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Research Article

# HISTOLOGICAL EFFECT OPTIMIZATION COMBINATION OF *BACILLUS SPHAERICUS* 2362 AND *BACILLUS THURINGIENSIS* SUBSP. *ISRAELENSIS* IN MIDGUT OF *CULEX QUINQUEFASCIATUS* RESISTANCE

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**ABSTRACT**

**Objective:** The objective of the study was to analyze pore-forming at *Culex quinquefasciatus* resistant larvae midgut and analyze optimization ratio after treatment using combination *Bacillus thuringiensis var. israelensis* (*Bti*) and *Bacillus sphaericus* (*Bs*) 2362.

**Methods:** This research was an experimental study. *C. quinquefasciatus* larvae were divided into 10 groups. The Group I until VII had treatment by the various concentration of combination *Bti* and *Bs* 2362, treatment Group VIII as a positive control, Group IX as a negative control, and Group X as a single *Bs* 2362. All of the treatment groups were examined for the histological effect of *C. quinquefasciatus* larvae midgut by hematoxylin eosin. The lowest lethal concentration 50% ($LC_{50}$) was a standard optimization ratio of combination *Bti* and *Bs* 2362. $LC_{50}$ was analyzed by probit.

**Results:** The lowest $LC_{50}$ was 2.274 part a million (ppm) at Group I was the optimization ratio. Various combination treatments Group I until VII were shown pores at *C. quinquefasciatus* larvae midgut after treatment by a combination of *Bti* and *Bs* 2362.

**Conclusion:** Combination of *Bti* and *Bs* 2362 was shown pores at *C. quinquefasciatus* larvae midgut, and optimization ratio was shown in Group I.

**Keywords:** *Bacillus thuringiensis var. israelensis*, *Bacillus sphaericus* 2362, *Culex quinquefasciatus midgut.*

## INTRODUCTION

Mosquitoes are vector many kind mosquito-borne diseases such as malaria, filariasis, dengue, yellow fever, chikungunya, and multiple viral encephalitis [1-3]. *Culex pipiens quinquefasciatus* transmit of filariasis, West Nile Virus, and Japanese Encephalitis [4]. Approximately 250 million people have been the world burden of lymphatic filariasis [5]. Almost all of province in Indonesia endemic of lymphatic filariasis such as North Sumatera, South Sumatera, Bangka Belitung, Papua, East Kalimantan, Central Java, Tangerang and more than 17 districts of West Java [6]. Therefore, adequate mosquito control strategies are important for interrupt mosquito-borne diseases transmission [7].

Chemical insecticides have been used during four decades, extensive used of synthetic insecticides have caused the development of resistance in mosquito, environmental pollution, harmful effects on beneficial non-target animals, food chain contamination [1,5,8]. Therefore, we need the alternative environment-friendly control agents, the bacterial larvicide that can be save used such as *Bacillus sphaericus* (*Bs*) and *Bacillus thuringiensis serovar israelensis* [1,9].

Over the past two decades, biological larvicide has been used as a mosquito vector control programs in the world [10,11]. The continuous exposure of *Bs* has been result in development of moderate to the high-level resistance of *C. quinquefasciatus* [8]. *Bacillus thuringiensis subsp israelensis* produced crystal protein toxin (Cry11Aa, Cry4Aa, and Cry4Ba), cytolitic protein toxin (Cyt1Aa) [12]. Cyt1Aa have strong binding affinity to the apical brush border of midgut epithelial cells of dipterans, which the Cyt1Aa toxin have the ability to perforate cells membrane [13]. Cry4Ba toxin and Cry11Aa toxin were shown to form pores in the cells membrane of dipterans [14]. Mixture combination of *Bs* with purified Crys of Cyt1Aa at a 10:1 ratio were completed suppressed resistance in *C. quinquefasciatus* population in the field [15]. Combination of Cry powders Cyt1Aa with *Bs* powders were resulting toxicity was much greater than *Bs* alone against *Aedes aegypti* [16]. *B. thuringiensis* Cry and Cyt toxins known as pore-forming toxins [12]. The aim of this study was to analyze pore-forming at *C. quinquefasciatus* resistant larvae midgut after treatment using optimization combination *Bacillus thuringiensis susp israelensis* and *Bs* 2362, which we used a new combination *Bs* 2362 and *Bacillus thuringiensis var israelensis* (*Bti*), following the single *Bs* 2362 have not been used in the laboratory yet in Indonesia and this research investigated to overcoming new resistance *C. quinquefasciatus* larvae to *Bs* 2362 in the laboratory at Department Parasitology of Gadjah Mada University, Yogyakarta in Indonesia.

