South Asian Journal of Parasitology

2(3): 1-7, 2019; Article no.SAJP.48776

Biocontrol Potential of *Bacillus thuringiensis* Isolated from Soil against Mosquito Larva

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Authors' contributions

This work was carried out in collaboration among all authors. Author MLRS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author MKN managed the analyses of the study. Author EEB managed the literature searches. All authors read and approved the final manuscript.

Article Information

Editor(s):
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Complete Peer review History: http://www.sdiarticle3.com/review-history/48776

Original Research Article

Received 26 February 2019
Accepted 09 May 2019
Published 30 May 2019

ABSTRACT

A major challenge for achieving successful mosquito control is overcoming insecticide resistance. This study was carried out to assess the larvicidal activity of *Bacillus thuringiensis* isolated from different soil samples within Sokoto metropolis using standard methods. Confirmatory identification of the organism was made based on biochemical characterization and microscopic observation. The larvicidal activity of *Bacillus thuringiensis* isolated were tested against the larva of mosquito using three dilutions of the Bacillus culture in a bioassay. The isolated organisms were confirmed as *Bacillus thuringiensis*. The result of the bioassay showed variation in the level of efficacy of the bacteria and depended on the time of the exposure. Mortality rate greater than 20% was observed after 60 minutes and increased to 100% after time of exposure was increased for all dilutions of *B. thuringiensis* used. The results showed that *Bacillus thuringiensis* toxins can be bacteriocidal to mosquito larvae in a matter of minutes depending on the concentration ingested by the larvae. This, in essence, proved that *Bacillus thuringiensis* is an effective bio-larvicide that can be used to reduce and possibly eradicate the nuisance of disease-causing mosquitoes and aid in the rollback of malaria.

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Keywords: Mosquito control; Bacillus thuringiensis; bioassay; larvicidal activity; mosquito larva.

1. INTRODUCTION

Mosquitoes are associated with the transmission of pathogens to humans and other vertebrates. Some of these include the causative agents of malaria, filariasis and dengue, as well as other mosquitoe-borne zoonotic arbovirus, like Saint Louis Encephalitis Virus (SLEV), West Nile Virus (WNV) and Eastern Equine Encephalitis Virus (EEEV) [1]. Significant morbidity and mortality are recorded as results of these diseases are as a result of the inherent difficulty of controlling mosquitoes [2,3]. Increase in the distribution of mosquitoes was associated with the emergence of viruses and diseases in new areas [4]. WHO [3] reported malaria to be the world most important vector-borne disease. Cases of these diseases have been reported in more than 100 countries, with approximately more than 3 billion people living in endemic areas. More than 200 million cases of malaria and eight hundred thousand (800,000) malaria-related deaths are recorded every year [5]. The increase in a number of malaria cases is as a result of deteriorating health systems, increase the resistance of anopheline to insecticides, time taken to develop an effective vaccine and as well resistance of plasmodium to antimalarial drugs [1].

Dengue, including dengue hemorrhagic fever and Dengue Shock Syndrome (DSS) transmitted by *Aedes* mosquitoes is rapidly becoming a worldwide disease, threatening a third of the world population, with an estimate of 50-100 million cases every year [6,7]. So also, *Lymphatic filariasis* caused by *Wuchereria bancrofti*, which is transmitted by mosquitoes, affects more than 120 million people around the world [8]. Lack of effective vaccine against these diseases has left the control of the mosquito population as the most effective way to prevent vector-borne diseases [9,10].

Chemical insecticides have been used in the last century to successfully control mosquitoes of the genus *Aedes* and *Anopheles*. Current ecological and environmental protection standards halt the use of these chemicals, because of their adverse effects on non-target species, including humans, environmental impact, contamination soil and water and development of mosquito resistance to insecticides [11]. New strategies were created to replace the use of chemical insecticides. They include Integrated Pest Management (IPM) that has guidelines. Guidelines of which are based on environmental planning, public awareness and biological control that control the mosquitoes more efficiently while preserving the environment from contamination [1]. Commercial preparations of *Bacillus thuringiensis (Bt)* as a biocontrol agent has been the greatest success in microbial pesticides, with more than 95% of the microbial pesticides sold being of this bacterial agent [12]. *Bacillus thuringiensis* (*Bt*) is a gram-positive, rod-shaped and spore-forming bacteria that is mostly found in the soil and produces polymorphic crystal proteins [13]. The insecticidal activity of *Bt* is due to the proteic parasporal inclusions that are produced during sporulation [14]. The insecticidal proteinaceous crystals (ICPs) comprised one or more crystal (Cry) and Cytolytic (Cyt) proteins recognized as δ-endotoxin. When ingested by the target insect, the ICPs dissolve in the midgut of the larva releasing proto-toxins that eventually lead to the formation of pores that causes cell-cytolysis [15,16].

