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Aims & Scope

Asian Pacific Journal of Tropical Biomedicine (APJTB) is administrated by Hainan Medical University and sponsored by Asian Pacific Tropical Medicine Press, and aims to establish an international academic communicating platform for researchers of tropical biomedicine and public health workers, especially specialists and scholars of the Asian Pacific region and worldwide on tropical biomedicine research, to prevent and control the prevalence of global tropical disease.

APJTB is an international academic journal and is distributed worldwide. The journal first reports the latest findings in both basic and clinical researches on tropical biomedicine by studying biology (botany, zoology and microbiology)–especially tropical plants, animals, microorganisms and marine organisms, analyzing biological substances and utilizing biotechnologies to assist the diagnosis and treatment of tropical diseases. Detailed areas of focus include biology, bacteriology, biochemistry, biotechnology, cell biology, environmental biology, microbiology, medical microbiology, pharmacology, physiology, pathology, immunology, virology, toxicology, epidemiology, vaccinology, hematology, histopathology, cytology, genetics and tropical agriculture.

Original article, review, epidemiological investigation, clinical research, management and decision–making, case report, editorial, short communication, letter to editor related to the aim and scope of APJTB are encouraged.

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APJTB will allow us to seek opportunities to work with others who share mutual goals, and to enhance our work through partnership, and to uphold the standards of our profession and contribute to its advancement.

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Information for Authors
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Evaluation of entomopathogenic *Bacillus sphaericus* isolated from Lombok beach area against mosquito larvae

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ABSTRACT

Objective: To isolate, characterize and evaluate toxicity of *Bacillus sphaericus* (*B. sphaericus*) from beach area of Lombok Island.

Methods: Soil was collected from determined locations and suspended in sterile physiological saline water. After heat shock was applied, suspension was spread on NYSM agar medium. Colonies grown were then observed and isolated. Colony, cell morphology, and biochemical/physiological characteristics were tested and compared to *B. sphaericus* 2362 as standard. Initial toxicity testing was done against three species of mosquito larvae (*Culex quinquefasciatus*, *Anopheles aconitus* and *Aedes aegypti*) and isolates that showed more than 50% larvae killing will be assayed to obtain LC_{50} and LC_{90} values within 48 h. PCR technique were conducted to obtain 16s rDNA amplicon for sequencing and to detect toxin-expressing genes (using multiplex PCR).

Results: Twenty isolates of *B. sphaericus* have been collected from 20 determined locations and their characteristics were in agreement with standard *B. sphaericus* characteristics. Bioassay testing showed that four isolates (namely isolate MNT, SLG, TJL2 and PLG) were mildly toxic against all larvae. The rests were either low toxic or non-toxic at all. Phylogenetic analysis showed that all four isolates were clustered with other known mildly and highly toxic strains. The multiplex PCR result showed four toxic isolates owned 1–2 bands from Bin toxin genes and three bands from Mtx toxin genes, whereas 16 isolates with low to non-toxic characteristics showed only three bands from Mtx toxin genes.

Conclusions: Four toxic isolates of *B. sphaericus* were isolated from beach area of Lombok Island. They showed mild toxicity against larvae of three mosquito species.

1. Introduction

Lombok Island is one of the popular tourism destinations in Indonesia after Bali. In Lombok Island, some mosquito-borne diseases are still becoming health problems. The most popular mosquitoes are *Anopheles* and *Aedes*. *Anopheles* is known as the vector of malaria, whereas *Aedes* is the vector of dengue hemorrhagic fever. In Lombok Island there are 6400 people infected by malaria, while 760 people are reported suffering from dengue hemorrhagic fever, respectively [1]. Other mosquito genus, *Culex* is known for spreading filariasis and Japan encephalitis virus. Diseases spread by *Culex* is less common in this island [2].

In many countries mosquito control relies on chemical pesticides. Although showing high efficacy, the usage of chemical pesticides in long term will result in malicious effect on human and environment. There are many health problems related to pesticides, from abdominal pain, dizziness, headaches, nausea, vomiting, as well as skin and eye problems to cancer and developmental defect as well [3]. Side effects on environment are ranging from non-target organism killing (non-harmless insects, birds, amphibians and fishes) to increasing resistance to mosquito [4]. To prevent unwanted effects of chemical pesticides, biological-agent-based pesticides (biopesticide) should be used as an alternative. This includes microorganisms or natural products [5].

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One popular biocontrol agent is *Bacillus sphaericus* (*B. sphaericus*) (besides *Bacillus thuringiensis* that was previously popular). This rod-formed Gram positive bacteria that has terminal bulging endospore shows high toxicity against *Culex* and *Anopheles*, while it shows the lowest susceptibility against *Aedes*. The bacteria has been used extensively in some countries. Many strains and their potentials have been studied as well [6]. However, little is known about *B. sphaericus* isolated from Lombok Island and its potential against *Culex*, *Anopheles* and *Aedes* larvae.