## METHODS

### Materials

*Bs* 2362 water dispersible granule (WDG) and *Bti* WDG (Institut Pasteur Paris), temephos 1% (Gadjah Mada University), aquadest, plastic cups 200 ml, pasteur pipette, anatomical forceps, buffer formalin 10%, measuring glass 100 ml, beaker glass, Erlemeyer glass 500 ml, analytic scale, plastic cups, 10% sucrose solution, buffer formalin 10%, liquid paraffin, tissue processor (Sakura, Torrance, CA), embedding machine (Sigma-Aldrich, St.Louis), Microm HM355s microtome (Thermo Scientific, Kalamazoo, MI), Fisherbrand Superfrost Plus slides, water bath, hot plate, xylol I, II, III, alcohol 100%, 95%, 80%, 70% Mayer-Hematoxylin solution, Eosin solution, glass object, cover glass, light microscope, mosquito cages, fish food, plastic trays.

### Mosquitoes

A laboratory colony of *C. quinquefasciatus* was obtained from the Department of Parasitology, Medicine Faculty, Gadjah Mada University, Yogyakarta, Indonesia. 750 early fourth-instar larvae were used in the bioassays. 25 *C. quinquefasciatus* larvae were put into each 7 treatment groups combination plastic cups, following one single *Bs* 2362 treatment plastic cups, temephos treatment plastic cups as a positive

control, and aquadest treatment plastic cups as a negative control [17]. Seven concentration combinations were used for this research; the formula of replication was used [18]:

$7 (r - 1) \geq 6$
$7r - 7 \geq 6$
$7r \geq 6 + 7$
$7r \geq 13$
$r \geq 1.85 \approx 3$

Each sample replication was 3 times. Amount of plastic cups for this study were 30 cups.

**Methods**

The research was a true experimental laboratory. *C. quinquefasciatus* early fourth-instar larvae were divided into 10 groups. The concentration treatment Group I was combination of *Bs* 2362 and *Bti* (8:2) part a million (ppm), the combination treatment Group II was (5:5) ppm, the combination treatment Group III was (7:3) ppm, the combination treatment Group IV was (6:4) ppm, the combination treatment Group V was (2:8) ppm, the combination treatment Group VI was (3:7) ppm, combination treatment Group VII was (4:6) ppm, treatment Group VIII used temephos 1% as a positive control, treatment Group IX used 100 ml aquadest as a negative control, and the treatment Group X as a single *Bs* 2362. *C. quinquefasciatus* fourth-instar larvae were dryed at tissue paper than the *C. quinquefasciatus* early fourth-instar larvae were put in a tube which contains 10% buffer formalin, thereafter *C. quinquefasciatus* larvae put in the freezer at − 70°C. The *C. quinquefasciatus* early fourth-instar larvae were examined for the histological effect of midgut by coloring hematoxylin eosin. The lowest lethal concentration 50% ($LC_{50}$) was a standard optimization ratio of combination *Bti* and *Bs* 2362. $LC_{50}$ was analyzed by probit.

**RESULTS**

The combination treatment Group I *Bs* 2362 with *Bti* (8:2) ppm was shown many pores at midgut of *C. quinquefasciatus* early fourth-instar larvae. The optimization combination was the treatment Group I (8:2) ppm which has given the result the lowest $LC_{50}$ 2.274 ppm. The result of treatment Group I was shown in Fig 1.

The combination of treatment Group V *Bs* 2362 with *Bti* (2:8) ppm and combination treatment Group VII (4:6) ppm were also shown pores at *C. quinquefasciatus* early fourth-instar larvae midgut. The $LC_{50}$ of combination treatment Group V and VII were 2.276 ppm and 2.279 ppm, respectively, the results were almost the same as with treatment Group I. The picture of midgut *C. quinquefasciatus* early fourth-instar larvae from combination treatment V and VII was shown in Figs 2 and 3.

