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Isolation, Characterization and Larvicidal Potential of *Bacillus thuringiensis* Toxin against Mosquito Larvae

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Abstract:-Malaria is a significant sub-Saharan African health challenge and control strategies mainly target the adult mosquitoes that transmit the malaria causing parasite. Unlike adults, mosquito larvae are not known to evade control strategies aimed at them. This study determined the larvicidal potential of *Bacillus thuringiensis* toxin (Bt) against mosquito larvae.Soil samples werecollected in triplicates using a hand-held soil auger from six different locations and pooled into six composite samples. Standard cultural method was used to isolate *Bacillus thuringiensis*from thecomposite soil sample in addition to molecular identification. Following identification, sporulation and Bt toxin were carried out using standard methodologies.Mosquito larvae in their 3rd and 4th instarsstages were collected, exposed to differentconcentrations (0.1, 0.01, 0.001, 0.0001 and 0 mg/mL) ofBt and monitored for 9 hours. The results of the first hour of exposure to Bt, showed a concentration dependent mortality of 30, 20, 10, 10 and 0 %, respectively across all concentrations (0.1, 0.01, 0.001, 0.0001 and 0 mg/mL) and 30% mortality for the positive control. However, 100% mortalities rates were recorded from the 5th hour for both the 0.1 mg/mL Bt concentration and the positive control.The results are of particular interest because Bt has shown similar larvicidal effect as the commercial based insecticide that is of a chemical origin. The excellent toxic effective activity shown by *Bacillus thuringiensis* shows a promising potential and should be exploited further towards our drive for malaria eradication.

Keywords:-*Bacillusthuringiensis, Malaria, Mosquito Larvae, BtToxin.*

I. INTRODUCTION

Historical evidence indicates that malaria is an ancient infectionthat occupies a unique place in human history (Panosian *et al.*, 2004). However, *Plasmodium* parasite the causative agentwas first discovered in 1880-1802 by Laveran and a decade and half years later, its life cycle and transmission by Anopheles mosquito vectorwas described (Gachelin *et al.*, 2018). Sub-Saharan African countries continue to bear the brunt of the infection (WHO, 2018; Khanam, 2017). In 2017 alone, there 219 million cases and 435,000 deaths reportedin 87 countries (WHO, 2018). Africa continues to bear the highest brunt of the scourge of malaria as it accounted for 92 % of cases and 93% of deaths despite huge fundsmade available (WHO, 2018).

Current vector control methods such as the use of insecticide-treated nets (ITNs) and chemical insecticides indoor residual sprays (IRS) targeted at the adult mosquitoes have shown some successes (Manu et al., 2015; Wilson et al., 2014; Bhatt et al., 2015). However, low utilization (Manu *et al.*,2017), abuse of the ITNs and increasing resistance is fast reversing the gains (Zogo *et al.*, 2019; Riveron *et al.*, 2018; WHO, 2018). Unlike adults, mosquito larvae are not known to evade control strategies aimed at them (Killeen et al., 2002). Furthermore, the indiscriminate use of chemicals is known to damage the environment and build up resistance (Hawkins *et al.*, 2019).

A potential and viable alternative or complimentary option to ITN and IRS used in the control of mosquitoes remains biological control (Aramideh *et al.*, 2010). Bt a biological agent is a viable alternative in vector and pest management (Aramideh *et al.*, 2010). Itis a Gram positive bacterium that on sporulation produces delta- endotoxin that becomes solubilized in insect guts under alkaline conditions and eventually leads to death (Eswarapriya *et al.*, 2010). Bioinsecticide based on Btdominates the bio-pesticidesglobal market for a number of reasons including biodegradability, specificity, safety, and wide spectrum of activity (El-Kerch et al., 2011; Bagari et al., 2013).

