Original Research Article

BIOCONTROL POTENTIAL OF *Bacillus thuringiensis* AGAINST MOSQUITO LARVA FROM ISOLATED SOIL

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.

Keywords: Mosquito control, *Bacillus thuringiensis*, bioassay, larvicidal activity, mosquito larva

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 *Aedes caspius* and *Culex pipiens* were isolated [17]. In this study, the larvicidal activity of *Bt* isolated from soil samples will be evaluated on mosquitoes.

MATERIALS AND METHODS

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 5cm 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.

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.

Nutrient Agar

Twenty-eight grams (28g) of nutrient agar was weighed and dissolved in 1000ml 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^{o}C for 15 minutes. The medium was cooled at about 45 - 50^{O}C after sterilization and then poured into sterile petri dishes (about 20ml per plate) under aseptic conditions. The plates were allowed to solidify and incubated at 37^{O}C for 24 hours and the sterility of the medium was checked [19].

3.2.2 Luria-Bertani Agar

This media is made up of Tryptone, Yeast extract, NaCl, NaOH and Bacto agar for jelling. Ten grams (10g) of Tryptone, ten grams (10g) of NaCl, five grams (5g) of NaOH and ten grams (10g) of yeast extract were weighed and dissolved in 950ml 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 1000ml and fifteen grams (15g) 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^{o}C for 15 minutes. The medium was cooled at about 45 – 50^{o}C after sterilization and then poured into sterile petri dishes (about 20ml per plate) under aseptic conditions. The plates were allowed to solidify and incubated at 37^{o}C for 24 hours and the sterility of the medium was checked [20].

Isolation of *Bacillus thuringiensis* from Soil

Five grams (5g) of each soil sample was weighed and added to 100ml 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].

Culturing of *Bacillus thuringiensis*

The diluted samples were cultured on nutrient agar plates for 24 hours at 37^{o}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^{o}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.

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].

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.).

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 48hrs. 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].

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].

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^{O}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].

Methyl Red Test (M.R.)

A speck of the isolate will be inoculated into the medium, which would be incubated at 37^{O}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].

Vogues-Proskauer Test (V.P)

A speck of the isolate will be inoculated into glucose phosphate water medium and incubated at 37^{O}C for 2 days. Ethanoic solution of 5% α-naphthol (1.2 ml) and 0.4 ml potassium hydroxide solution will be added to 2ml 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].

Coagulase Test

About 2 or 3 colonies were emulsified in 0.05ml of saline contained in a serological tube. 1ml of plasma was added and incubated at 35^{0}-37^{0}C and it was checked after 1hour, 2hours, 3hours and 4hours 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].

Motility Test

A small quantity of each isolate was stabbed into triple sugar iron agar and incubated at 37^{0}C for 24 hours. Motility was observed by the spread of the organism outwards from the stabbed area.

Urease Test

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

Citrate Utilization Test

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

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 45ml 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^{o}C – 30^{0}C for 6 hours. At intervals of 30 minutes, each cup was observed for the larval presence and larval mortality rate was calculated.

RESULTS

The microscopic and biochemical characteristics of the isolated organisms are shown in table 4.1. The characteristics of which confirmed the isolated organism to be *Bacillus thuringiensis*. Table 4.2 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 4.3 shows the bioassay for the 10^{-3} diluents of *B.*

185 *thuringiensis* on mosquito larvae. The mortality rate of 52% was recorded after 180 minutes,
186 after which a 100% mortality rate recorded after 360 minutes. Illustrated in table 4.4 is the
187 bioassay of the 10^{-5} diluents of *B. thuringiensis* against mosquito larvae. The mortality rate of
188 60% was recorded after 210 minutes, after which a mortality rate of 100% was recorded after
189 360 minutes.
190
191
192

193 **Table 4.1: 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	+	+	-	-	-	-	+	+	+	-	+	+

194

195 **Key:** + = Positive

196 - = Negative

197

198

199 **Table 4.2: 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

200

201

202

203 **Table 4.3: 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

204

Table 4.4: 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

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 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 4.1 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]. Thereby 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].

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.

References

1. Wilke ABB Marelli MT. Paratransgenesis: A Promising New Strategy for Mosquitoe Vector Control. *Parasites and Vectors*. 2015; 8:342. DOI 10.1186/s 13071-015-0959-2.

2. World Health Organization (WHO). Progress Report 2000-2009 and Strategic Plan 2010-2020 of the Global Program to Eliminate Lymphatic filariasis: Halfway Towards Eliminating Lymphatic Filariasis. 2010; ISBN 978-92-4-150072-2.

3. World Health Organization (WHO). WHO Global Malaria Programme. New Report Signals Showdown in The Fight Against Malaria, 2012. Geneva.

4. Fonseca DM Smith JL Wilkerson RC Fleischer RC. Pathways of Expansion and Multiple Introduction Illustrated by large Genetic Differentiation Among World Wide Populations of the Southern House Mosquitoe. *American Journal of Tropical Medicine and Hygiene.* 2006; 74: 284-289.

5. Bremen JG Egan A Keusch GT. The Intolerable Burden of Malaria: A new Look at the Numbers. *American Journal of Tropical Medicine and Hygiene*. 2001; 64: iv-vii.