The combination of treatment *Bs* 2362 with *Bti* Group II (5:5) ppm, Group III (7:3) ppm, Group IV (6:4) ppm, and Group VI (3:7) ppm was also shown pores forming. The consecutive $LC_{50}$ of combination treatment Group II, III, IV, and VI were 9.193 ppm, 4.146 ppm, 3.191 ppm, and 3.122 ppm, respectively. The picture of midgut *C. quinquefasciatus* larvae in Group II, III, IV, and VI was shown in Figs 4-7.

While the treatment Group X with single *Bs* 2362 was not shown pores at midgut of *C. quinquefasciatus* early fourth-instar larvae. The $LC_{50}$ single *Bs* 2362 was 32.675 ppm, which biggest than treatment Group I until VII. The histologic picture of single *Bs* 2362 was shown in Fig. 8.

The results of $LC_{50}$ of various combination of *Bs* 2362 with *Bti* were shown in Table 1.

**DISCUSSION**

The combination of *Bs* 2362 with *Bti* Group I (8:2) ppm was the optimization combination with the lowest $LC_{50}$ was 2.274 ppm and shown the most pores at midgut of *C. quinquefasciatus* fourth-instar

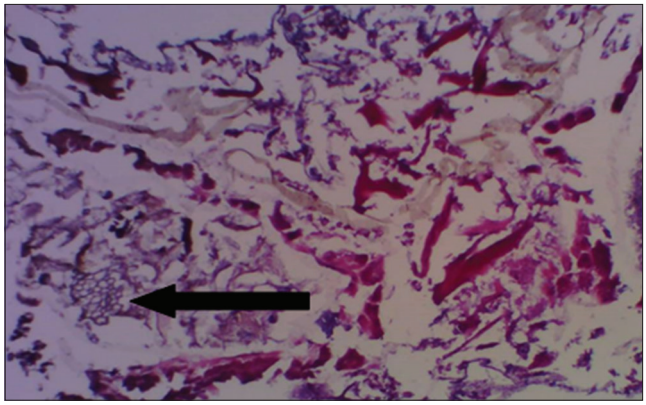

**Fig. 1: Midgut pores of *Culex quinquefasciatus* larvae in treatment Group I (black arrow shown pores of midgut)**

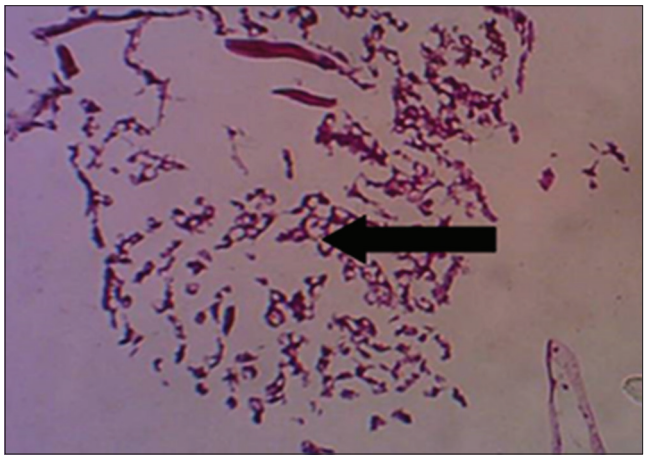