Development of local strain-based biopesticide/bacterial agent is very important to suppress cost from importing from foreign countries and also to promote local industry capability. Some researchers have developed medium formulation and production system to propagate potential bacterial agent using local and low-cost ingredient [7]. This capability will enable biopesticide production on community and low industrial level, without sacrificing effectiveness of the bacterial agent in controlling mosquito larvae.

In this study, we collected *B. sphaericus* from 20 locations near beach area around Lombok Island. We also evaluated toxicity of isolated *B. sphaericus* against *Culex*, *Anopheles* and *Culex* larvae. Toxin gene detection was also performed to support the toxicity attribute of isolated *B. sphaericus*.

2. Materials and methods

2.1. Bacterial isolation and characterization

Twenty locations near beach area around Lombok Island were determined for soil collection. Locations were chosen based on closeness to village (Kampung) and predictive mosquito breeding habitat (small ditch, river opening, *etc*.). The sampling locations are presented in Figure 1.

Five hundred grams of soil from those areas were collected compositely and stored at sterile screw capped container. The isolation procedure was done as follows. Suspension was made from 25 g of soil with 225 mL sterile physiological salt solution. Then it was heated at 80 °C for 30 min and diluted into 10^{-1}–10^{-5} dilutions. The dilution was then spread on NYSM agar (nutrient agar with 0.5 g/L yeast extract, 0.2 g/L $MgCl_2$, 0.01 g/L $MnCl_2$, and 0.1 g/L $CaCl_2$). Antibiotic was also added to the medium (streptomycin, 100 μg/mL) to selectively inhibit other bacteria [8].

Characterization of the bacteria was done morphologically and biochemically/physiologically. Morphological characteristics observed were colony morphology, cell structure, Gram staining and endospore form and position. Biochemical/physiological characteristics tested were catalase, starch hydrolysis, nitrate reduction, sugar utilization, indole, H_2S, urease, oxidase, casein hydrolysis and aerobicity. Sensitivity against antibiotics (mainly streptomycin and chloramphenicol) were tested as well [9].

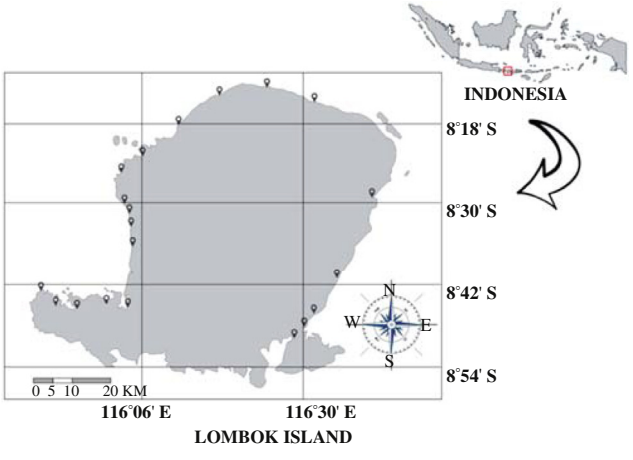

Figure 1. Sampling locations around Lombok Island, Indonesia.

2.2. Bioassay

2.2.1. Larva preparation

Culex quinquefasciatus (*Cx. quinquefasciatus*), *Anopheles aconitus* (*An. aconitus*) and *Aedes aegypti* (*Ae. aegypti*) larvae were obtained from rearing facility at Institute for Vector and Reservoir Control Research and Development (IVRCRD), Salatiga, Central Java, Indonesia. All eggs were hatched and reared in untreated water (well water) intensively for 6–8 days to reach 3rd instar stage.

2.2.2. Liquid culture preparation

A loop of single colony from chosen isolate(s) and *B. sphaericus* 2362 as standard were taken and inoculated on 100 mL NYSM liquid medium supplemented with 0.2 g/L $MgCl_2$, 0.01 g/L $MnCl_2$ and 0.1 g/L $CaCl_2$ [8]. The cultures were incubated at 30 °C, shaken at 170 r/min for 72 h.

2.2.3. Initial toxicity testing

This testing was based on procedures mentioned by Dulmage *et al*. [10]. *Cx. quinquefasciatus*, *An. aconitus* and *Ae. aegypti* larvae were prepared in containers (20 larvae per container in three replications) filled with well water (untreated water) and mixed with bacterial culture (*B. sphaericus* isolates) reached 10% (v/v) in 200 mL total volume. Larvae death was observed in 48 h after application. Average larvae death was calculate for each *B. sphaericus* isolates tested.

2.2.4. Bioassay

Third instar larvae of *Cx. quinquefasciatus*, *An. aconitus* and *Ae. aegypti* were prepared in 63 containers (21 containers for each species). Each container was filled with 200 mL untreated water mixed with liquid culture of isolated *B. sphaericus* (in 7 dilutions with 10-fold different concentrations in 3 replications) [10]. This procedure was done for every isolate that showed more than 50% larvae killing in initial toxicity testing. Dead larvae observation from each dilution was made at 48 h after application. Probit analysis was made from the observation and lethal values (LC_{50} and LC_{90} on 48 h observation) were calculated with 0.05 of significance level using Minitab statistical software version 16 for Windows.

