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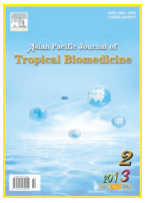

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Toxicity studies for indigenous *Bacillus thuringiensis* isolates from Malang city, East Java on *Aedes aegypti* larvae

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Comments
This paper provides a potential solution for controlling the vector of dengue, *Ae. aegypti*, in Indonesia. It finds that the Indonesia indigenous bacterium *B. thuringiensis* can reduce the *Ae. aegypti* larvae, with bright prospect of further promotion. This discovery benefits local residents, what's more, Indonesiaas an important tropical port of tourism, commerce and trade, cultural communication, promoting biological control of dengue will reduce the spread of dengue and influence of population outside the island, which means a great significance of public health.
(Details on Page 116)

ABSTRACT

Objective: To investigate the toxicity of indigenous *Bacillus thuringiensis* (*B. thuringiensis*) isolates from Malang City for controlling *Aedes aegypti* (*Ae. aegypti*) larvae. **Methods:** Soil samples were taken from Purwantoro and Sawojajar sub-districts. Bacterial isolation was performed using *B. thuringiensis* selective media. Phenotypic characteristics of the isolates were obtained with the simple matching method. The growth and prevalence of spores were determined by the Total Plate Count method, and toxicity tests were also performed on the third instar larval stage of *Ae. aegypti*. The percentage of larval mortality was analysed using probit regression. The LC_{50} was analysed by ANOVA, and the Tukey HSD interval was 95%. **Results:** Among the 33 selected bacterial isolates, six were obtained (PWR4-31, PWR4-32, SWJ4-2b, SWJ4-4b, SWJ-4k and SWJ5-1) that had a similar phenotype to reference *B. thuringiensis*. Based on the dendrogram, all of the bacterial isolates were 71% similar. Three isolates that had a higher prevalence of reference *B. thuringiensis* were PWR4-32, SWJ4-4b and SW5-1, of which the spore prevalence was 52.44%, 23.59%, 34.46%, respectively. These three indigenous isolates from Malang City successfully killed *Ae. aegypti* larvae. The PWR4-32 isolates were the most effective at killing the larvae. **Conclusions:** Six indigenous *B. thuringiensis* isolates among the 33 bacterial isolates found in the Sawojajar and Purwantoro sub-districts were toxic to the third instar larvae of *Ae. aegypti*. The PWR4-32 isolates were identical to the reference *B. thuringiensis* and had 88% phenotype similarity. The PWR4-32 isolates had the highest spore prevalence (52.44%), and the early stationary phase occurred at 36 h. The PWR4-32 isolates were the most effective at killing *Ae. aegypti* larvae (LC_{50}-72 h=2.3×10^8 cells/mL).

KEYWORDS
Aedes aegypti, *Bacillus thuringiensis*, LC_{50}, Toxicological studies

1. Introduction

Dengue haemorrhagic fever (DHF) is one of the most serious public health problems in Indonesia and many other tropical countries around the world. DHF outbreaks have occurred in Indonesia since 1986; the first case was in Surabaya. In 2010, there were more than 26059 reported DHF cases in East Java[1]. Dengue fever remains a serious health problem in both urban and rural areas of East Java. This is because dengue fever is a disease that causes high yearly mortality. Malang city is one of the cities in East Java that has endemic DHF. Sawojajar, Purwantoro, Jatimulyo, Klojen and Sukun are sub-districts of Malang city that became DHF-endemic areas in the last three years[2]. DHF is transmitted predominantly by *Aedes aegypti* (*Ae. aegypti*) mosquitoes that have adapted to living near human-inhabited areas[3].

Ae. aegypti (Linnaeus) is the major urban vector of the dengue virus worldwide[4]. This mosquito species has cosmo-tropical distribution and is widely distributed throughout Indonesia. Therefore, an effective environmental management system is necessary to avoid the spread of human disease.

Bacillus thuringiensis (*B. thuringiensis*) is an important insect

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pathogen that is highly toxic to mosquito larvae and related dipterans[5]. The toxicity is attributed to δ-endotoxin, which is made of proteins that are produced and assembled when the bacteria sporulate[6,7]. In Indonesia, some insecticides use active microbial *B. thuringiensis* imported from the countries such as Belgium (Bactospeine), the United States (mop) and Switzerland (Thuricide). The original *B. thuringiensis* exploration efforts in Indonesia were carried out, because the *B. thuringiensis* crystal protein had an arrow host spectrum. Therefore, the ideal effort for controlling Indonesian mosquitoes would be using *B. thuringiensis* isolated from Indonesia.

The objective of this study was to investigate the toxicity of indigenous *B. thuringiensis* isolates from Malang city on *Ae. aegypti* larvae.