**Fig. 2: Midgut pores of *Culex quinquefasciatus* larvae in treatment Group V (black arrow shown pores of midgut)**

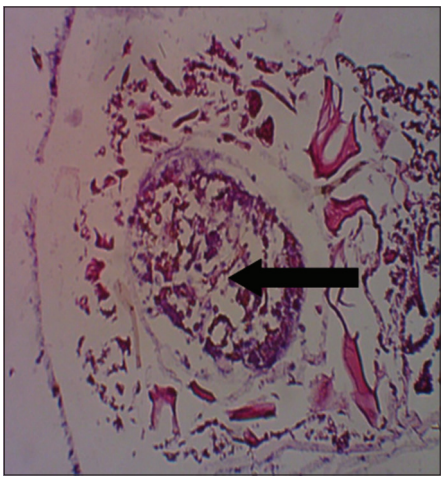

**Fig. 3: Midgut pores of *Culex quinquefasciatus* larvae in treatment Group VII (black arrow shown pores of midgut)**

larvae at Laboratory Parasitology Gadjah Mada University, Yogyakarta, Indonesia. Laurence Depres, *et al.* have written at their book about *Bti* have four toxins *Cry4Aa, Cry4Ba, Cry11A,* and *Cyt (Cytolitc) 1Aa*, that the function of *Cry4Aa* and *Cry4Ba* were making pores at midgut of epithel mosquitoe larvae [12,14]. *Cyt1Aa* was strongly affinity of unsaturated fatty acids on epithel mosquitoe larvae midgut; therefore, it is having ability midgut membrane perforation [13]. *Bs* produced mosquitocidal

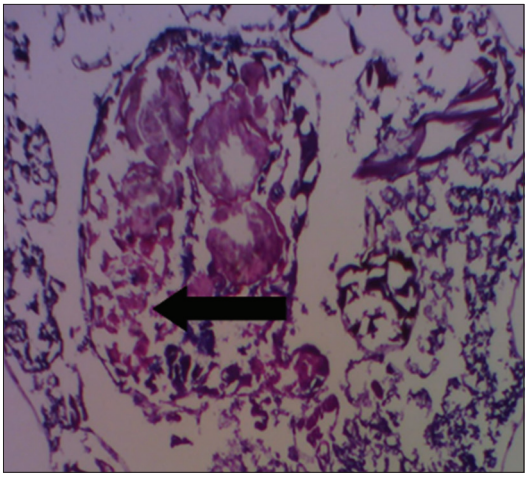

**Fig. 4: Midgut of *Culex quinquefasciatus* larvae in combination treatment Group II was (5:5) ppm (black arrow shown pores of midgut)**

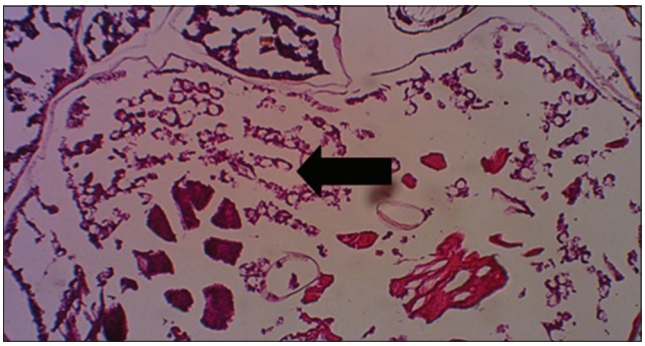

**Fig. 5: Midgut of *Culex quinquefasciatus* larvae in combination treatment Group III was (7:3) ppm (black arrow shown pores of midgut)**

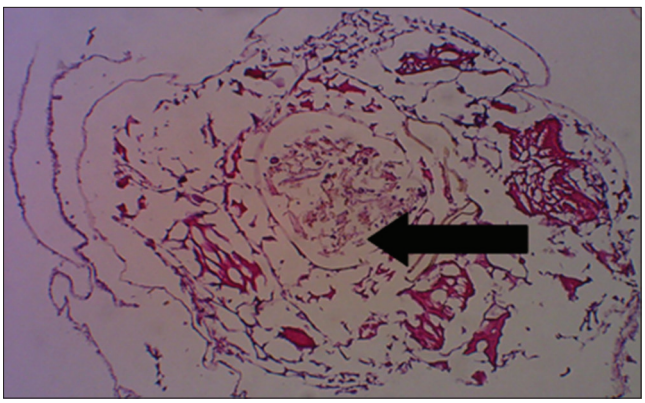

**Fig. 6: Midgut of *Culex quinquefasciatus* larvae in combination treatment Group IV was (6:4) ppm (black arrow shown pores of midgut)**