2.3. Phylogenetic analysis and toxin gene detection

2.3.1. DNA extraction

The method was described by Ausubel *et al*. [11] with slight modification. A full loop of *B. sphaericus* isolates from solid NYSM agar was added into 560 μL of TE buffer (pH 7.6). Ten microliters of lysozyme (concentration of 30 mg/mL) was added and the mixture was then incubated at 37 °C for 1 h. Thirty microliters of 10% SDS and 10 μL of 20 mg/mL Proteinase K were added. The mixture was then incubated at 37 °C for 1 h. One hundred microliters of 5% NaCl and 80 μL of cetyl trimethyl ammonium bromide (concentration of 10% in NaCl) were then added to the mixture and incubated at 65 °C for 1 h. Extraction with phenol: chloroform (1:1 v/v) was done and the mixture was stirred at 10 000 r/min for 5 min. Upper phase was collected and mixed with 2× volume of absolute ethanol. After being incubated at −20 °C overnight, the mixture

was then stirred at 12 000 r/min for 20 min and supernatant was carefully discarded. Pellet obtained was washed with 75% ethanol. After being stirred at 12 000 r/min for 30 min, DNA pellet was dissolved with 50 µL of TE buffer (pH 7.6).

2.3.2. Phylogenetic analysis

Genomic DNA of isolated *B. sphaericus* was amplified using 16s rDNA primers (namely 27f and 1492r). The sequence of 27f primer was 5′-AGA GTT TGA TCM TGG CTC AG-3′ and the sequence of 1492r primer was 5′-GGT TAC CTT GTT ACG ACT T-3′ [12]. The amplification parameter was as follows. Pre-denaturation was at 94 °C for 5 min, denaturation was at 94 °C for 20 s, primer annealing was at 52 °C for 30 s, and elongation was at 72 °C for 90 min. Cycle number was 35 cycles and post-elongation was at 72 °C for 5 min. Amplicon resulted was then sequenced and contigs were developed from the sequences using BioEdit Program for Windows. Contig alignment and tree construction were performed using MEGA V5 for Windows [13].

2.3.3. Toxin gene identification

The existence of toxin genes owned by *B. sphaericus* was detected with multiplex PCR applying *bin* and *mtx* gene primer pairs [14] presented in Table 1. Amplification parameter was as follows. Pre-denaturation was at 94 °C for 5 min, followed by denaturation at 94 °C for 30 s, primer annealing at 45 °C for 30 s and elongation at 72 °C for 60 s. The cycle were 35 cycles with final elongation at 72 °C for 5 min. Amplicon was analyzed using agarose gel electrophoresis in 2% gel.

Table 1
Sequence of primers used in multiplex toxin detection PCR.

Sequences	Target	Size (bp)
F: 5′-TTG CCA ATA TTG AGT GTG C-3′ R: 5′-TGC CTT CAC TTC CAG AAA AC-3′	*binA*	200
F: 5′-TAG TGT GAA TTC TCT AGC C-3′ R: 5′-CAC TCA GTT AGG AGA AAG A-3′	*binB*	100
F: 5′-TGT GTC TTC TAC TGG AGA T-3′ R: 5′-ACT GTT TAT GCT TCA CCT A-3′	*mtx1*	300
F: 5′-CTC CCT ATT GCT CGT ACT CT-3′ R: 5′-TTT CGG TTT CCC AGT TAT C-3′	*mtx2*	850
F: 5′-TAC GAA ATG ATA CCG ATA G-3′ R: 5′-GAT ACC CAC TTA AGT CCT C-3′	*mtx3*	400

3. Results

3.1. Bacterial isolation and characterization

From all sampling locations, 20 isolates of *B. sphaericus* were isolated. The characteristics of *B. sphaericus* isolated are presented in Figure 2. Detail characterization (colony, cell and biochemical/physiological) of the *B. sphaericus* isolates is presented in Table 2.

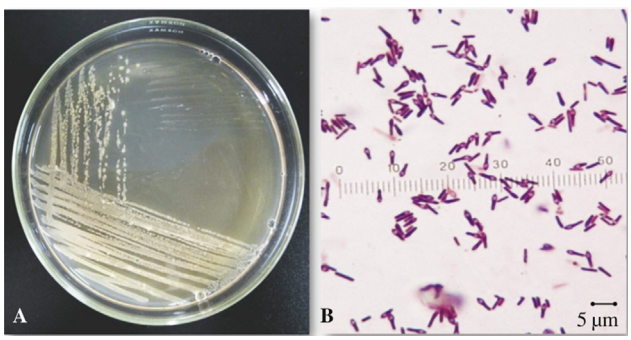

Figure 2. Colony and cell morphology.
A: Colony on NYSM agar 72 h later; B: Gram staining of isolated *B. sphaericus* (1 000×).