Despite the use of *Bacillus thuringiensis* as a biocontrol agent for over 30 years, no significant resistance was recorded. However, the search for natural *Bt* isolates with increased activity against mosquito and other insect is still encouraged. Recently, *Bt* with increased activity against *Aedescaspius* and *Culexpipiens* were isolated [17]. In this study, the larvicidal activity of *Bt* isolated from soil samples will be evaluated on mosquitoes.

2. MATERIALS AND METHODS

2.1 Sample Collection

A total of 4 soil samples were collected from Tamaje, Mabera, Arkilla and Kantin Daji areas of Sokoto state, where there is no previous record of application of *Bacillus thuringiensis* based insecticides. The soil samples were collected aseptically from top of 5 cm depth. The samples were placed immediately in plastic bags and labelled appropriately [18]. The soil samples were transported to the laboratory and stored at room temperature. In the same vein, mosquito larva was collected from stagnant waters around Sokoto metropolis.

2.2 Media Preparation

The media to be used to culture the bacteria such as Nutrient agar and Luria-Bertani will be prepared according to the manufacturer's instruction.

2.3 Nutrient Agar

Twenty-eight grams (28 g) of nutrient agar was weighed and dissolved in 1000 ml of distilled water in a sterile conical flask. The mixture was heated using a hot plate to dissolve the medium. The conical flask was plugged with cotton wool stoppers and wrapped with aluminium foil. It was then sterilized using an autoclave at 121°C for 15 minutes. The medium was cooled at about 45 - 50°C after sterilization and then poured into sterile petri dishes (about 20 ml per plate) under aseptic conditions. The plates were allowed to solidify and incubated at 37°C for 24 hours and the sterility of the medium was checked [19].

2.4 Luria-Bertani Agar

This media is made up of Tryptone, Yeast extract, NaCl, NaOH and Bacto agar for jelling. Ten grams (10 g) of Tryptone, ten grams (10 g) of NaCl, five grams (5 g) of NaOH and ten grams (10 g) of yeast extract were weighed and dissolved in 950 ml of distilled water in a sterile conical flask. The mixture was heated using a hot plate to dissolve. The final volume was added up to 1000 ml and fifteen grams (15 g) of Bacto agar was added. The conical flask was plugged with cotton wool stoppers and wrapped with aluminium foil. It was then sterilized using an autoclave at 121°C for 15 minutes. The medium was cooled at about 45 – 50°C after sterilization and then poured into sterile petri dishes (about 20 ml per plate) under aseptic conditions. The plates were allowed to solidify and incubated at 37°C for 24 hours and the sterility of the medium was checked [20].

2.5 Isolation of *Bacillus thuringiensis* from Soil

Five grams (5 g) of each soil sample was weighed and added to 100 ml of distilled water. The samples were heated on a hot plate for 10 minutes to eliminate all bacteria incapable of producing endospores. Since it is known that *Bacillus thuringiensis* produces spores, it will be safe to assume that if it was present in the soil, it would be in our heated sample. The samples were then diluted 5 fold to eliminate all humic materials within the samples and to reduce the overall colony forming units within each sample [18].

2.6 Culturing of *Bacillus thuringiensis*

The diluted samples were cultured on nutrient agar plates for 24 hours at 37°C in to order to give the spores chance to germinate on media with adequate nutrients and optimal temperature [18]. The media, however, offers favourable growth for a wide range of bacteria as well as *Bacillus thuringiensis*. The colonies were sub-cultured onto Luria-Bertani plates and incubated at 37°C for 24 hours, so as to obtain pure cultures of *B. thuringiensis*. Series of tests which include gram staining and biochemical tests were further employed to identify *Bacillus thuringiensis* after the formation of colonies with smooth round shape and earthy odour.

2.7 Gram Staining Techniques

A smear of colonies isolated after the identification was made on clean glass slides using a sterile wire loop. They were air dried and fixed. The smears were flooded with crystal violet for about 60 seconds and were washed with tap water. They were then tipped off with Lugol's iodine for 30 seconds and then washed with tap water. They were decolourized with acetone and washed off with tap water. The fixed smears were counterstained with safranin and allowed for 60 seconds and then washed off with tap water and allow to air dry. Oil immersion was added to the stained slides and viewed under a microscope using x100 objective for the morphological characteristics of the isolates [21].