toxin (*Mtx*) and binary (*Bin*) toxin, Bin binds with a single receptor of brush border membrane epithel *Culex* larvae by digestive enzyme *Culex pipiens maltase 1*, Bin toxin making pores at receptor of brush border membrane epithel *Culex* larvae [19]. *Mtx* has 3 types of toxin *Mtx* 1, *Mtx*, 2, and *Mtx* 3, while *Mtx* 3 has a role in the formation of pores at midgut *Culex* larvae [20]. The research of Poopathi and Abidha was shown the Bin toxin of *Bs* and multiple toxin of *Bti* after being ingested into mosquito larvae midgut; their effects were shown disruption, separation, and ploughing of columnar epithelial cells midgut, which causing the death of mosquitoe larvae [21]. The study of Wirth *et al*.

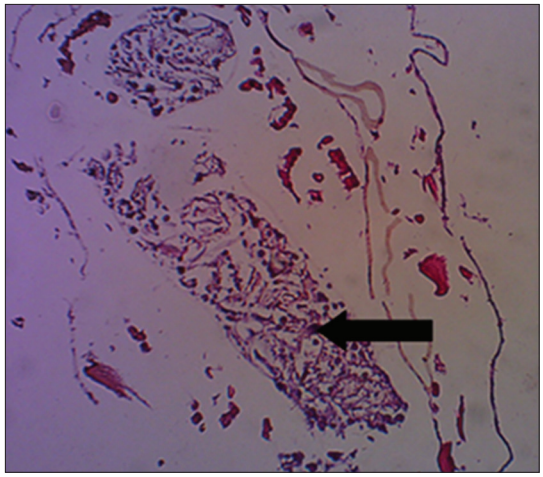

**Fig. 7: Midgut of *Culex quinquefasciatus* larvae in combination treatment Group VI was (3:7) ppm (black arrow shown pores of midgut)**

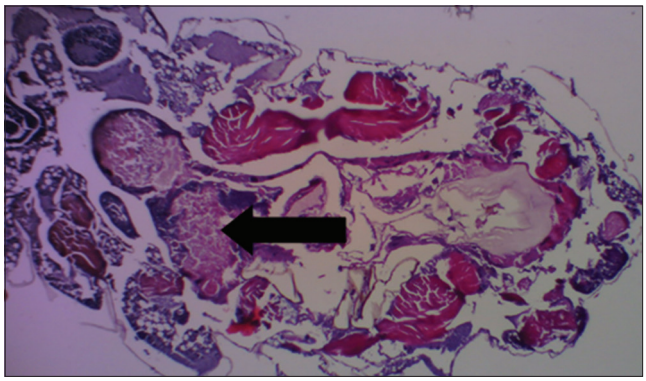

**Fig. 8: Midgut of *Culex quinquefasciatus* larvae in treatment Group X single *Bacillus sphaericus* 2362 (black arrow shown pores of midgut)**

**Table 1: $LC_{50}$ of seven combination treatment group**

| Combination *Bs* 2362 with *Bacillus thuringiensis subsp israelensis* | $LC_{50}$ |
|---|---|
| I ( 8:2) | 2.274 |
| II (5:5) | 9.193 |
| III (7:3) | 4.146 |
| IV (6:4) | 3.191 |
| V (2:8) | 2.276 |
| VI (3:7) | 3.122 |
| VII (4:6) | 2.279 |
| Mean | 3.783 |
| Standard deviation | 2.483 |

$LC_{50}$: Lethal concentration 50%, *Bacillus sphaericus*

combined the mixture of *Bs* 2362 with *Mtx 2* and *Cyt1Aa* from *Bacillus thuringiensis* (8:1:1) was also synergistic with synergy factor at the $LC_{50}$ value 44 for the resistant colonies *C. quinquefasciatus* to *Bs* 2362 (Bs-R), which combined have distinct mode of action when Cyt1Aa was lipophilic and lyses cell, while *Mtx 2* was making pore at target cells, which the mixture toxins with both mechanism mode of action could suppress resistance to *Bs* 2362 (*Bs*-R) [22].

## CONCLUSION

The combination of *Bs* 2362 and *Bti* was shown pores at *C. quinquefasciatus* larvae midgut and optimization ratio was shown in Group I with the concentration was (8:2) ppm.

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