not *B. thuringiensis*, but the variety of results have been observed before. Gonzalez et al. (2011) discovered several *B. thuringiensis* that had slight variance in their biochemical characteristic. From biochemical point of view, these 2 samples were the promising candidates for *B. thuringiensis*. However, all gram-positive samples were further analysed by PCR and ARDRA to confirm their species.

Sample II-212 biochemical assay and staining were done after all sample had undergone ARDRA analysis. This sample was taken to consideration due to its colony shape that closely resembles *B. thuringiensis*. The result of biochemical assay and staining confirmed the similarity with the positive control, and thus, were analysed further.

16S rRNA Amplification

DNA of bacterial samples was extracted using heat-shock method to make the cells undergo lysis and DNA can be extracted from supernatant. The bacterial DNA samples were amplified using 27F and 1492R primers. Bacterial samples from first batch extraction were all tested, and all samples showed band with similar size (Figure 3A). Positive control band was around 1500 bp in size, similar to sample
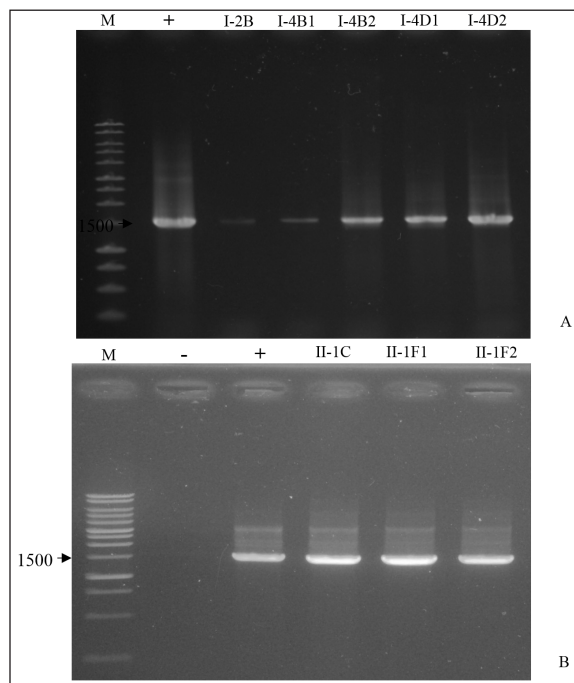

Figure 3. PCR result of 16S rRNA amplification for samples from first batch extraction (A) and second batch extraction (B). DNA ladder (M) used was 1000 bp ladder.

bands which around 1500 bp as well. Both first and second batch samples showed similar band size. Figure 3A showed I-2B and I-4B1 had smaller bands compared to other 3 samples, which might be caused by low DNA concentration prior to PCR.

Samples from the second batch were selected based from staining results. 3 gram positive, bacilli-shaped bacteria (II-1C, II-1F1, and II-1F2) had their DNA extracted and used for 16S rRNA amplification. Figure 3B shows the PCR amplification result for 3 samples, having bands in the same size as positive control, and all were around 1500 bp in size. 16S rRNA amplification in previous research (Atallah, El-Shaer, & Abd-El-Aal, 2014; Shishir et al., 2014) showed similar 16S rRNA size of 1500 bp.