2. Materials and methods

2.1. Study area

Malang city is the second largest city in the East Java province, Indonesia. In 2008, it was established as the fourth largest city in Indonesia. It is located approximately 400 m above sea level, which makes the city have cool weather and a temperate climate. Malang city is located between 07°46′48″-08°46′42″ north latitude and 112°31′42″-112°48′48″ east longitude[8]. The Malang city district borders Mojokerto and Pasuruan to the north, the Lumajang district to the east, the Blitar district to the west, and the Indian Ocean to the south. Soil sample sites were determined based on endemic DHF data from the Malang city health office in a preliminary study using search-sampling methods. Each soil location in the five-point set was sampled (Figure 1).

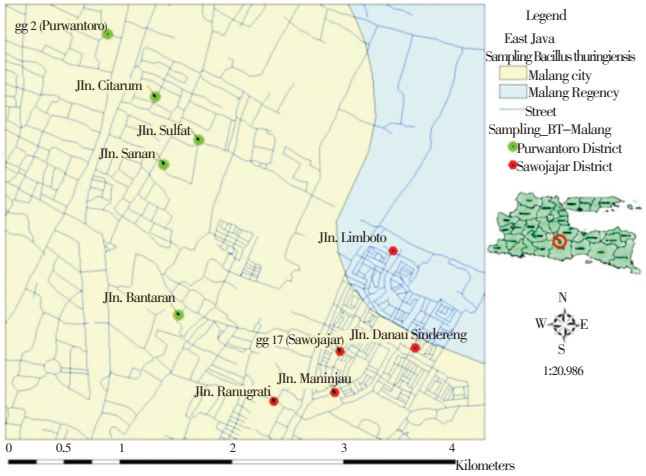

Figure 1. The map of Malang city.

2.2. Isolation of bacteria

Five soil samples were taken from 10 cm below the soil surface at each location[9]. The soil samples were 25 g and were placed into 225 mL sterile physiological saline solution (8.5 g/L NaCl). Serial 10^{-1} to 10^{-6}-fold dilutions of the sample suspensions were made in NaCl. Then, about 0.1 mL of the suspension was inoculated into a Petri dish containing *B. thuringiensis* selective media. Cultures were incubated for 48-72 h at 30 °C. Any isolates that grew were purified on a quadrant streak plate. Single colonies were inoculated into *B. thuringiensis* selective media. Cultures were incubated for 48-72 h at 30 °C. Isolates were stored on nutrient agar and *B. thuringiensis* selective media at 4 °C and in glycerol at -80 °C. The reference *B. thuringiensis* isolates used in this study were isolated from the swamp in Rancak Upas, Bandung District, and other bacteria were from the Bandung Institute of Technology (ITB) collection.

2.3. Classification of bacteria based on phenotypic attribute similarity

The phenotypes of the bacterial isolates were characterised by morphological observation and biochemical and physiological tests[10]. Morphological observations were performed on single cells or single colonies. Single colonies were observed for colour, surface texture, colony structure, Gram staining and the presence of endospores. Biochemical tests were performed including the catalase test, growth on Simmons citrate agar, urea hydrolysis, methyl red and acid butanediol (Voges Proskauer) fermentation, carbohydrate fermentation, motility, tryptophan hydrolysis, the oxidase test and the nitrate test. Observed physiological test parameters consisted of temperature (30 °C and 60 °C) and pH (4.0, 7.0 and 9.0). Bacterial density was determined with a spectrophotometer at a 600 nm wavelength[11]. Phenotypic data with a plus (+) or minus (-) sign were compiled using Microsoft Excel. The data that changed were used to construct a CLAD97 dendrogram reflecting operational taxonomic units based on the similarity index value as determined by simple matching methods. The unweighted pair group method of arithmetic averages algorithm was used.

2.4. Bacteria and spore number determination

The total number of live bacteria was calculated from stock bacterial suspension cultures that were inoculated into nutrient broth and homogenised. A total of 0.1 mL of the stock bacterial suspension from each dilution was inoculated on nutrient agar and incubated at 30 °C. The number of *B. thuringiensis* was based on the number of colonies that grew after incubating for 12, 24, 36, 48, 72 and 96 h.

2.5. Endospore screening

Isolated colonies were inoculated on sporulation media (10 g glucose, 7.5 g peptone, 6.8 g KH_2PO_4, 123 mg $MgSO_4 \cdot 7H_2O$, 2.33 mg $MnSO_4 \cdot 4H_2O$, 14 mg $ZnSO_4 \cdot 7H_2O$, and 320 mg $FeSO_4$ dissolved in 1 mol/L NH_4Cl). The bacterial suspensions were incubated at 30 °C and shaken at

120 r/min for 24 h; then, 3.75 mL of the suspension was inoculated into 25 mL of sporulation media and incubated at 30 °C, where it was shaken at 120 r/min for 24 h. Spore prevalence was calculated based on the number of live spores divided by the number of live cells times 100%[12].