6. Reiter P. Oviposition, Dispersal and Survival in *Aedes aegypti*: Implications for the Efficacy of Control Strategies. *Vector-Borne Zonotic*. 2007; 7: 261-274.

7. Mairiang D Zhang H Sodija A Murali T Suriyaphol P Malasit P *et al*. Identification of New Protein Interactions Between Dengue Fever Virus and its Hosts, Human and Mosquitoes. Plos One. 2013; 8, ee53535.

8. Pidiyar VJ Jangid K Patole MS Shouche YS. Studies on Cultured and Uncultured Microbiota of *Culex quinquefasciatus* Mosquito Midgut Based on 16s ribosomal RNA gene Analysis. *American Journal of Tropical Medicine and Hygiene*. 2004; 70: 597-603.

9. Pates H Curtis CF. Mosquito Behavior and Vector Control. Annual Review of Entemology. 2005; 50: 53-70.

10. Vreysen M Robinson AS Hendrichs J. Area-Wide Control of Insect Pests: From Research t Field Implementation. The Netherlands: Springer. 2007; P. 325-361.

11. Dorta DM Vasuki V Rajavel A. Evaluation of Organophosphorus and Synthetic Pyrethroid Insecticides Against Six Vector Mosquito Species. *Rev Saude Publica*. 1993; 27:391-397.

12. Federici BA Hyun-Woo P Dennis KB. Overview of the Basic Biology of Bacillus thurigiensis with Emphasis on Genetic Engineering of Bacterial Larvicides foe Mosquitoe Control. The Open Toxicology Journal. 2010; 3: 83-100.

13. Cruz D Ian NB Bautista JR Teves FG. Isolation and Identification of *Bacillus thuringiensis* from *Harpaphe haydeniana* and its Entemotoxic Evaluation Against *Aedes* and *Culex* Larvae. *International Research Journal of Biological Sciences*. 2015; 4(3): 21-26.

14. Federici BA. *Bacillus thuringiensis in Biological Control*. In: Handbook of Biological Control. F Fosher (Ed.) Academic Press (Ed.). 1999; 577-593. ISBN 10: 0-12-257305-6.

15. Otieno-Ayayo ZN Zaritsky A Wirth MC Manasherob R Khasdan V Cahan R Ben-Dov E. Variations in the Mosquito Larvicidal Activities of Toxins from *Bacillus thuringiensis* Spp. *Israelensis*. *Environmental Microbiology*. 2008; 10: 2191-2199.

16. Angolo-Falho RC Loguercio LL. *Bacillus thuringiensis* is an Environmental Pathogen and Host-Specificity has Developed as an Adaptation to Human Generated Ecological Niches. *Insects*. 2014; 5: 62-91.

17. El-Kersh TA Al-akeel RA Al-Sheikh YA. Isolation and Distribution of Mosquitoes Cry genes in *Bacillus thuringiensis* Strains native to Saudi Arabia. *Tropical BioMed*. 2014; 31: 616-632.

18. Travers RS Martin PAW Reichelderfer CF. Selective Process for Efficient Isolation of Soil Bacillus sp. *Applied Environmental Microbiology*. 1987; 53: 1263-1266.

19. Cheesbrough M. District Laboratory Practices in Tropical Countries (Part 2). Cambridge University Press. 2002; 137-139.

20. Sambrook J Russell DW. Molecular Cloning a Laboratory Manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY. 2001.

21. Oyeleke SB Manga SB. Essentials of Laboratory Practicals in Microbiology 1st Edition. To Best Publisher Minnaa, Niger State, Nigeria. 2008; 36-58.

22. Ahmed HA Ali SG Abdul-Raouf UM. Isolation, Characterization and Molecular Identification of *Bacillus thuringiensis* Alex-13 isolated from Egypt Against *Spodoptera littoralis*. *International Journal of Microbiology and Allied Sciences*. 2015; 2(2): 34-44.

23. Oei C Hindley J Berry C. Binding of *Bacillus sphaericus* Binary Toxin and its Deletion Derivatives to *Culex Quinquefasciatus* Gut: Elucidation of Functional Binding Domains. *Journal of General Microbiology.* 1992; 138: 1515-1526.

24. Wei D Cai Q Yuan Z. Mosquitocidal Toxin From *Bacillus sphaericus* Induces Stronger Delayed Effects than Binary Toxin on *Culex quenquefasciatus* (Diptera; Culcidae). *Journal of Medical Entemology*. 2006; 43: 726-730.

25. Berry C JacksonTap J Oei C Hindley J. Nucleotide Sequence of Two Toxin Genes from *Bacillus sphaericus* IAB59: Sequence Comparisons Between Five Highly Toxigenic Strains. *Nucleic Acid Research*. 1989: 17(18): 7516.

26. Darboux I Nielsen-Leroux C Charles JF Pauron D. The Receptor of *Bacillus sphaericus* Binary Toxin in *Culex pipens* (*Diptera culcidae*) midgut: Molecular Cloning and Expression. *Insect Biochemical and Molecular Biology*. 2001; 31(10): 981-990.

27. Aissaoui L Boudjelida H. Larvicidal Activity and Influence of *Bacillus thuringiensis* (Vectobac G), on Longevity and Fecundity of Mosquito species. *European Journal of Experimental Biology*. 2014; 4(1): 104-109.