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Despite bearing the highest brunt of malaria deaths and health related issues, and WHO recommendation that larviciding be used side by side with ITNs and IRS, the use and successes recorded of larvicides remain elusive in the same region (Fillinger and Lindsay, 2011). Despite accounting for the bulk of the brunt of malaria, Nigeria is yet to join the list of the few African countries currently exploring the potentials of Bt. Thus, the study was aimed at determining the potential of isolated *B*t on mosquito larva. The specific objectives of the study included isolation and molecular characterization of B. *thuringiensis*, and comparison of their larvicidal potential with those of the well-known permethrin insecticide powder.

II. MATERIAL USED IN THE STUDY

- *Sample Collection*

Soil samples were collected at a depth of 100mm using hand held soil auger from six locations inObong University community in triplicates and then pooled into 6 composite samplestransferred into sterile polyethylene bags and then transported immediately to the laboratory for further analyses (Asitok et al., 2017).

- *Isolation of Bacillus thuringiensis*

Bt was isolated using the heat treatment method previously described (Edwards and Soares, 1988). This done using 0.25g of soil which were dissolved in test tubes containing nutrient broth and supplemented with sodium acetate and incubated overnight in a water bath. After which the samples were subjected to heat treatment and then standard microbiological techniques for isolation and characterization.

- *DNA Extraction, Amplification and Identification of Isolates*

DNA extraction and purification was done using ZR Fungal/Bacterial DNA MiniPrep™50 Preps Model D6005 (Zymo Research, California, USA) by strictly following the manufacturer's instructions as previously described (Edet et al., 2019). The DNA was then sequenced using Sanger sequencing techniques and the resulting sequence used to identify the isolate.

- *Sporulation of Bacillus thuringiensis*

For the sporulation of the*Bacillus thuringiensis*, the overnight broth was sub-cultured onto 2XSG broth medium (Composition Difco nutrient broth 16g, KCl 2g, MgSO4.7H2O 0.5g were dissolved in 1L of distilled water. The pH was adjusted to 7.0 with the addition of 1M NaOH and then autoclaved (121^{o}C at 15 psi). The broth was then cooled to 55^{o}C and 1M Ca $(NO_3)_2$ 1ml, 0.1M $MnCl_2$.H2O, 1mM FeSO4 1ml and glucose 50% (w/v) filter sterilized 2ml added to it..

- *Isolation of BtCrystal Protein*

This was done as previously described (Bagari et al., 2013). Briefly, theBt culture broth was centrifuged at low speed (1000 x g for 1 minute). After centrifugation, the pellet at the bottom was discarded while the supernatant was collected into another tube. The supernatant was then centrifuged again at low speed (1000 x g for 60 seconds) and the resulting residue discarded. The supernatant was againcentrifuged at 5000 x g for 60 minutes. After the third centrifugation, the pellets were retained and the supernatant discarded. The cells in the pellet were disrupted via repeated freezing and drying as previously described (Johnson and Hecht, 1994).

- *Collection of Mosquito Larvae*

Mosquito larvae were obtained from the university campus hotels. A total of 7 stainless bowls were filled with distilled water and allowed to stand in the open but away from rain for 2 weeks prior to the start of the bench work. From each of the basin, active mosquito larvae were collected and transferred into labelled sterile universal bottles containing distilled water and then transferred immediately to the laboratory for Btbioassay. The collected mosquito larvae were in their 3rd and 4th instar larval stages.

- *Preparation of BtAssay*

Following molecular characterization, a simple Btbioassay was carried out. The resulting Bt toxin was then used to prepare various concentrations (0.1, 0.01, 0.001 and 0.0001mg/L). To each of these concentrations, a total of 10 active mosquito larvae were introduced. Positive and negative controls were also setup using distilled water and an Insecticide based on permethrin with a concentration (0.1g/ml), respectively. The setups were monitored for mortality of the larvae after every 1hour for 9 hours.