2.6. Testing of Ae. aegypti

Ae. aegypti larvae were provided by the Central Council of Vector and Disease Reservoir Research and Development (B2P2VRP), Salatiga, Indonesia. The larvae were transferred to the laboratory of Ecology and Diversity, Department of Biology, Brawijaya University, where self-perpetuating colonies were established and maintained for the present study.

Toxicity studies were performed on test compounds as described by Wright and El-kersh *et al.* with some modifications[13,14]. Mortality data were analysed by using log-probit analysis to estimate the probit regression line and calculate LC_{50}[15].

2.7. Evaluation of the potential bacteria toxicity on Ae. aegypti larvae

B. thuringiensis toxicity against *Ae. aegypti* larvae was evaluated by randomised factorial design. Tests on each isolate were repeated three times. For each *B. thuringiensis* isolate, the effects on larval mortality were observed after 24, 48, and 72 h, respectively. One single and isolated colony was sub-cultured on nutrient broth media, incubated for 24 h at 30 °C and centrifuged at 3 000 r/min for 10 min at 4 °C. The supernatant was discarded, and the pellets were added to a 10 mL bacterial culture followed by another centrifugation, which was repeated three times. The subsequent pellets were then added to 1 mL 0.85% (v/v) NaCl and homogenised. The pellets were inserted into a tube, and NaCl was added until the volume reached 10 mL. Suspension test samples were made by adding 10 mL of the suspension pellets to 90 mL of sterile NaCl. Dilutions of bacterial suspension were made based on different bacterial cell densities, such as 1:0, 1:1, 1:3, 1:5, 1:7, 1:10, and 1:20. Each dilution was tested in a tube that contained 20 mosquito larvae, and each isolate was tested three times. Each isolate was exposed for 24, 48 or 72 h, and it was observed whether there was an effect of *B. thuringiensis* on larval mortality. As a control, 10 mosquito larvae were tested in a tube without *B. thuringiensis*.

2.8. Statistically analysis

The percentage of larval mortality was analyzed using probit regression. The LC_{50} was analyzed by analysis of variance (ANOVA) and it will be continued the TUKEY HSD interval was 95%.

3. Results

Of the 33 selected bacterial isolates, six were obtained (PWR4-31, PWR4-32, SWJ4-2b, SWJ4-4b, SWJ-4k and SWJ5-1) that had a similar phenocopy to reference *B. thuringiensis*.

3.1. Phenotypic characteristics and similarity among B. thuringiensis isolates

All of the bacterial isolates had similarity values of 71%. According to the dendrogram and phenotype similarity values greater than 71%, isolates were classified into two groups based on colony colour (Figure 2). The first group, containing PWR4-32, PWR4-31, SWJ5-1, and SWJ4-4b, had a similarity value of 78% compared with *B. thuringiensis*. The second group, containing SWJ4-4k and SWJ4-2b, had a similarity value of 82%. Subgroup classification was based on endospore location: one subgroup had a sub-terminal spore location, and the other subgroup had a central spore location.

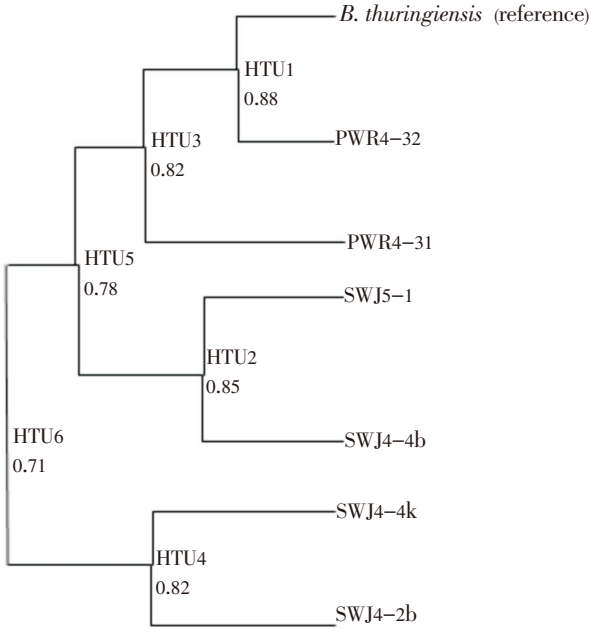

Figure 2. Dendrogram of relationships between indigenous *B. thuringiensis* isolates in Malang city and reference *B. thuringiensis*.