All characteristics of isolated *B. sphaericus* have showed similarity to those mentioned in Bargey's Manual of Determinative Bacteriology [9], except for some characteristics that were varied. All isolates were collected from area that was unexposed directly and indirectly by sea water (in form of salt dam/pool or rip-tide). Sampling points were sand/soil covered/shaded by foliage and rich in organic matters.

Table 2
Culture characteristics of the isolated *B. sphaericus*.

Characteristics	Isolated	Standard
Form	Round	Round
Margin	Entire	Entire
Surface	Flat and smooth	Flat and smooth
Color	White-cream	Opaque (grown on nutrient agar)
Form	Rod	Rod
Gram reaction	+	V
Size (L × W)	3.0–5.0 µm × 0.5–0.75 µm	1.5–5.0 µm × 0.6–1.0 µm
Endospore	+	+
Endospore position	Terminal	Terminal/subterminal
Bulging endosporangium	+	+
Catalase	+	+
Starch hydrolysis	–	–
Acid production from sugar	–	–
Nitrate reduction	–	–
Indole	–	–
H_2S	–	–
Urease	+	V
Oxidase	+	+
Casein	+	+
Aerobicity	Aerobe	Aerobe
Sensitivity to streptomycin	Resistant	Resistant
Sensitivity to chloramphenicol	Sensitive	V

+: Positive; –: Negative; V: Variable.

Table 3
Isolates of *B. sphaericus* from many location/origin with their toxicity against 3 species of mosquitoes.

Isolate code	Origin	Initial toxicity against (%)		
		Culex	*Anopheles*	*Aedes*
AMP	West Lombok	0.00	0.00	0.00
MNT	West Lombok	100.00	72.00	36.70
SDK	West Lombok	0.00	0.00	0.00
BTL	West Lombok	0.00	0.00	0.00
MLB	West Lombok	0.00	0.00	0.00
SRE	North Lombok	0.00	0.00	0.00
GDG	North Lombok	0.00	0.00	0.00
SLG	North Lombok	100.00	100.00	60.00
LCR	North Lombok	0.00	0.00	0.00
OBL	North Lombok	0.00	0.00	0.00
BGK	South Lombok	0.00	0.00	0.00
LBP	South Lombok	0.00	0.00	0.00
PLG	South Lombok	0.00	0.00	0.00
SKT	South Lombok	100.00	100.00	50.00
LBR	South Lombok	0.00	0.00	0.00
LBL	East Lombok	0.00	0.00	0.00
LBH	East Lombok	0.00	0.00	0.00
TJL1	East Lombok	20.00	8.33	0.00
JRW	East Lombok	20.00	8.33	0.00
TJL2	East Lombok	100.00	100.00	31.70

3.2. Toxicity of isolated *B. sphaericus*

Table 3 presents toxicity testing results of isolated *B. sphaericus* [in 10^{-1} final whole culture (FWC) dilution] against 3 species of mosquitoes.

From 20 locations sampled, there were 4 locations gave isolates that were toxic against 3 kinds of mosquitoes' larvae. Others showed very low to none toxicity. The values of LC_{50} and LC_{90} of the isolates from the four locations in 48 h observation are presented in Table 4.

Both toxic and low toxic isolates showed similar pattern, *Culex* was the most susceptible to all isolates and *Anopheles* was the second after *Culex*. Toxic isolates and low toxic isolates only killed *Aedes* larvae in small percentages. Compared to 3 other isolates, isolate SLG was the most toxic isolates. This was followed by isolate TJL2, SKT and MNT, respectively.

3.3. Phylogenetic analysis and toxin gene detection

Phylogenetic tree where isolated *B. sphaericus* and some strains of known *B. sphaericus* as comparison is presented in Figure 3. Isolates PLG and MNT were in the same cluster with some known highly and mildly toxic strains of *B. sphaericus*. Isolates TJL2 and SLG were close to each

Table 4
LC values of isolated *B. sphaericus* in 48 h.

Isolates	Concentration unit	*Culex*		*Anopheles*		*Aedes*	
		LC_{50}	LC_{90}	LC_{50}	LC_{90}	LC_{50}	LC_{90}
MNT	Cell/mL	3.70×10^{5}	6.40×10^{5}	1.76×10^{7}	4.57×10^{7}	4.45×10^{7}	8.04×10^{7}
	FWC dilution	8.71×10^{-4}	1.50×10^{-3}	2.54×10^{-2}	6.57×10^{-2}	6.40×10^{-2}	1.16×10^{-1}
SKT	Cell/mL	1.13×10^{5}	4.08×10^{5}	2.85×10^{5}	1.52×10^{6}	1.78×10^{7}	3.18×10^{7}
	FWC dilution	3.50×10^{-4}	1.27×10^{-3}	8.84×10^{-4}	4.71×10^{-3}	5.51×10^{-2}	9.88×10^{-2}
TJL2	Cell/mL	1.03×10^{5}	3.79×10^{5}	8.94×10^{4}	3.75×10^{5}	1.72×10^{7}	3.32×10^{7}
	FWC dilution	3.17×10^{-4}	1.16×10^{-3}	2.74×10^{-4}	1.15×10^{-3}	5.29×10^{-2}	1.02×10^{-1}
SLG	Cell/mL	9.41×10^{5}	3.32×10^{6}	2.39×10^{5}	5.11×10^{5}	2.08×10^{7}	4.14×10^{7}
	FWC dilution	2.71×10^{-3}	9.56×10^{-3}	6.88×10^{-4}	1.47×10^{-3}	6.01×10^{-2}	1.19×10^{-1}