III. RESULTS

Results are as presented in Table 1. From the result, across all the concentrations, a concentration dependent mortality was observed. After an hour, mortality rates of 30, 20, 10, 10 and 0 % respectively for concentrations of 0.1, 0.01, 0.001, 0.0001 and 0mg/mL, The Bt toxin were prepared by dissolving the appropriate concentrations (mg) in 10ml of sterile distilled water. Similar hourly mortality rates were observed for the highest concentration of Bt used in this study and the positive control. Mortalities of 30, 60, 100, 100 and 100% were observed for both 0.1mg/ml Bt and 0.1mg/ml permethrin based insecticide after 1, 3, 5, 7 and 9th hours, respectively. The lowest concentration of 0.0001 mg/ml did not give 100% mortality but 70% after 9 hours of exposure.

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Concentrations (mg/mL)	Time (hours) 1	3	5	7	9
*BT*0.1	3 (30%)	6(60%)	10 (100%)	10 (100%)	10 (100%)
BT 0.01	2 (20%)	3(30%)	6 (60%)	8 (80%)	10 (100%)
*BT*0.001	1 (10%)	2 (20%)	4 (40%)	6 (60%)	10 (100%)
*BT*0.0001	1 (10%)	2 (20%)	3 (30%)	4(40%)	7 (70%)
Distilled water	0 (1000%)	0(100%)	0 (100%)	0 (100%)	0 (100%)
Insecticide based on permethrin	3 (30%)	6 (60%)	10 (100%)	10 (100%)	10 (100%)

Table 1:- Mortality of Mosquito Larvae with *Bacillus thuringiensis*

IV. DISCUSSION

The two major ways malaria can be controlled are prompt treatment and vector control. Conventional or orthodox antimalarial drugs are usually based on artemisinin and sadly, there have been reported cases of resistance to both drugs (White, 2010).In the area of control, currently, two major strategies for vector control targetting the adult mosquitoes and have shown some success are ITNs and IRSwhich target adult mosquitoes. (Manu et al., 2015; Wilson et al., 2014; Bhatt et al., 2015) but there are some worrying challenges such as abuse of the ITN and increasing resistance (Manu et al., 2017; Zogo *et al.*, 2019; Riveron *et al.*, 2018; WHO, 2018).

The best target for the control of malaria remains the larvae which have not been shown thus far to develop any form of resistance to chemical and biological based control agents (Killeen et al., 2002). Furthermore, indiscriminate use of chemical insecticides has some adverse effects (Hawkins et al., 2019). A potential and viable alternative or complimentary option to ITN and IRS used in the control of mosquitoes remains biological control (Aramideh *et al.*, 2010).

Earlier reports have shown that Bt is pathogenic to mosquitoes, blackflies, other Dipterans and the midges and this confirms the larvacidal activity recorded in our study. Furthermore, the study also reported that they usually do not have any cidal effect on human, plants and other non-target organisms. Elsewhere, *Bacillus thuringiensis* is harmless to birds, fish, and shrimp and does not cause disease in animals (Land and Milijand, 2014).The results from this study revealed that Bt gave a concentration dependent mortality. As the concentrations of the Bt reduced, the mortality also reduced as evident in the 70% at the least concentration of 0.0001mg/mL of Bt.

Across several African countries field trials of commercial products of*Bacillus thuringiensis* var. *israelensis*and *Bacillus sphaericus*have been reported. In an earlier study, Romi et al (1993)obtained larval reduction of 89 to 100 % and 67-100 % against *Anopheles arabiensis* and with residual effect of 2 days in Mahitsy, Madagascar. In Mbita, Kenya Larvicidal reduction of 37-100% and 99 % was reported against *A. gambiae* and *A. funestus*, respectively with residual effects that ranged from 2 to 23 days (Fillinger and Lindsay, 2006). In another Kenya study, 89-99 and 77-100 % Larvicidal reduction was reported with 8 residual effect against *A. gambiae* (Kahindi et al., 2008). However, in the flood plains of River Gambia, 100 % *Anopheles* species Larvicidal reduction have been reported with residual effect of 7 days (Majambere et al., 2007). Bobirwa, Botswana and Buhera, Zimbabwe, 47-95 % reduction have been reported against Anopheles species with residual effect of 14 days (79). These findings especially for those of *Anopheles* species are within range of our findings.