The similarity value between *B. thuringiensis* and the PWR4-32 isolate was 88%. Both of the isolates and PWR4-31 were in different groups, which had a similarity value of 82%. A distinguishing characteristic among the three bacteria was the oxidase enzyme. *B. thuringiensis* and the PWR4-32 isolate had oxidase, but the PWR4-31 isolate did not (Table 1). Distinguishing factors between the PWR4-32 isolate and *B. thuringiensis* were related to motility and nitrate. PWR4-32 isolates are not motile, and they can not reduce nitrates. In contrast, *B. thuringiensis* are motile, and they can reduce nitrate. Based on the phenotypic characteristics and literature searches, it was determined

Table 1

Phenotypic characteristics of different bacterial isolates.

Phenotypic characteristics		PWR4-31	PWR4-32	SWJ4-2b	SWJ4-4b	SWJ4-4k	SWJ5-1	Reference *B. thuringiensis*
Colony	*circular	+	+	+	+	+	+	+
Observation	*entire	+	+	+	+	+	+	+
Elevation	*effuse	+	+	–	+	–	–	+
	*convex	–	–	+	–	+	–	–
	*umbonate	–	–	–	–		–	–
	*opaque	+	+	+	+	+	+	+
Colour	*dark white	+	+	–	+	–	+	+
	*yellow	–	–	+	–	+	–	–
Cell	*shape: basil	+	+	+	+	+	+	+
Observation	*colour: purple/violet	+	+	+	+	+	+	+
Endospore	*central	–	–	+	+	–	+	–
	*terminal	–	–	–	–	+	–	–
	*sub terminal	+	+	–	–	–	–	+
	catalase	+	+	+	+	+	+	+
	oxidase	–	+	–	+	–	+	+
Motility	*rhizoid	–	–	+				+
	*arborescent	–	–	–	+	–	–	–
	*immobile	+	+	–	–	+	+	–
	Simmon´s citrate	–	–	–	–	–	+	–
	MR	–	–	–	–	–	–	–
	VP	–	+	+	–	–	–	+
Carbohydrate	*glucose	+	+	+	+	+	+	+
fermentation	*maltose	–	+	+	+	+	+	+
	*lactose	+	+	+	+	+	+	+
	*sucrose	+	+	+	+	+	+	+
	*mannitol	+	+	+	+	+	+	+
	urea hydrolysis	–	–	–	+	+	+	–
	tryptophan hydrolysis							
	nitrate	+	–	+	+	+	+	+
Temperature	*30 °C (mesophyl)	+	+	+	+	+	+	+
	*60 °C (thermophile)	+	–	+	–	+	–	–
pH	*acid (pH 4.0)	+	+	+	+	+	+	+
	*neutral (pH 7.0)	+	+	+	+	+	+	+
	*alkali (pH 9.0)	+	+	+	+	+	+	+

that *B. thuringiensis* (reference) had similar characteristics to the *B. thuringiensis* subspecies aizawai[16].

3.2. Spore prevalence in *B. thuringiensis* isolates

Determination of the isolates which would be used for testing mosquito larvae toxicity was based on the highest toxic-prevalence among the six isolates. The three isolates, PWR4-32, SWJ4-4b and SW5-1 were more prevalent than *B. thuringiensis*, and these isolates had a spore prevalence of 52.44%, 23.59%, and 34.46%, respectively. The results are presented in Table 2.

Relationship between the cell density and the spore number of the reference *B. thuringiensis* is illustrated in Figure 3, and that of the three isolates, PWR4-32, SWJ4-4b and SW5-1 are illustrated in Figures 4-6, respectively.

Increasing the cell density and the spore number of all the isolates or increasing the exposure time showed a proportional increase in mortality rate or decrease in LC_{50}, respectively (Figure 7).

Table 2

Percentage of spore prevalence in *B. thuringiensis* isolates at 48 h.

Isolates	Percentage of spore prevalence (%)
PWR4-31	9.28±11.49
PWR4-32	52.44±40.09
SWJ4-2b	0.82±0.55
SWJ4-4b	23.59±9.91
SWJ4-4k	4.34±21.38
SWJ5-1	34.46±12.28
Reference *B. thuringiensis*	10.02±36.96

3.3. Toxicological evaluation of indigenous *B. thuringiensis* isolates against *Ae. aegypti* larvae

The ANOVA and the LC_{50} results indicate a significant effect ($P<0.05$) among the tested isolates. The three indigenous isolates of Malang City (PWR4-32, SWJ 4-4b and SWJ 5-1) killed *Ae. aegypti* larvae. Among them, the PWR4-32 isolates were the most effective, as (22.79×10^7) cells/mL of which were required to kill 50% of the *Ae.*

