Biolarvicidal efficacies of entomopathogenic microorganisms isolated from the breeding sites of mosquitoes in Osogbo town, southwestern Nigeria

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The high rate of insecticide resistance in mosquito vectors has been a topical issue dominating discussion in different parts of the world (Oduola *et al.* 2010) and the urgent need to search for alternative methods has been seriously advocated (Howard *et al*. 2010). The search for alternative methods becomes imperative when considering the degree of morbidity and mortality associated with mosquito-borne diseases (Adeleke *et al*. 2010). Malaria is one of the leading infectious diseases threatening over 40 % of the world's population. The disease has been known to account for over 500 million clinical cases annually, with over 2.7 million deaths (WHO 2008). Apart from malaria, other mosquito-borne diseases such as filariasis, dengue fever, and yellow fever are also of considerable public health significance on different continents of the world (Adeleke *et al*. 2008).

Prominent among the alternative sources of mosquito control is the use of entomopathogenic microorganisms (Schotle *et al*. 2004). The entomopathogenic microorganisms have been reported to be highly toxic to the mosquitoes and safe to non-target organisms, thus making them environmental friendly (Howard *et al*. 2010). The use of microbial control agents, especially those that are effective against mosquito larvae, is now being revived after the discovery of highly mosquitocidal *Bacillus sphaericus* (Singer 1974) and *B. thuringiensis* serotype H-14 (Goldberg & Margalit 1977). Several advantages, *i.e.* high larvicidal activity, non-toxic to non-target organisms, cost-effectiveness and long shelf life of these agents have enabled their widespread use in different mosquito control strategies (Scholte *et al.* 2004).

However, there have been some dissenting opinions on the effectiveness of the conventionally produced biolarvicides against the indigenous or locally bred mosquitoes in different geographic areas (Bella *et al.* 1985; Obeta & Okafor 1986). The recent report of tolerance of local mosquitoes to *B. sphaericus* imported for the control of mosquitoes in Sokoto State, Nigeria, lends support to these divergent opinions (Manga *et al*. 2008). There is therefore need for concerted efforts to isolate and evaluate the local strains of biolarvicides for the control of locally bred mosquitoes. The present study was undertaken to identify the entomopathogenic microorganisms associated with the breeding sites of mosquitoes and their potential for biocontrol of *Culex* vectors in Osogbo town.

The study was carried out in Osogbo town, the Osun state capital (4°34'E 7°46'N). The Local Government Area has an area of 47 km^2 and a population of 155 507.

Water samples from breeding sites were collected into sterilized plastic containers. Collection of water samples was about 2–3 cm below the surface of the water where the effect of ultraviolet light was minimal.

The physicochemical parameters of each positive site were taken *in situ* using a digital meter (Genway, Germany) and tape measure. The parameters taken were temperature, pH, electrical conductivity, size of water body and type of water body.

Three culturing media used were nutrient agar (containing sodium chloride, beef extract, peptone, yeast extract, agar number 2), chocolate agar and MacConkey agar. All media were prepared following the manufacturers' specification. Each sample was inoculated with 0.25 ml into each plate containing one of the above media using a sterile standard wire loop and the inocula were streaked out to obtain distinct colonies. All plates were incubated at room temperature (25 ± 2 °C) for 24 hours. After incubation, plates were observed for growth after 24 hours.

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Table 1. Physicochemical parameters of the mosquito breeding sites encountered during the study.

Location	Temperature (°C)	pH	Electrical conductivity (μS)	Size of water body*
Gbomi	33.2	9.40	180.5	Small
Sussiy area	32.7	9.07	134.4	Medium
Oke-Baale	30.7	10.05	97.4	Small
Olorunkemi	29.3	11.65	68.5	Small
OmoodoAgba	30.0	11.54	103.5	Medium
Average	30.98	10.34	116.86	

*Small ≤10 × 10 m; medium >10 × 10 m but ≤20 × 20 m.

identification were carried out, followed by Gram staining for bacteria. Spore-producing entomopathogenic *Bacillius* identified in the water samples (*B. thuringiensis* and *B. sphaericus*) were sub-cultured on yeast-enriched nutrient agar at room temperature for 24 hours

Microbial concentration was prepared using a haemocytometer**.** The surfaces of the haemocytometer and coverslip were cleaned with 70 % ethanol. The coverslip was placed over the counting platform, and a small amount of water was placed on the ground glass area to improve the seal. The cells were then mixed thoroughly to disperse clumps and produce a uniform suspension. The cell suspension was used directly and the exact dilution was determined for the type of cells and initial concentration. A maximum cell count of between 20 and 50 cells/mm^2 was used (Fresken & Goren 2000). The sample was transferred to the edge of the haemocytometer and left to be drawn under the coverslip by capillarity. The cells were left for a few minutes before counting. The slide was viewed at ×400 magnification. The central area of the grid occupied the centre of the microscope field and the cells were evenly distributed without any clumps. Outer four squares were counted and a counter was used to record the number of cells in the grid (the more squares and cells, the better the precision but more time-consuming).

