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New Effective Strains of Bacteria *Bacillus thuringiensis* ssp. *israelensis* for Bloodsucking Mosquito Control

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***Abstract*—**Five original strains of entomopathogenic bacteria with insecticidal activity against mosquito larvae of the genera *Aedes*, *Culex* and *Anopheles* have been isolated from natural conditions in Armenia and characterized. According to morphological, physiological and biochemical parameters, all isolates were identified as *Bacillus thuringiensis* spp. *israelensis* (*Bti*). High larvicidal activity has been showed by three strains *Bti*. These strains can be recommended for industrial production of bacterial preparations.

***Keywords*—**Armenia, *Bacillus thuringiensis* ssp. *israelensis*, bloodsucking mosquito control, new effective strains of bacteria.

I. Introduction

CURRENTLY, chemical insecticides remain the main way for bloodsucking insect control, to which the insects quickly develop resistance which requires either increasing of dosages, or insecticide rotation. Lacking the selectivity of impact, chemical insecticides cause the death of non-target and often useful organisms. The accumulation of insecticides in natural constituents (water, soil, etc.) makes them environmentally hazardous. These shortcomings make it necessary to find new environmentally friendly methods of bloodsucking insect control. For adult insect control there is still no alternative to chemical agents, however for destruction of larvae the biologics are increasingly used.

The use of bacterial agents against insects of medical importance (primarily, against mosquitoes and black flies) began in the late 70's of the XX century. It was connected with the discovery of the bacteria *Bacillus thuringiensis* ssp. *israelensis* (*Bti*). The main advantage of biological agents when compared to chemical ones is selectivity. Agents based on *Bti* have been used for over 30 years, and during that time there were no cases of their negative effect on other organisms. There was no occurrence of insect resistance to these agents also. Given the ability of *Bti* to synthesize 4 types of protein toxins, it is hardly possible to predict the emergence of resistance in the future.

The disadvantages of bacterial insecticides should include a relatively short residual effect. Lack of reproduction of bacteria *Bti* in nature necessitates repeated treatments of ponds. According to expert estimates, in future the share of biological agents for bloodsucking insect control should grow by isolation of new effective strains of bacteria, improvement of formulations, cost reduction and development of sustainable tactics of their use. These considerations confirm the relevance of the research.

Research Objective. The objective of the present studies was the selection of new strains of entomopathogenic bacteria and study of their larvicidal activity.

II. Materials and Methods

Bacteria were isolated from insects dead in natural environment, in particular from butterflies and grasshoppers. Insects were homogenized in sterile water, and the homogenate filtered under sterile conditions was heated in a water bath (was pasteurized) at 75-80 ^{0}C for 15-20 minutes. Cultivation of bacteria was carried out on the following agarized nutrient media in Petri dishes at 35 ^{0}C for 24-48 hours:

1. Fish-peptone agar (g / l): fish paste - 20.0, yeast extract - 5.0, agar - 20.0, pH 7.2 -7.4.
2. BEB medium (g / l): peptone 10.0, NaCl – 5.0, yeast extract - 10.0, agar - 20.0, pH 7.2 - 7.4.

Strains of bacteria forming toxin crystals were selected from grown colonies by means of microscopy.

The identification of bacterial strains was carried out by morphological, physiological and biochemical parameters.

When tested on the larvae of mosquitoes, maintenance of bacterial cultures was carried out on LB agar in Petri dishes with a 10 day passage. In 40 hours, a good growth of colonies of all strains was observed. In 72 hours scraped from Petri dishes with a sterile buffer solution, suspension of bacteria was tested on the third instar mosquito larvae: *Aedes aegypti*, *Culex pipiens*, and *Anopheles stephensi*. Before setting, each test bacterial suspension was titrated. Titer ranged from 1 to 1.5 x 10^9 spores/ml.

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For screening of entomopathogenic bacteria for mosquito larvae a technique proposed by A. A. Voytsik and S. P. Rasnitsyn was used [4]. The third instar larvae were placed in 25 individuals in Petri dishes with 50 ml of bacterial suspension. Larval mortality was observed in 24 hours. Four-fold series of at least 6 concentrations were undertaken. Four replicates of each concentration were performed. LC_{50} for mosquito larvae of the tested species were calculated by the number of spores in the samples and expressed as spores/ml.