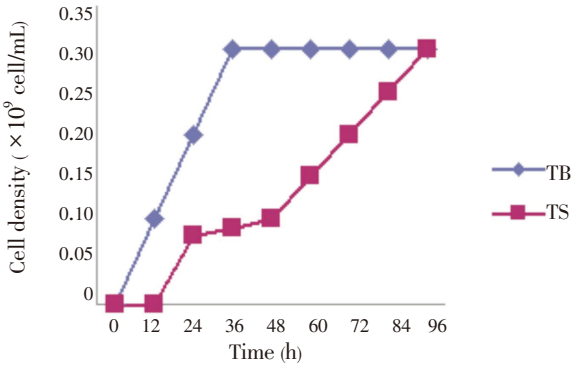

Figure 3. The number of total bacteria (TB) and total spore (TS) of the reference *B. thuringiensis*.

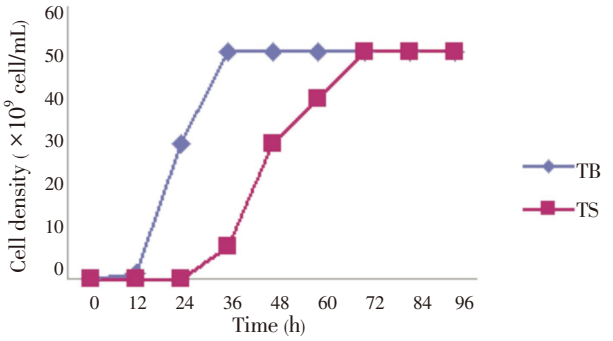

Figure 4. The number of total bacteria (TB) and total spore (TS) of the PWR4-32 isolate.

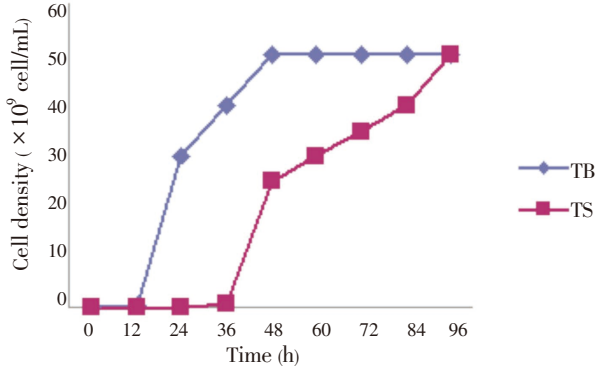

Figure 5. The number of total bacteria (TB) and total spore (TS) of the SWJ4-4b isolate.

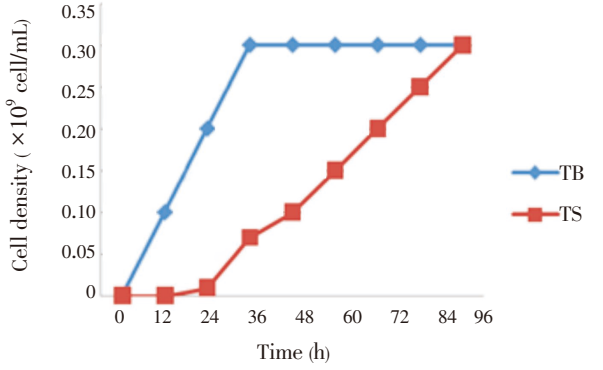

Figure 6. The number of total bacteria (TB) and total spore (TS) of the SWJ5-1 isolate.

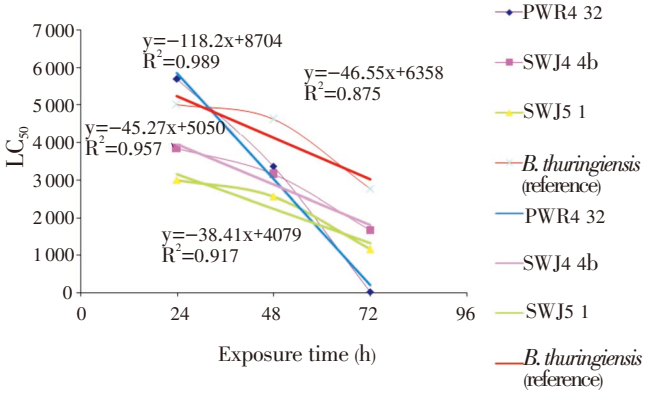

Figure 7. Relationship between the LC_{50} and the *B. thuringiensis* exposure time.

Table 3

ANOVA among the isolatess based on LC_{50}.