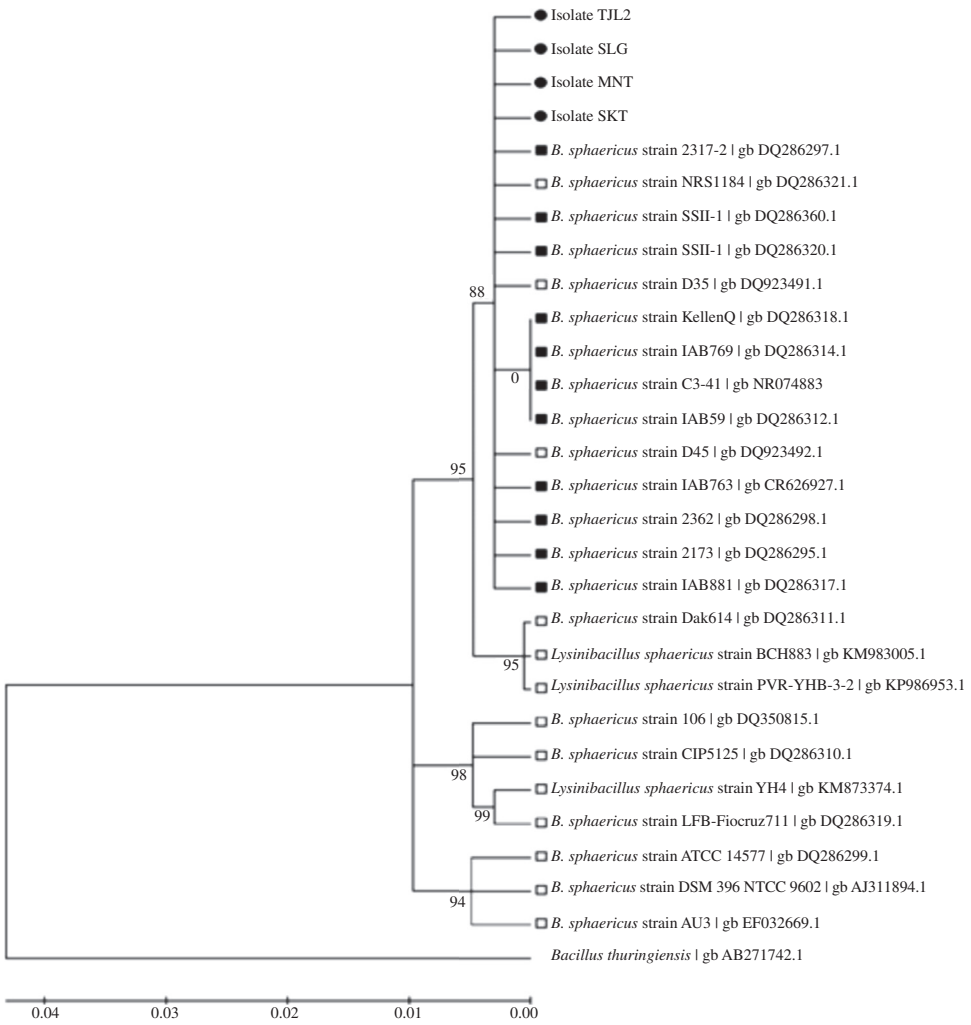

Figure 3. Neighbor-joining cladogram of isolated *B. sphaericus* (marked with dot symbol) with known strains of toxic *B. sphaericus* (marked with solid square symbol) and non-toxic *B. sphaericus* (marked with hollow square symbol) and with *B. thuringiensis* as outgroup species.

other, but not in the same cluster with first two isolates. Grouping *B. sphaericus* using 16s rRNA approach only showed strain closeness by 16s rRNA sequence similarity, but it cannot group their toxicity.

Toxin gene detection PCR result is presented in Figure 4. The existence of binary toxin components (represented by 100 bp for *binB* and 200 bp for *binA* PCR product) can be related to the high anti-larval effect of *B. sphaericus* strain (shown by *B. sphaericus* 2362, SLG, TJL2 and PLG). Both Bin components showed higher anti-larval effect compared to single component (showed by isolate MNT). Mosquitocidal toxin (represented by 300 bp for *mtx1*, 400 bp for *mtx3* and 850 bp for *mtx2*) showed very low anti-larval effect (or none at all).