III. Results and Discussion

Initially, four strains isolated from insects dead in natural environment, especially butterflies and grasshoppers, were tested, and only one of them, strain BT2 had larvicidal activity. Then studies of another four strains were conducted. The effectiveness of these strains was performed in comparison with the strain BT2. The results of the tests are shown in Table I.

Strains Bti 1, Bti 2 and BT2 showed high efficacy against the larvae of all mosquito species.

For the larvae of *Aedes aegypti*, strain Bti 1 was the most active, it exceeded larvicidal activity of strain Bti 2 5.4 times, and larvicidal activity of BT2 - 12.3 times (Table I).

TABLE I
Effectiveness of Entomopathogenic Bacteria Strains for the Third Instar Larvae of Laboratory Culture of Bloodsucking Mosquitoes

Mosquito species	LC_{50} (spores/ml) of strains				
	BT 2	Bti 1	Bti 2	Bti 3	Bti 4
Aedes aegypti	$2.9x10^3$ a $4.8x10^3$ b $1.8x10^3$ c	$3.9x10^2$	$2.1x10^3$	$6.3x10^4$	$6.5x10^4$
Culex pipiens	$6.8x10^3$ a $4.2x10^2$ b $4.0\ x10^2$ c	$4.5x10^2$	$3.4x10^2$	$9.0x10^3$	$1.1x10^4$
Anopheles stephensi	$5.3x10^4$ a $2.6x10^3$ b $2.4x10^3$ c	$3.0x10^3$	$3.7x10^3$	$6.2x10^4$	$6.4x10^4$

Notation: a initial tests, b tests compared to Bti 1and Bti 2, c tests compared to Bti 3 and Bti 4.

For larvae *Anopheles stephensi* and *Culex pipiens* effectiveness of strains was practically identical. However, it should be noted that, activity of the strains in relation to these mosquito species increased, most likely as a result of selective work. If during the initial experiments LC_{50} of strain BT2 for the larvae of *Anopheles stephensi* was 5.3×10^4spores/ml, then during these experiments the effectiveness in relation to strain BT2 has increased 14-fold. Similar results were obtained for the larvae of *Culex pipiens* (Table I).

The work with the strains Bti 3 and Bti 4 was held by the same methods and also in comparison with the strain BT2. However, the effectiveness of strains Bti 3 and Bti 4 was significantly lower than in the strains previously tested. Even in the highly sensitive to *Bti* mosquito larvae of *Aedes aegypti* and *Culex pipiens* LC_{50} for the Bti 3 was 6.3×10^4 spores/ml and 9.0×10^3 spores/ml, respectively. This concentration exceeds the concentration of BT2 35 times for *Aedes aegypti*, and 21 times for *Culex pipiens*. For Bti 4, this ratio is 36 and 26 times, respectively. Against the larvae of *Anopheles stephensi* activity of strains was also low (Table I).

Larvae of *Ae. aegypti* and *C. pipiens* were the most sensitive to the effects of spore-crystal mixture of strain BT2, and larvae of mosquitoes *An. stephensi* were less sensitive to the effects of the cultures. Different sensitivity of the mosquitoes owes to the fact that the larvae feed on different levels of water. Mosquito larvae of genus *Culex* feed on the entire surface of the water filtering particles in the layer under the surface film, genus *Aedes* spends a lot of time in the water column and at the bottom of reservoirs, scraping submerged objects, genus *Anopheles* filters particles from the surface of water. *Bti* remains a very short time on the surface of water [3]. The quantity of water filtered per hour by different species of mosquitoes varies: *Ae. aegypti* absorbs 632 ml, *Cx. quiquefasciatus* - 515 ml, *An. albimanus* – 83.9 ml [1]. So, apparently, the mosquitoes of genus *Anopheles* are less sensitive to the influence of *Bti*. Mahmood showed that larvae of *Ae. aegypti* swallow spores in 11.5 times more than larvae of *An. albimanus* do [2].

A complete study of the morphological, physiological and biochemical parameters of strain BT2 showing high insecticidal activity, have been carried out.