Isolates	Cell density (×10^9 cells/mL)		
	LC_{50}-24 h	LC_{50}-48 h	LC_{50}-72 h
Control	–	–	–
PWR4-32	56.99 (dA)	33.61 (bB)	0.23 (aC)
SWJ4-4b	38.32 (cA)	31.42 (bA)	16.59 (abB)
SWJ5-1	29.97 (bA)	25.55 (bA)	11.53 (abB)
The reference *B. thuringiensis*	49.98 (dA)	46.11 (cA)	27.63 (bA)

The same letters after the numbers denote no significant difference (significant at the 0.05 level). The capital letters indicate the time between trials, while the lowercase letters indicate tests among the isolates.

aegypti larvae within 72 h. The 72-h exposure time was more effective than that of the 24-h and the 48-h exposure times (Table 3).

4. Discussion

Similar characteristics among the six selected isolates were: rod-shaped bacteria; gram-positive; exhibiting oval endospore production; circular in shape along the entire edge; and having an opaque inner structure. None of the isolates were able to convert glucose into acid in the methyl red test. The Voges-Proskauer test revealed that the isolates PWR4-32 and SWJ4-2b could convert glucose to acetoin. These results were similar to those of the reference *B. thuringiensis*, which had negative MR and positive Voges-Proskauer test results. All of the six isolates grew well at 30 °C, but three of the isolates (PWR4-32, SWJ4-4b, and SWJ5-1) could not grow at 60 °C. All of the isolates grew at pH 4.0, 7.0 or 9.0. In general, neutral (pH 7.0) media are used for optimum bacterial growth[17]. These data can be used as a basis for bacterial toxicity tests against mosquito larvae. Bacteria can survive for a long time and have high efficacy against mosquito larvae at pH 7.0[18].

If bacterial spore prevalence was increasing, it could be assumed that the amount of toxin produced was also growing. As the number of bacterial toxins increases, one may expect the bacteria to be more effective at killing mosquito

larvae. There are differences in spore prevalence which is associated with the individual characteristics of the spore-forming isolates. *B. thuringiensis* has two developmental phases: germination and sporulation[19]. During sporulation, parasporal crystals are released by autolysis. These crystals are toxic and will damage the mosquito larval digestive tract, thus causing larval mortality.

The early stationary phase is marked by vegetative cell death, followed by toxin accumulation, because the cells metabolise the available nutrients resulting in nutrient shortage and competition. The bacteria will then synthesise secondary metabolites that are used to maintain life. In *B. thuringiensis*, this stationary phase is associated with spore and toxin formation. Toxins from *B. thuringiensis* cells are formed after the formation of cells endospores[20].

Toxicity tests were conducted using various dilutions of the bacterial suspension (1:0, 1:1, 1:3, 1:5, 1:7, 1:10, 1:15, and 1:20) and exposure times (24, 48, and 72 h). The toxin effectiveness of *B. thuringiensis* isolates was determined. The bacteria form spores and parasporal crystals during the stationary phase, which is a nutritionally deficient state; at that time, the parasporal crystals would be toxic and could kill the *Ae. aegypti* larvae[21]. The mosquito third instar larvae were selected, because at this stage, the larvae have a complete anatomical structure and the body is divided into three parts (head, thoracic, abdomen). Therefore, damage to the larvae can be easily observed within each section. A previous study demonstrated that the numbers of intestinal epithelial cells and peritrophic cells were increased in accordance within creasing larval toxin resistance[22].

The ANOVA and the LC_{50} results indicate a significant effect ($P<0.05$) among the tested isolates. The three isolates (PWR4-32, SWJ 4-4b, SWJ 5-1), which are indigenous of Malang city, killed *Ae. aegypti* larvae. Among the isolates, the PWR4-32 isolates were the most effective, as (22.79×10^7) cells/mL were required to kill 50% of the *Ae. aegypti* larvae within 72 h. The 72-h exposure time was more effective than that of the 24-h and the 48-h. Once the bacterial toxin enters the mosquito larvae digestive tract, the increased time allows the toxin to accumulate, and the toxin's (δ-endotoxin) effects increase in accordance with exposure. The toxin binds to receptors located on the apical microvillus membrane of the epithelial midgut cell wall. After the toxin binds to its receptor, there is a change in the toxin's conformation, allowing toxin insertion into the membrane. Electrophysiological and biochemical evidence suggest that the toxins generate pores in the cell membrane, which disturbs the osmotic balance; consequently, the cells swell and lyses. The gut becomes paralysed, and the mosquito stops feeding. Most mosquito larvae generally show reduced activity after 2 h and die within 6 h of toxin injection[5,23]. Mosquito larvae mortality is also influenced by factors such as bacterial concentration, larval age and the bacterial strain used[24,25].