ARDRA Analysis

Based on staining and biochemical activity assay, only several samples which showed similar characteristics to *B. thuringiensis* were tested for ARDRA. All first batch samples (I-2B, I-4B1, I-4B2, I-4D1, I-4D2) were tested using *Pst*I, *Eco*RI, and *Hind*III restriction enzymes. Figure 4 shows the restriction results of the samples. *Hind*III and *Eco*RI restriction enzymes cleaved both the positive control and samples into similar restriction patterns, but *Pst*I restriction shows a different result. Positive control *B. thuringiensis* was not cleaved by *Pst*I, giving a band size of 1500 bp while all samples were cleaved into 2 bands around 900 and 700 bp.
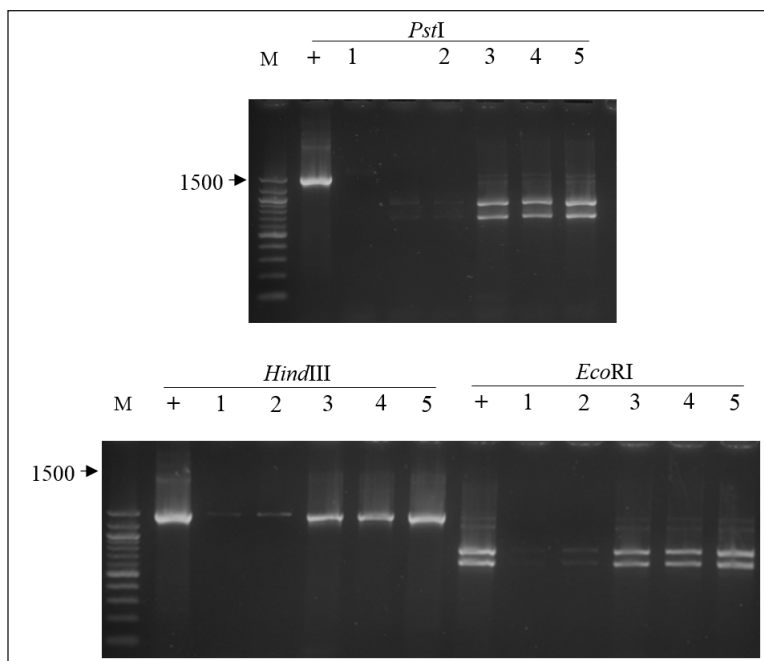

Figure 4. ARDRA analysis result using *Pst*I, *Hind*III, *Eco*RI restriction enzyme. Marker used is 100 bp ladder. Sample 1 (I-2B), 2 (I-4B1), 3 (I-4B2), 4 (I-4D1), and 5 (I-4D2).

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ARDRA result from second batch bacteria showed a different restriction pattern on sample 2 (II-1F1). As can be inferred from Figure 5, sample II-1F1 was cleaved into 2 fragments of 900 and 700 bp in size respectively. In this ARDRA analysis, *Eco*RI enzyme (data not displayed) gave no distinct restriction patterns, so *Hae*III was used instead. *Hae*III restriction of II-1F1 sample yielded different patterns (indicated by white arrow), albeit less profound compared with *Pst*I.

This result indicated the difference between positive controls with bacterial samples, namely 16S rRNA of *Bacillus thuringiensis*. All first batch samples restriction patterns were different from positive control; therefore, the bacterial samples may not *B. thuringiensis*. In second batch bacterial samples, only II-1F1 sample had different restriction patterns. All samples with different restriction patterns were omitted from next process, except sample I-4D2 which have similar biochemical assay result with positive control.
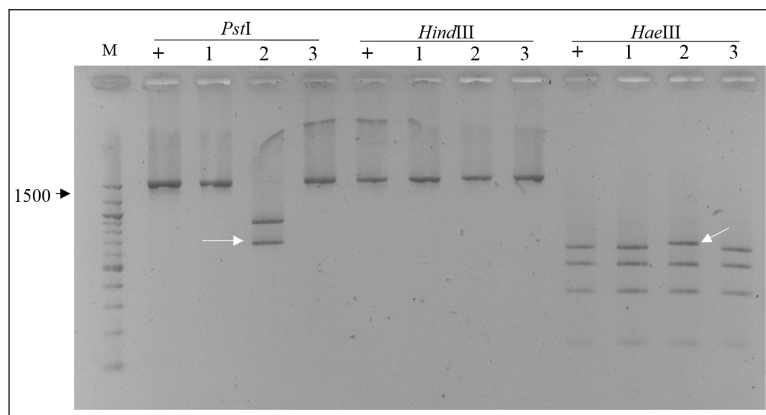

Figure 5. ARDRA analysis result using *Pst*I, *Hind*III, and *Hae*III restriction enzyme. Sample 1(II-1C), 2 (II-1F1), 3 (II-1F2) is each cleaved by 3 restriction enzymes. Different restriction bands are indicated by white arrows.