Semi-field trials using commercial products of *Bacillus thuringiensis* var. *israelensis*and *Bacillus sphaericus*against Anophles and Culicinae gave larvicidal reductions of 51 -100 % with application 0-2 to 0.4 mg/L in Kumasi, Ghana has been reported (Nartey et al., 2013). In another study, 70 to 100 % malaria vector reduction was reported at an application rate of 0.5 to 1.0 mg/l (Baffour-awuah et al., 2014). Compared to our findings, the results were similar even though our concentrations were much lower.

V. CONCLUSION

The findings in this study are of particular interest because given the fact it mortality rate was comparative to the premethrin based insecticide that is a toxic xenobiotic in its cidal action. The excellent toxic effective activity shown by *Bacillus thuringiensis* shows a promising potential of eliminating the nuisance caused by insects.

REFERENCES

[1]. Baffour-awuah S, Owusu-Dabo E, Kruppa T, Annan A, Nartey R, Dogbe J, et al. *Lysinibacillussphaericus*biolarvicide, an efficacious tool in the control of *Anopheles gambiae*in Kumasi, Ghana. East Afr J Public Health. 2014;11:851–61.

[2]. Nartey R, Owusu-Dabo E, Kruppa T, Baffour-Awuah S, Annan A, Oppong S, et al. Use of *Bacillus thuringiensis* var. *israelensis*as a viable option in an integrated malaria vector control programme in the