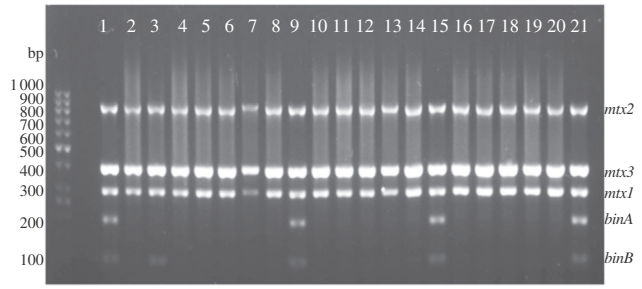

Figure 4. Toxin genes (*bin* and *mtx*) multiplex amplification result in 2% agarose gel.
Lane 1: *B. sphaericus* 2362; Lane 2: Isolate AMP; Lane 3: Isolate MNT; Lane 4: Isolate SDK; Lane 5: Isolate BTL; Lane 6: Isolate MLB; Lane 7: Isolate SRE; Lane 8: Isolate GDG; Lane 9: SLG; Lane 10: Isolate LCR; Lane 11: Isolate OBL; Lane 12: Isolate BGK; Lane 13: Isolate LBP; Lane 14: Isolate PLG; Lane 15: Isolate SKT; Lane 16: Isolate LBR; Lane 17: Isolate LBL; Lane 18: Isolate LBH; Lane 19: Isolate TJL1; Lane 20: Isolate JRW; Lane 21: Isolate TJL2.

4. Discussion

This report was the first report of *B. sphaericus* isolated from Lombok Island, Indonesia. The most-frequently used strain of *B. sphaericus* that has been applied in some countries is *B. sphaericus* 2362 that was firstly isolated in Nigeria. This strain was isolated from black fly [15]. This strain becomes one standard for comparison with newly isolated *B. sphaericus* from many countries.

Since providing all nutrients and growing factors for bacteria that live in it, soil becomes a potential habitat for *B. sphaericus*. However, this study showed that area that was rich in organic matters did not always become habitat for toxic *B. sphaericus*. From all locations that were explored, toxic isolates of *B. sphaericus* were obtained only from 4 locations. Other isolates from the rest 16 locations were low toxic on no toxic at all. This demonstrated that soil rich in organic matters was not the key factor to obtain toxic *B. sphaericus*. Any chances that enable bacteria contact with mosquito larvae should be considered to gain toxicity. That was described in some reports in the discovery of *Bacillus thuringiensis* and *B. sphaericus* [16,17].

It was mentioned by de-Barjac [18], that toxicity of *B. sphaericus* can be categorized into 3 classes. The highest toxicity showed LC_{50} value of 10^{-6}–10^{-8} (based on final whole concentration/FWC dilution). The examples of high toxicity strains are *B. sphaericus* strain 2362, 1593 and LB24. While mild toxicity showed LC_{50} value of 10^{-4}–10^{-5} of FWC dilution. The examples of strains having this toxicity are *B. sphaericus* strain SSII-1, ISPC5 and LB29. The lowest value is ranging from 10^{-2} to 10^{-3} (of FWC dilution), and *B. sphaericus* strain K and strain Q are low toxic strains discovered in the United States. All those LC values were against *Culex* larvae. If compared to that category, *B. sphaericus* isolated from Lombok Island can be grouped into lowly to mildly toxic isolates.

Larvae death occurred within some hours after *B. sphaericus* endospores have been ingested by a larval. After ingested, protease released in the midgut will process the endospores into toxins subunit components. Toxin related to sporulated *B. sphaericus* is binary toxin. The binary toxin will be broken into two components, namely BinA (51 kDa) and BinB (42 kDa). This toxin will make interaction with specific receptor along *Culex* and *Anopheles* midgut. However, in *Aedes* midgut, there are no interaction between toxin and toxin receptor. Hence, binary toxin will give the highest anti-larvicidal activity only against *Culex* and *Anopheles*. On the other hand, *Aedes* showed the least susceptibility against *B. sphaericus* [19].

Besides binary toxin, *B. sphaericus* also synthesizes non-sporulation related toxin, called mosquitocidal toxin (Mtx toxin). The toxin is produced when the bacteria is on vegetative life stage. This toxin comprises of Mtx1 (100 kDa), Mtx2 (32 kDa) and Mtx3 (36 kDa) subunits [20,21]. In contrast with binary toxin, mosquitocidal toxin does not interact with receptor inside larvae midgut and only give low anti-larvicidal effect to *B. sphaericus* [22]. The low toxicity of mosquitocidal toxin was caused by proteolytic degradation on Mtx toxin [23]. When intact, Mtx toxin (mainly Mtx1 subunit) shows ability to kill larvae better than binary toxin [24]. This was shown that when *mtx* genes were cloned into protease-free non-toxic *B. sphaericus* strain, *mtx1* gene expression in protease-free *B. sphaericus* could kill mosquito larvae as good as *B. sphaericus* strain harboring Bin toxin protein and naturally toxic *B. sphaericus* strain [25]. *B. sphaericus* toxins activities will cause collapse of larvae nervous and muscle system. In turn, this will make the larvae loss their ability to move along water surface and undergo asphyxia by drowning [26].