2.8 Characterization of the Isolated Bacteria

The colonies that form on the T3 agar will again be confirmed by biochemical tests based on Indole test, Catalase test, Triple Sugar Iron test (T.S.I), Methyl Red test (M.R.), Vogues-Proskauer (V.P.).

2.9 Indole Test

A test tube of sterile peptone water, enriched with 1% tryptophan will be inoculated with a young culture of isolates and incubated at 37^{O}C for 48 hrs. About 4 drops of kovac's reagent will be added and shaken gently. Red colour will occur immediately at the upper part of the test tube indicating a positive test. A yellow colour at the surface will denote a negative result [21].

2.10 Catalase Test

The container containing 3% hydrogen peroxide solution will be shaken to expel the dissolved oxygen. One drop of the solution will be placed on a clean glass slide. Presence of gas bubbles

indicates a positive test while the absence of gas bubbles indicates a negative reaction [19].

2.11 Triple Sugar Iron Agar (T.S.I test)

A speck of the isolate will be inoculated by streaking and stabbing into the medium and will be incubated at 37°C for 24 hours. Fermentation of any of the sugar will be indicated by a change in colour, from red to yellow and crack or raised in the medium indicates gas production [21].

2.12 Methyl Red Test (M.R.)

A speck of the isolate will be inoculated into the medium, which would be incubated at 37°C for 48 hours. Few drops of methyl red would be added to the culture. M.R positive test will indicate a red colour while no changes denote negative [21].

2.13 Vogues-Proskauer Test (V.P)

A speck of the isolate will be inoculated into glucose phosphate water medium and incubated at 37°C for 2 days. Ethanoic solution of 5% α-naphthol (1.2 ml) and 0.4 ml potassium hydroxide solution will be added to 2 ml of culture and will be shaken vigorously. It will be placed in a sloping position (for maximum exposure of the culture to air) and will be examined after 30 to 60 minutes. The evolution of red colour indicates a positive test for Voges-Proskauer [21].

2.14 Coagulase Test

About 2 or 3 colonies were emulsified in 0.05 ml of saline contained in a serological tube. 1 ml of plasma was added and incubated at 35°-37°C and it was checked after 1 hour, 2 hours, 3 hours and 4 hours of incubation for signs of clotting of the plasma. Increase in viscosity or complete clotting indicates a positive coagulase test, while the absence of viscosity or clotting indicates a negative coagulase test [21].

2.15 Motility Test

A small quantity of each isolate was stabbed into triple sugar iron agar and incubated at 37°C for 24 hours. Motility was observed by the spread of the organism outwards from the stabbed area.

2.16 Urease Test

A speck of each isolate was inoculated into Christensen's urea agar and incubated at 37°C for 24 hours. The liberation of red colour indicates urease positive test while the initial yellow colour indicates a negative test.

2.17 Citrate Utilization Test

A speck of each isolate was inoculated into Koser's citrate medium and was incubated at 37°C for 72 hours. Positive citrate is confirmed by the promotion of blue colour while the initial green colour denotes a negative result [21].

2.18 Bioassay

The *Bacillus thuringiensis* isolates selected were tested against larva of mosquito. The stock cultures of *Bacillus thuringiensis* from slant bottles were picked using a sterile wire loop and diluted five-fold 10^{-1}- 10^{-5} in sterile distilled water in five test tubes. Five (5) ml each of the cultures in the first, third and fifth test tubes was added to three (3) disposable cups containing 45 ml of sterile distilled water, providing each cup with different dilution factors. Twenty-five (25) larvae were transferred into each of the disposable cups. The cups were kept at 25°C – 30°C for 6 hours. At intervals of 30 minutes, each cup was observed for the larval presence and larval mortality rate was calculated.

3. RESULTS

The microscopic and biochemical characteristics of the isolated organisms are shown in Table 1. The characteristics of which confirmed the isolated organism to be *Bacillus thuringiensis*. Table 2 shows the result for the bioassay of 10^{-1} diluents of *Bacillus thuringiensis* against mosquito larvae. The mortality rate was found to increase as the incubation time increases. A mortality rate of 52% was recorded after 150 minutes and a 100% mortality rate was recorded after 330 minutes. The result illustrated in Table 3 shows the bioassay for the 10^{-3} diluents of *B. thuringiensis* on mosquito larvae. The mortality rate of 52% was recorded after 180 minutes, after which a 100% mortality rate recorded after 360 minutes. Illustrated in Table 4 is the bioassay of the 10^{-5} diluents of *B. thuringiensis* against mosquito larvae. The mortality rate of 60% was recorded after 210 minutes, after which a mortality rate of 100% was recorded after 360 minutes.