Cry1 Gene Amplification

Inconclusive results of ARDRA meant it was difficult to identify *B. thuringiensis* sample. Therefore, another set of primers Lep1A and Lep1B were used to amplify *cry1* gene of *B. thuringiensis*. Therefore, these pairs of primers can detect *B. thuringiensis* based on their *cry1* gene. From the result of previous biochemical assay and ARDRA, 4 samples were amplified using Lep1A

and Lep1B primers. Sample I-4D2 was tested for biochemical characteristic with positive control *B. thuringiensis*. Another two samples were taken from ARDRA result (II-1C and II-1F1), with sample II-212 tested because it has similar biochemical characteristics.

Lep1A and Lep1B amplify Lepidopteran-specific crystal toxic genes, which produce crystal proteins to kill

Leptidoptera insects (Bravo et al., 2007). PCR amplification of *cry1* gene using Lep1A/Lep1B primers showed a 357 bp band, similar to 363 bp*B. thuringiensis* positive control (Figure 6). Other samples didn't produce any amplification bands, and II-1F2 showed a faint band, but it was too little to be considered.
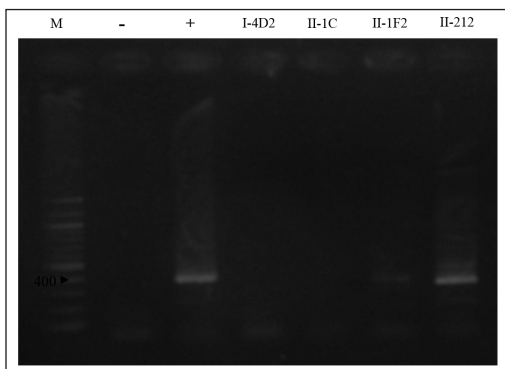

Figure 6. cry1A gene amplification. Sample II-212 (white arrow) shows a band similar to positive control. Marker used is 100 bp ladder.

Abdulreesh, Osman and Assaeedi (2012) found that Lep1A/Lep1B primers amplified *cry1Aa, cry1Ab*, and *cry1Ac* genes in *B. thuringiensis*, resulting in a 490 bp band. Ammouneh, Harba, Idris and Makee (2011) also had 490 bp band using Lep1A/Lep1B primers. Rijzaani and Bahagiawati

(2003) showed Lep1A/Lep1B primers produced several kinds of bands aside from 490 bp, depending on the *B. thuringiensis* isolate. In this research, Lep1A/Lep1B amplification resulted in 363 bp for positive control and 357 bp for sample II-212.

According to Abdulreesh et al. (2012), 490 bp indicated amplification of *cry1Aa, cry1Ab*, and *cry1Ac* gene. The smaller amplicon band in this research might because the bacteria don't possess all 3 *cry1Aa, cry1Ab*, and *cry1Ac* genes, for both positive control and sample II-212. This indicated that crystal protein produced by sample II-212 might had reduced efficiency in killing Lepidopteran insects, since it did not have a complete 3 genes. But its efficacy towards mosquito still requires further testing.