Grouping *B. sphaericus* using 16s rRNA could not cluster this species based on strain toxicity, so other approaches should be made. Grouping using flagella agglutination by de-Barjac *et al.* [27] has clustered *B. sphaericus* in serotype H1A; H2; H5; H5A, 5B; H2 and H5. This grouping clustered *B. sphaericus* toxicity very well. Other approach using phage typing [28], resulted in four phagetypes groups, which was able to cluster *B. sphaericus* based on their toxicity as well. This phylogenetic analysis, besides showing closeness of these toxic *B. sphaericus* isolates to other known *B. sphaericus* strains, also supports identification of the bacteria, since there are some species show similar characteristics to *B. sphaericus*. The other technique, such as multiplex PCR which we used in this study, was able to detect toxin-expressing genes (binary and mosquitocidal toxins).

Multiplex PCR is variant of PCR technique applying more than one primer pairs to amplify more than one locus/positions simultaneously [29]. In this study, multiplex PCR succeeded to detect the existence of Bin and Mtx toxins altogether. This supports toxicity testing and bioassay results. Mild to high toxicity effect of *B. sphaericus* can be expected if two PCR

products present (100 and 200 bp). PCR products of 100 bp and 200 bp are related to existence of *binB* and *binA* genes, respectively. These genes express binary toxin in non-vegetative stage of *B. sphaericus* that are most active in killing mosquito larvae. Other PCR products, 300, 400 and 850 bp which are related to *mtx1*, *mtx3* and *mtx2* genes, showed very little effect of killing mosquito larvae (or none at all). If all PCR products exist altogether, high toxicity effect can be expected.

Many strains of *B. sphaericus* (either toxic or not toxic) have been studied and collected in some countries, but local strain is still promising for local-ingredient-based biopesticide candidates. Along with more toxic *B. sphaericus* strain search, growth and production medium formulation should be explored as well.

Twenty *B. sphaericus* isolates from beach areas around Lombok Island have been isolated and 4 isolates showed low to mild toxicity against 3 species of mosquito larvae. The rest 16 isolates have showed either very low toxicity or non-toxicity at all.

Conflict of interest statement

We declare that we have no conflict of interest.

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References

[1] Ministry of Health of Indonesia. [Health profiles of West Nusa Tenggara Province in 2012]. Jakarta: Ministry of Health of Indonesia; 2013. Indonisia. [Online] Available from: http://dinkes.ntbprov.go.id/sistem/data-dinkes/uploads/2013/10/Data-Kesehatan-Provinsi-NTB-2012.pdf [Accessed on 23rd January, 2015]

[2] World Health Organization. WHO campaign: about vector-borne diseases. Geneva: World Health Organization; 2015. [Online] Available from: http://www.who.int/campaigns/world-health-day/2014/vector-borne-diseases/en/ [Accessed on 7th August, 2015]

[3] Lorenz ES. Potential health effects of pesticides. Pennsylvania: Penn State's College of Agricultural Sciences; 2009. [Online] Available from: http://extension.psu.edu/pests/pesticide-education/applicators/fact-sheets/pesticide-safety/potential-health-effects-of-pesticides/extension_publication_file [Accessed on 7th August, 2015]

[4] Denholm I, Devine GJ, Williamson MS. Evolutionary genetics. Insecticide resistance on the move. *Science* 2002; **297**(5590): 2222-3.

[5] United States Environmental Protection Agency. What are biopesticides. Washington: United States Environmental Protection Agency; 2015. [Online] Available from: http://www2.epa.gov/ingredients-used-pesticide-products/what-are-biopesticides [Accessed on 20th November, 2015]

[6] Poopathi S, Abidha S. Mosquitocidal bacterial toxins (*Bacillus sphaericus* and *Bacillus thuringiensis* serovar *israelensis*): mode of action, cytopathological effects and mechanism of resistance. *Glob Sci Res J* 2013; **1**(1): 53-69.

[7] Yadav K, Dhiman S, Baruah I, Singh L. Development of cost effective medium for production of *Bacillus sphaericus* strain isolated from Assam, India. *Microbiol J* 2011; **1**(2): 65-70.

[8] Vanlalhruaia N, Kumar S, Gurusubramanian G. *Bacillus sphaericus* in the biological control of mosquito vector complex. *Sci Vis* 2011; **11**(2): 61-71.

[9] Holt JG. *Bergey's manual of determinative bacteriology*. 9th ed. Baltimore: The Williams & Wilkins Company; 1994.