Morphology and Cultural Characteristics: Cells of the isolated strain BT2 are rods measuring 0.8 to 1.0 x 2.0 to 6.0 micron, with rounded ends. They are gram-positive with active mobility. Spores are oval, oblong, measuring 0.8 to 1.0 x 1.0 to 1.5 micron; the location is central. However, the developing parasporal crystal inclusions push a spore to one of the poles of the cell. In the process of sporogenesis, sporangium does not become inflated.

In the process of sporogenesis, large crystalline inclusions are formed in the cell of the strains studied, which strongly refract light. The crystals are intensely colored by basic dyes; the shape of the crystals is rhombic (Fig. 1).

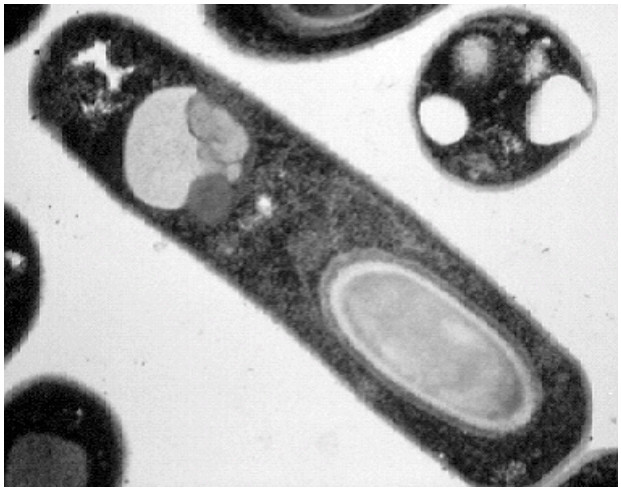

Fig. 1 Spore-forming cells during the formation of the crystal of *Bacillus thuringiensis* spp. *israelensis*

On meat-peptone agar (MPA) and compositionally similar agarized media, the studied strain forms large, smooth colonies of whitey-gray color. The colonies do not grow into the medium. They grow on the surface in an agar column, when inoculating stab. Gelatin deliquates rather intensively.

When growing on meat-peptone broth (MPB), a strong turbidity and dense sediment were observed.

Physiology: Isolated strain BT2 has active proteolytic, amylolytic and hemolytic properties; it characterized also by formation of phospholipase C.

With the formation of organic acids without gas the isolated strain assimilates: glucose, fructose, maltose, cellobiose, and glycerol. It does not assimilate: arabinose, xylose, galactose, lactose, mannitol, dulcitol, and inulin (Table II).

TABLE II
ASSIMILATION OF SUGARS, ALCOHOLS, ORGANIC ACIDS BY STRAIN BT2

Assimilation			
Sugars		Alcohols, organic acids, amino acids	
Glucose	+	Mannitol	-
Fructose	+	Sorbitol	-
Sucrose	+	Dulcitol	-
Maltose	+	Citrate	+
Cellobiose	+	Glycerol	+
Trehalose	+	Asparagine	+
Lactose	-	Glutamine	+
Arabinose	-	Leucine	+
Xylose	-	Isoleucine	+
Galactose	-	Serine	+
Rhamnose	-	Valine	+
Inulin	-	Arginine	+
Sorbose	-	Ornithine	+
Dextrin	+	Lysine	+
Salicin	+		

The strain does not have invertase activity. Denitrification and reaction of BT2 are positive; the strain does not form indole. Reactions of AMC (acetylmethylcarbinol), DHA (dihydroxyacetone), lecithin-vitelline are positive. Serotype determination was performed with the help of flagellar antigen. Serotyping by H-antigen showed that this strain belongs to serotype H14.

The biochemical properties of the remaining strains correspond in main to those of strain BT2. All isolated strains that have insecticidal activity against mosquito larvae were identified as *Bacillus thuingiensis* spp. *israelensis*.

IV. Conclusion

Five original strains of entomopathogenic bacteria *Bacillus thuingiensis* spp. *israelensis* were isolated from natural conditions and characterized. Three of the strains are highly effective against of mosquito larvae and can be recommended for industrial production of bacterial preparations.

Acknowledgment

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