Kumasi Metropolis, Ghana. Parasit Vectors. 2013;6:116.
[3]. Majambere S, Lindsay SW, Green C, Kandeh B, Fillinger U. Microbial larvicidesfor malaria control in The Gambia. Malar J. 2007;6:76.
[4]. Romi R, Ravoniharimelina B, Ramiakajato M, Majori G. Field trials of *Bacillus thuringiensis* H-14 and *Bacillus sphaericus*(strain 2362) formulations against *Anopheles arabiensis* in the central highlands of Madagascar. J Am Mosq Control Assoc. 1993;9:325–9.
[5]. Kahindi SC, Midega JT, Mwangangi JM, Kibe LW, Nzovu J, Luethy P, et al. Efficacy of vectobac DT and Culinexcombi against mosquito larvae in unused swimming pools in Malindi, Kenya. J Am Mosq Control Assoc. 2008;24:538–42.
[6]. Fillinger U, Lindsay SW. Suppression of exposure to malaria vectors by an order of magnitude using microbial larvicides in rural Kenya. Trop Med Int Health. 2006;11:1629–42.
[7]. Bagaari, L., Syedy, M., Sharma, G. P., Sharma, P. and Soni, P. (2013). Isolation of Crystal Protein from *Bacillus thuringiensis*. International Journal of Pure & Applied Bioscience, 1(2), 44-47.
[8]. Fillinger U, Lindsay SW. (2011). Larval source management for malaria control in Africa: myths and reality. Malaria Journal, 10:353.
[9]. El-kersh, T. A., Al-sheikh, Y. A., Al-akeel, R. A., Alsayed, A. A. (2011). Isolation and characterization of native *Bacillus thuringiensis* isolates from Saudi Arabia. African Journal of Biotechnology Vol. 11(8), pp. 1924-1938,
[10]. Edet UO, Anika OC, Umoren E (2019). Toxicity profile of crude oil degrading bacterial isolate s. International Journal of Scientific & Engineering Research. 10(7): 1131-1135.
[11]. Eswarapriya B, Gopalsamy B, Kameswari B, Meera R, Devi P (2010). Insecticidal Activity of Bacillus thuringiensis IBT- 15Strain against Plutellaxylostella. Int. J. Pharm. Tech Res. 2: 2048-2053.
[12]. Hawkins, N. J., Bass, C., Dixon, A. &Neve, P (2019). The evolutionorigins of pesticide resistance. Biology Reviews of the Cambridge Philosophical Society, 94(1), 135-155. doi: 10.1111/brv.12440.
[13]. Aramideh S, Saferalizadeh MH, Pourmirza AA, Bari MR, Keshavarzi M, Mohseniazar M (2010). Characterization and pathogenic evaluation of Bacillus thuringiensis isolates from West Azerbaijan province-Iran. Afr. J. Microbiol. Res. 4(12): 1224-1229.
[14]. Bhatt S, Weiss DJ, Cameron E, Bisanzio D, Mappin B, Dalrymple U, et al. The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. Nature. 2015;526:207–11.
[15]. Killeen GF, Fillinger U, Knols BGJ. Advantages of larval control for African malaria vectors: low mobility and behavioural responsiveness of immature mosquito stages allow high effective coverage. Malar J. 2002;1:8.
[16]. Lu, G., Traore, C., Meissner, P., Kouyate, B., Kynast-Wolf, G., Beiersmann, C., Coulibaly, B., Becher, H. &Muller, O. (2015). Safety of insecticide-treated mosquito nets for infants and their mothers: randomized controlled community trial in Burkina Faso. Malaria Research, 14:527, DOI 10.1186/s12936-015-1068-6.
[17]. Manu, G., Boamah-Kaali, E. A., Febir, L. G., Ayipah, E., Owusu-Agyei, S. and Asante, K. P. (2017). Low Utilization of Insecticide-Treated Bed Net among Pregnant Women in the Middle Belt of Ghana. Malaria Research and Treatment. https://doi.org/10.1155/2017/7481210.
[18]. Gachelin, G., Garner, P., Ferroni, E., Verhave, P. Opinel, A. (2018). Evidence and strategies for malaria prevention and control: a historical analysis. Malaria Journal, *17:96*https://doi.org/10.1186/s12936-018-2244-2
[19]. Saving Lives, Buying Time: Economics of Malaria Drugs in an Age of Resistance Kenneth J. Arrow, Claire Panosian, and HellenGelband, Editors, Committee on the Economics of Antimalarial Drugs. ISBN: 0-309-53233-7, 388 pages, 6 x 9, (2004) This PDF is available from the National Academies Press at: http://www.nap.edu/catalog/11017.html.
[20]. Khanam, S. (2017). Prevalence and Epidemiology of Malaria in Nigeria: A Review. International Journal of Research in Pharmacy and Biosciences 4(8): 10-12.
[21]. Moiroux N, Gomez MB, Pennetier C, Elanga E, Djènontin A, Chandre F,et al. Changes in *Anopheles funestus*biting behavior following universalcoverage of long-lasting insecticidal nets in Benin. J Infect Dis.2012;206:1622–9.
[22]. Ranson H, Lissenden N. Insecticide resistance in African Anophelesmosquitoes: a worsening situation that needs urgent action to maintainmalaria control. Trends Parasitol. 2016;32:187–96.
[23]. Zogo, B., Tcheieko, B. N., Koffi, A. A., Dahounto, A., Alou, L. P. A., Dabire, R. K., Baba-Moussa, L., Moiroux, N. Pennetier, C. (2019). Impact of sunlight exposure on the residual efficacy of biolarvicides*Bacillus thuringiensisisraelensis*and *Bacillus sphaericus*against the main malaria vector, *Anopheles gambiae*. Malaria Journal, 18:55, https://doi.org/10.1186/s12936-019-2687-0.
[24]. Riveron, J. M., Tchouakui, M., Mugenzi, L., Menze, B. D., Chiang, M. C. and Wondji, C. S. (2018). Insecticide Resistance in Malaria Vectors: An Update at a Global Scale http://dx.doi.org/10.5772/intechopen.78375.
[25]. Derua, Y. A., Kwela, E. ., Kisinza, W. N., Githeko, A. K. &Mosha, F. W. (2019). Bacterial larvicides used for malaria vector control in sub-Saharan Africa: review of their effectiveness and operational feasibility. Parasite and vectors, 12, 426. https://doi.org/10.1186/s13071-019-3683-5.
[26]. Johnson, B. H. & Hecht, M. H. (1994). Recombinant Proteins Can Be Isolated from *E. coli* Cells by Repeated Cycles of Freezing and Thawing. Biotechnology, 12, 1357-1361.