[10] Dulmage T, Yousten AA, Singer S, Lacey LA. Guidelines of production of *Bacillus thuringiensis* H-14 and *Bacillus sphaericus*. Geneva: World Health Organization; 1990. [Online] Available from: https://extranet.who.int/iris/restricted/handle/10665/61645 [Accessed on 23rd January, 2015]

[11] Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, et al. *Current protocols in molecular biology*. New York: John Wiley & Sons Inc; 1995.

[12] James G. Universal bacterial identification by PCR and DNA sequencing of 16S rRNA gene. In: Carter IWJ, Schuller M, James GS, Sloots TP, Halliday CL, editors. *PCR for clinical microbiology*. Netherlands: Springer; 2010, p. 209-14.

[13] Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011; **28**(10): 2731-9.

[14] Prabhu DI, Sankar SG, Vasan PT, Piriya PS, Selvan KB, Vennison SJ. Molecular characterization of mosquitocidal *Bacillus sphaericus* isolated from Tamil Nadu, India. *Acta Trop* 2013; **127**(3): 158-64.

[15] Weiser J. A mosquito-virulent *Bacillus sphaericus* in adult *Simulium damnosum* from northern Nigeria. *Zentralbl Mikrobiol* 1984; **139**(1): 57-60.

[16] Goldberg LH, Margalit J. A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii*, *Uranotaenia unguiculata*, *Culex univitatus*, *Aedes aegypti* and *Culex pipiens*. *Mosq News* 1977; **37**(3): 355-8.

[17] Kellen WR, Clark TB, Lindegren JE, Ho BC, Rogoff MH, Singer S. *Bacillus sphaericus* Neide as a pathogen of mosquitoes. *J Invertebr Pathol* 1965; **7**(4): 442-8.

[18] de-Barjac H. Classification of *B. sphaericus* strains and comparative toxicity to mosquito larvae. In: de Barjac H, Sutherland DJ, editors. *Bacterial control of mosquitoes & black flies*. Netherlands: Springer; 1990, p. 228-36.

[19] Poopathi S, Tyagi BK. The challenge of mosquito control strategies: from primordial to molecular approaches. *Biotechnol Mol Biol Rev* 2006; **1**(2): 51-65.

[20] Arapinis C, de la Torre F, Szulmajster J. Nucleotide and deduced amino acid sequence of *Bacillus sphaericus* 1593M gene encoding a 51.4 kD polypeptide which act synergistically with the 42 kD protein for the expression of the larvicidal toxin. *Nucleic Acid Res* 1988; **16**(15): 7731-9.

[21] Baumann L, Broadwell AH, Baumann P. Sequence analysis of the mosquitocidal toxin genes encoding 51.4- and 41.9-kilodalton protein of *Bacillus sphaericus* 2362 and 2297. *J Bacteriol* 1988; **170**(5): 2045-50.

[22] Nielsen-LeRoux C, Raghunatha Rao D, Murphy JR, Carron A, Mani TR, Hamon S, et al. Various levels of cross-resistance to *Bacillus sphaericus* strains in *Culex pipiens* (Diptera: Culicidae) colonies resistant to *B. sphaericus* strain 2362. *Appl Environ Microbiol* 2001; **67**(11): 5049-54.

[23] Yang Y, Wang L, Gaviria A, Yuan Z, Berry C. Proteolytic stability of insecticidal toxins expressed in recombinant bacilli. *Appl Environ Microbiol* 2007; **73**(1): 218-25.

[24] Wei S, Cai Q, Yuan Z. Mosquitocidal effect of *Bacillus sphaericus* induces stronger delayed effect than binary toxin on *Culex quinquefasciatus* (Diptera: Culicidae). *J Med Entomol* 2006; **43**(4): 726-30.

[25] Thanabalu T, Porter AG. A *Bacillus sphaericus* gene encoding a novel type of mosquitocidal toxin of 31.8 kDa. *Gene* 1996; **170**(1): 85-9.

[26] Litaiff EC, Tadei WP, Porto JIR, Oliveira IMA. Analysis of toxicity on *Bacillus sphaericus* from amazonian soils to *Anopheles darlingi* and *Culex quinquefasciatus* larvae. *Acta Amazon* 2008; **38**(2): 255-62.

[27] de-Barjac H, Larget-Thiéry I, Cosmao Dumanoir V, Ripouteau H. Serological classification of *Bacillus sphaericus* on the basis of toxicity to mosquito larvae. *Appl Microbiol Biotechnol* 1985; **21**: 85-90.

[28] Yousten AA, de-Barjac H, Hendrick J, Cosmao Dumanoir V, Myers P. Comparison between bacteriophage typing and serotyping for differentiation of *Bacillus sphaericus* strains. *Ann Microbiol (Paris)* 1980; **131B**(3): 297-308.

[29] Hayden MJ, Nguyen TM, Waterman A, Chalmers KJ. Multiplex-ready PCR: a new method for multiplexed SSR and SNP genotyping. *BMC Genomics* 2008; **9**: 80.