A negative relationship can be observed between the exposure time and the LC_{50} for the indigenous *B. thuringiensis* of Malang city and the reference *B. thuringiensis*. It means that with a longer exposure time, the LC_{50} value will decrease and the larval mortality level will increase[26,27]. All of the tested and reference isolates had the highest percentages for larval mortality at the highest cell density and the longest exposure time because the toxins were released by the bacteria and accumulated in the *Ae. aegypti* larvae's digestive tract. Increased mortality was also observed for the mosquito larvae. These combined conditions resulted in a higher mosquito larvae mortality rate.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Dengue haemorrhagic fever is a serious disesase that causes high yearly mortality in East Java and other parts of the world. DHF is transmitted predominantly by *Ae. aegypti* that has cosmo-tropical distribution throughout Indonesia. Therefore, an effective biological control agent for the mosquito vector is a critical component in control of the disease. The ideal environmental management effort for controlling *Ae. aegypti* is to use *B. thuringiensis* isolated from local area that has a similar location with the mosquito vector.

Research frontiers

The first investigation of local strains of *B. thuringiensis* are isolated, identified and tested on the efficacy of killing *Ae. aegypti* larvae. The research will be great importance and innovation in regard to biological control technology. Indonesia has many endemic regions of dengue, especially in East Java.

Related reports

The general principal of the methodology had appeared in some publications, but modifications of the methodology seem to be the first only in this paper. Some publications still use bacterial isolates from outside the mosquito habitat itself as in Wirth MC (2010), Poopathi S, (2010), and El-kersh TA (2012), whereas this research is the first study using *B. thuringiensis* isolates from the regions of the mosquito (East

Java). The study also used *Ae. aegypti* captured from East Java as the native habitat of the mosquito.

Innovations and breakthroughs

The isolation and characterization of B thuringiensis from East Java soils is appreciated and determining the larvicidal activity regarding to this still need more explanation.

Applications

Prospective application for controlling of *Ae. aegypti* and reducing transmission of DHF can be expected from this research.

Peer review

This paper provides a potential solution for controlling the vector of dengue, *Ae. aegypti*, in Indonesia. It finds that the Indonesia indigenous bacterium *B. thuringiensis* can reduce the *Ae. aegypti* larvae, with bright prospect of further promotion. This discovery benefits local residents, what's more, Indonesiaas an important tropical port of tourism, commerce and trade, cultural communication, promoting biological control of dengue will reduce the spread of dengue and influence of population outside the island, which means a great significance of public health.

References

[1] Provincial Health Office. Profile of Dengue patients in East Java in 2005-2010. Indonesia: Provincial Health Office; 2011. [Online] Available from: http://dinkes.jatimprov.go.id/userfile/dokumen/1312948638_Profil_Kesehatan_Provinsi_Jawa_Timur_2010.pdf. [Accessed in 2012]

[2] Malang City Health Office. Profile of Dengue patients in the city of Malang in 2008. Malang: Malang City Health Office; 2008. [Online] Available from: http://dinkes.jatimprov.go.id/userfile/dokumen/1311839621_Profil_Kesehatan_Provinsi_Jawa_Timur_2008.pdf. [Accessed in 2012].

[3] Mulyatono KC, Yamanaka A, Yotopranoto S, Konishi E. Vertical transmission of dengue virus in *Aedes aegypti* collected in Surabaya, Indonesia, during 2008-2011. *Jpn J Infect Dis* 2012; **65**: 274-276.

[4] Jansen CC, Beebe NW. The dengue vector *Aedes aegypti*: what comes next. *J Microbes Infect* 2010; **12**: 272-279.

[5] Poopathi S, Abidha S. Mosquitocidal bacterial toxins (*Bacillus sphaericus* and *Bacillus thuringiensis* serovar israelensis): mode of action, cytopathological effects and mechanism of resistance. *J Physiol Pathophysiol* 2010; **1**(3): 22-38.

[6] Sanahuja G, Banakar R, Twyman RM, Capell T, Christou P. *Bacillus thuringiensis*: a century of research, development and commercial applications. *J Plant Biotechnol* 2011; **9**: 283-300.

[7] Haggag KHE, Yousef HMA. Differentiation among Egyptian *Bacillus thuringiensis* strains at sporulation by whole cellular protein profiles. *J Agri Sci* 2010; **6**(2): 224-233.

[8] Malang City Health Office. Health Profile of Malang, East Java. Malang City in the 2008. Malang: Malang City Health Office; 2008. [Online] Available from: http://dinkes.jatimprov.go.id/userfile/dokumen/1311839621_Profil_Kesehatan_Provinsi_Jawa_Timur_2008.pdf. [Accessed in 2012].