The RFLP was performed to analyse similarity between sample and positive controls. Both were analysed using 4 restriction enzymes to detect any differences in gene. The restriction on both the sample and control yielded only 1 band size of 360 bp as can be seen on Figure 7. From this result it can be inferred that II-212 is closely related to positive control *B. thuringiensis*.
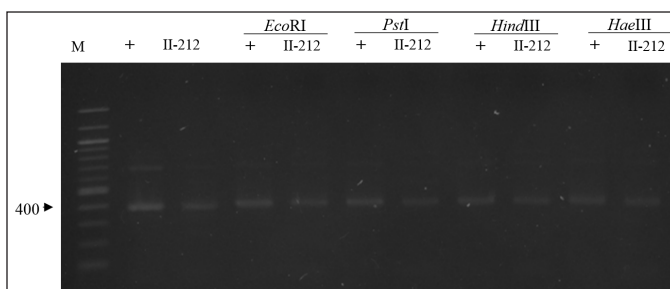

Figure 7. *cry1* gene amplicons RFLP. All samples show uniform sized bands to untreated PCR result (lane 2 and 3). Marker used is 100 bp.

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CONCLUSION

The bacterial isolation from *Aedes aegypti* larvae with impaired movement and slow response was performed to find *B. thuringiensis* using several biochemical assays, staining and molecular analysis. Bacterial samples were placed in selective medium to eliminate non-spore-forming gram-positive bacteria. Biochemical assay and staining were used to narrow the bacteria candidate for *B. thuringiensis* and as cross-reference for molecular analysis. ARDRA and *cry1* RFLP analysis indicated sample II-212 was *B. thuringiensis*. The efficacy of the crystal protein needs to be tested further to detect II-212 toxicity towards *Aedes aegypti* larvae.

ACKNOWLEDGEMENT

REFERENCES

Abdulreesh, H. H., Osman, G. E. H., & Assaeedi, A. S. A. (2012). Characterization of insecticidal genes of *bacillus thuringiensis* strains isolated from arid environments. *Indian Journal of Microbiology*, *52*(3), 500–503.

Ammouneh, H., Harba, M., Idris, E., & Makee, H. (2011). Isolation and characterization of native *Bacillus thuringiensis* isolates from Syrian soil and testing of their insecticidal activities against some insect pests. *Turkish Journal of Agriculture and Forestry*, *35*, 421–431.

Atallah, A. G., El-Shaer, H. F. A., & Abd-El-Aal, S. Kh. (2014). 16S rRNA characterization of a *Bacillus* Isolates from Egyptian soil and its plasmid profile. *Research Journal of Pharmaceutical, Biological and Chemical Sciences*, *5*(4), 1590–1604.

Bar, A., & Andrew, J. (2013). Morphology and morphometry of *Aedes aegypti* larvae. *Annual Review & Research in Biology*, *3*(1), 1–21.

Bergey, D. H. (2005). *Bergey's manual of systematic bacteriology* (2nd ed.) [E-Book version]. New York, NY: Springer Verlag. Retrieved on August 24, 2017, from https://books.google.co.id/

Bravo, A. (1998). Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. *Applied and Environmental Microbiology*, *64*(12), 4965–4972.

Bravo, A., Gill, S. S., & Soberón, M. (2007). Mode of action of *Bacillus thuringiensis Cry* and *Cyt* toxins and their potential for insect control. *Toxicon*, *49*(4), 423–435.

Clarke, P. H., & Cowan, S. T. (1952). Biochemical methods for bacteriology. *Microbiology*, *6*, 187–197.

De Vos, P., Garrity, G. M., Jones, D., Krieg, N. R., Ludwig, W., Rainey, F. A., … Whitman, W. B. (2009). *Bergey's Manual of Systematic Bacteriology* (2nd ed., Vol. 3). New York, NY: Springer Science.

Diniz, M. M. C. S. L, Henriques, A. D. S., Leandro, R. S., Aguiar, D. L., & Beserra, E. B. (2014). Resistance of *Aedes aegypti* to temephos and adaptive disadvantage. *Rev Saude Publica*, *48*(5), 775–782.

Gonzalez, A., Diaz, R., Diaz, M., Borrero, Y., Bruzon, R. Y., Carreras, B., & Gato, R. (2011). Characterization of *Bacillus thuringiensis* soil isolates from Cuba, with insecticidal activity against mosquitos. *Revista de Biologia Tropical*, *59*(3), 1007–1016.