4. DISCUSSION

Mosquitoes are a great nuisance and they pose a serious threat to human health in society. Many chemical insecticides have been produced for the control of mosquitoes in the past years, some of which have been very effective while others have done little or no good at all. Most of the insecticides used are made of synthetic

Table 1. Biochemical and morphological characteristics of *Bacillus thuringiensis*

Isolates	Gram reaction	Catalase	Coagulase	Glucose	Sucrose	Lactose	Gas	Motility	Citrate	MR	VP	Urease	Indole
A	+ve Rod	+	-	-	-	-	-	+	+	+	-	+	+
B	+ve Rod	+	+	-	-	-	-	+	+	+	-	+	+
C	+ve Rod	+	-	-	-	-	-	+	+	+	-	+	+
D	+ve Rod	+	+	-	-	-	-	+	+	+	-	+	+

Key: + = Positive - = Negative

Table 2. Bioassay of 10^{-1} diluents of *Bacillus thuringiensis* culture against mosquito larvae

Time (min)	0	30	60	90	120	150	180	210	240	270	300	330	360
No. of larva	25	24	20	17	14	12	10	7	4	2	1	0	0
Control	25	25	25	25	25	25	25	25	25	25	25	25	25
Mortality	0	1	5	8	11	13	15	18	21	23	24	25	25
Mortality rate (%)	0	4	20	32	44	52	60	72	84	92	96	100	100

Table 3. Bioassay of 10^{-3} diluents of *Bacillus thuringiensis* culture against mosquito larvae

Time (min)	0	30	60	90	120	150	180	210	240	270	300	330	360
No. of larva	25	24	23	21	19	16	12	9	7	6	3	1	0
Control	25	25	25	25	25	25	25	25	25	25	25	25	25
Mortality	0	1	2	4	6	9	13	16	18	19	22	24	25
Mortality rate (%)	0	4	8	14	24	36	52	64	72	76	88	96	100

Table 4. Bioassay of 10^{-5} diluents of *Bacillus thuringiensis* culture against mosquito larvae

Time (min)	0	30	60	90	120	150	180	210	240	270	300	330	360
No. of larva	25	25	24	23	23	21	18	10	6	3	1	0	0
Control	25	25	25	25	25	25	25	25	25	25	25	25	25
Mortality	0	0	1	2	2	4	7	15	19	22	24	25	25
Mortality rate (%)	0	0	4	8	8	16	28	60	76	88	96	100	100

chemicals and were found to have a negative effect on the diversity of many insects well being of humans and as well the environment. These, therefore, call the need for the search of biological control methods that cause less harm to human health, diversity of the insects and the environment. The efficacy of *Bacillus thuringiensis* as a larvicide for controlling mosquito larva yielded great results. The microscopic and the biochemical characteristics of the organisms as shown in Table 1 confirmed the basic characteristics of *Bacillus thuringiensis*, being Gram-positive and having a rod shape. The biochemical characteristics showed the motile nature of the organism among others. These characteristics are similar to what was reported by Ahmed et al. [22].

In the bioassay for the control of mosquito larvae, different diluents used showed varying degree of effectiveness with 100% mortality rate recorded after 360 minutes. This might be attributed to the ability of the organism to produce a binary toxin (Bin) which is a primary insecticidal component produced during sporulation and vegetative stage of *B. thuringiensis* in controlling the growth of the mosquito larvae. This is in agreement with what was reported by Oei et al. [23]. In all the diluents, very low mortality was recorded after 30 minutes of incubation, which could be attributed to the time of exposure of the larvae as well as the number of the organisms present in the container. But more than half of the larvae were death after 240 minutes, with a high mortality rate of over 70% recorded in all the diluents of the *B. thuringiensis*. This could also be attributed to the time of exposure of the larvae and as well the increase in number of the cells in the medium that could be attributed to the increase in the number of organisms ingested by the mosquito larvae, which causes damage in the midgut of the larvae [24]. There by releasing the crystallized binary toxins, that in turns are solubilized in the midgut, releasing two proteins [25], that are cleaved by endogenous proteins to form active toxins [26]. This is in agreement with what was reported by Aissaoui and Boudjelida [27].

5. CONCLUSION

Bacillus thuringiensis naturally found in the soil has proved to be a good larvicidal agent against mosquito larvae in the laboratory. The organism and it product can be further studied to search for novel compounds that can be used in the control of mosquito-borne diseases such as malaria.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle3.com/review-history/48776