[9] Chartterjee SN, Bhattacharya T, Dangar TK, Chandra G. Ecology and diversity of *Bacillus thuringiensis* in soil environment. *Afr J Biotechnol* 2007; **6**(13): 1587-1591.

[10] Zakeel MCM, Dissanayake DMD, Weerasinghe PA. Molecular characterization of *Bacillus thuringiensis* strains isolated from a selected site in Nochchiyagama, Anuradhapura in Srilanka. *J Trop Ag Res Ext* 2009; **12**(1): 31-34.

[11] Gong Y, Mingshun L, Dong X, Hui W, Jin H, Dandan W, et al. Comparative proteamic analysis revealed metabolic changes and the translational regulation of cry protein synthesis in *Bacillus thuringiensis*. *J Proteomics* 2012; **75**: 1235-1246.

[12] Jensen GB, Larsen P, Jacobsen BL, Madsen B, Smidt L, Andrup L. *Bacillus thuringiensis* in fecal samples from greenhouse workers after exposure to *B. thuringiensis*-based pesticides. *J Appl Environ Microbiol* 2002; **68**: 4900-4905.

[13] Wright JW. The WHO programmer for the evaluation and testing new insecticides. *Bull Wld Hlth Org* 1971; **44**: 11-12.

[14] El-kersh TA, Al-sheikh YA, Al-akeel RA, Alsayed AA. Isolation and characterization of native *Bacillus thuringiensis* isolates from Saudi Arabia. *Afr J Biotechnol* 2012; **11**(8): 1924-1938.

[15] El-Maghraby S, Nawwar GA, Bakr RFA, Helmi N, Kamel O. Toxocological studies for some agricultural waste extracts on mosquito larvae and experimental animals. *Asian Pac J Trop Biomed* 2012; **2**(7): 558-563.

[16] Dulmage HT, Yousten AA, Singer S, Lacey LA. Guidelines for production of *Bacillus thuringiensis* H-14 and *Bacillus sphaericus*. UNDP-WHO; 1990, p. 2-10.

[17] Das JB, Dangar TK. Microbial populations and *Bacillus thuringiensis* diversity in saline rice field soils of Coastal Orissa, India. *Afr J Microbiol* 2008; **2**: 326-331.

[18] Raina MM, Ian LP, Charles PG. *Risks from pathogen in biosolids. Wastewater treatment and biosolids reuse*. California: Elsevier Inc; 2009, p. 527-529.

[19] Manonmani AM, Geetha I, Bhuvaneswari S. Enhanced production of mosquitocidal cyclic lipopeptide from *Bacillus subtilis* subsp. Subtilis. *Indian J Med Res* 2011; **134**(4): 476-482.

[20] Muniady S, Rathinam X, Subramaniam S. Quick isolation and characterization for the confirmation of a novel *Bacillus thuringiensis* strains from chicken manure samples. *Afr J Micro Res* 2011; **5**(20): 3131-3137.

[21] Renganathan K, Rathinam X, Danial M, Subramaniam S. Quick isolation and characterization of novel *Bacillus thuringiensis* strain from mosquito breeding sites in Malaysia. *J Food Agric* 2011; **23**(1): 17-26.

[22] Wirth MC. Mosquito resistance to bacterial larvicidal toxins. *J Toxicol* 2010; **3**: 126-140.

[23] Poopathi S. Novel fermentation media for production of mosquito pathogenic bacilli in mosquito control. In: A Mendez-Vilas, editor. *Current Research Topics in Applied Microbiology and Microbial Biotechnology*. 2010, p. 349-358.

[24] Ramirez-Suero M, Valerio-Alfaro G, Bernal JS, Ramirez-Lepe M. Synergisitic effect of chitinases and *Bacillus thuringiensis* israelensis spore-toxin complex against *Aedes aegypti* larvae. *J Canadian Entomologist* 2011; **148**: 157-164.

[25] Poopathi S, Tyagi BK. The challenge of mosquito control strategies: from primordial to molecular approaches. *J Biotech Molecular Bio Rev* 2006; **1**(2): 51-65.

[26] Arivoli S, Tennyson S. Larvicidal and adult emergence inhibition activity of *Abutilon indicum* (Linn.) (Malvaceae) leaf extracts against vector mosquitoes (Diptera: Culicidae). *J Biopesticides* 2011; **4**(1): 27-35.

[27] Valadez-Lira JA, Alcocer-Gonzalez JM, Damas G, Nunuz-Mejia G, Oppert B, Rodriques-Padilla C, et al. Comparative evaluation of phenoloxidase activity in different larval stages of four lepidopteran pests after exposure to *Bacillus thuringiensis*. *J Insect Sci* 2011; **12**: 1-11.