Gram, H.C. (1884). *Fortschritte der medizin* [Advances in medicine] (T. D. Brock, Trans.). Washington DC, USA: ASM Press.

Hemraj, V., Diksha, S., & Avneet, G. (2013). A review on commonly used biochemical test for bacteria. *Innovare Journal of Life Science*. *1*(1), 1–7.

Karyanti, M. R., Uiterwaal, C. S. P. M., Kusriastuti, R., Hadinegoro, S. R., Rovers, M. M., Heesterbeek, H., & Bruijning-Verhagen, P. (2014). The changing incidence of dengue hemorrhagic fever in Indonesia: a 45-year registry-based analysis. *BMC Infectious Diseases*, *14*(412), 1–7. DOI: 10.1186/1471-2334-14-412

Kementerian Kesehatan Republik Indonesia [Ministry of Health of the Republic of Indonesia]. (2006). *Situasi DBD di Indonesia* [Dengue fever situation in Indonesia]. Jakarta, Indonesia: Pusat Data dan Informasi Kemenkes RI.

Kraemer, M. U. G., Sinka, M. E., Duda, K. A., Mylne, A. Q. N., Shearer, F. M., Barker, C. M., & Hay, S. I. (2015). The global distribution of the arbovirus vectors *Aedes aegypti* and *Aedes albopictus*. *eLife, 4*, 1–18. doi: 10.7554/eLife.08347

Mulyatno, K. C., Yamanaka, A., Ngadino., & Konishi, E. (2012). Resistance of *Aedes aegypti* (L.) larvae to temephos in Surabaya, Indonesia. *Southeast Asian Journal of Tropical Medicine and Public Health*, *43*(1), 29–33.

Regev, A., Keller, M., Strizhov, N., Sneh, B., Prudovsky, E., Chet, I., &Zilberstein, A. (1996). Synergistic activity of a *Bacillus thuringiensis* and a bacterial endochitinase against *Spodoptera littoralis* larvae. *Applied and Environmental Microbiology*, *62*(10), 3581–3586.

Rijzaani, H., & Bahagiawati (2003). Ekstraksi DNA *Bacillus thuringiensis* isolat lokal yang mengandung gen *Cry1* untuk pembuatan pustaka plasmid Bt [DNA extraction of *Bacillus thuringiensis* local isolate containing gen *Cry1* for plasmid Bt libraries creation]. *Hasil Penelitian Rintisan dan Bioteknologi Tanaman* (pp. 318–327). Bogor, Indonesia: Pusat Perpustakaan dan Penyebaran Teknologi Pertanian.

Schaeffer, A. B., & Fulton, M. C. (1933). A simplified method of staining endospores. *Science*, *77*, 194.

Shishir, A., Roy, A., Islam, N., Rahman, A., Khan, S. N., & Hoq, M. M. (2014). Abundance and diversity of *Bacillus thuringiensis* in Bangladesh and their *cry* genes profile. *Frontiers in Environmental Science*, *2*(20), 1–10. doi: 10.3389/fenvs.2014.00020

Travers, R. S., Martin, P. A. W., & Reichelderfer, C. F. (1987). Selective process for efficient isolation of soil *Bacillus spp*. *Applied and Environmental Microbiology*, *53*(6)*,* 1263–1266.

World Health Organization (2016). *Zika virus*. Retrieved on August 19, 2017, from http://www.who.int/news-room/fact-sheets/detail/zika-virus

Zettel, C., & Kaufman, P. (2012). *Yellow fever mosquito Aedesaegypti (Linnaeus) (Insecta: Diptera: Culicidae)*. Retrieved on August 22, 2017, from http://edis.ifas.ufl.edu/pdffiles/IN/IN79200.pdf

Zobell, C. E. (1932). Factors influencing the reduction of nitrates and nitrites by bacteria in semisolid

media. *Journal of Bacteriology*, *24*(4), 273-281.