The cell concentration was calculated as follows:

Cell concentration per millilitre = Total cell count in 4 squares × 2500 × dilution factor

The larvae of *Cx. quinquefasciatus* collected during breeding site prospection were reared in the laboratory to adults. Literature exist that *Cx. quinquefasciatus* is the most widely susceptible mosquito to *Bacillius* species and has been extensively controlled using *B. thuringiensis* and *B. sphaericus* (Manga *et al*. 2008). The mosquito colony was maintained in the laboratory of Biological Sciences, Osun State University, Osogbo, as previously described by (Anyaele & Amusan 2003).

Petri dishes were labelled based on the formulated concentrations of *B. thuringiensis* and *B. sphaericus*. Five replicates of five *Cx. quinquefasciatus* larvae were transferred into Petri dishes containing 40 ml of sterile distilled water each, and also into the control Petri dish. To each Petri dish, 0.6 ml of different concentrations of larvicide (10^{10}, 10^{11}, 10^{12} bacterial cell concentrations) was added in accordance with the labelling. The control Petri dish had no biolarvicide. The larvae showed no sign of weakness or sluggishness before the introduction of the biolarvicides.

The biolarvicides were also introduced (with the same concentrations) into plastic containers with tadpoles as non-target organisms. The larvae and tadpoles exposed to *Bacillus* concentrations were allowed to stand for 72 hours at room temperature (Obeta & Okafor 1986). After every 12 hours, the numbers of dead larva(e) in each Petri dish were counted and recorded accordingly.

The data were transformed by square root of $x + 0.5$ and subjected to *t*-test to determine the significant variation in efficacy of the tested concentrations of the bacteria over the period of exposure. LT_{50} was calculated using probit analysis.

The mosquito breeding sites encountered during the study were stagnant water, stream, discarded tyres and gutters. The size of the breeding sites ranged from small to medium. The means of temperature, electrical conductivity and pH of the breeding sites were 30.98 °C, 116.86 μS and 10.34, respectively (Table 1).

Six species of microorganisms were isolated and identified from the water samples collected from the breeding sites. The microorganisms encountered were *Listeria monocytogens, Bacillus sphaericus, B. cereus, B. thuringiensis, Staphylococcus aureus* and *Aspergillus fumigatus*.

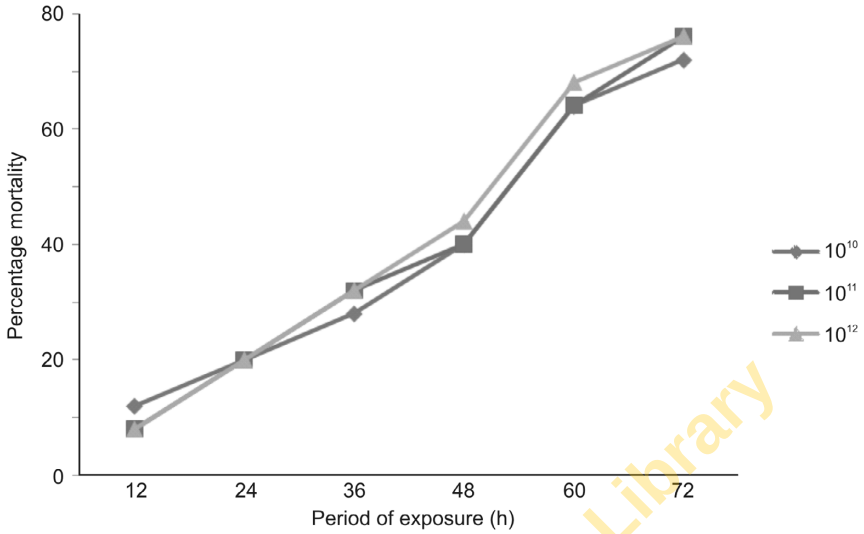

Fig. 1. Percentage mortality of *Culex quinquefasciatus* larvae at different concentrations of *Bacillius thuringiensis*.

For the purpose of biolarvicidal assay, the two spore-forming *Bacillus* species (*B. thuringiensis* and *B. sphaericus*) were selected for further studies. The results of bioassay of different concentrations of *B. thuringiensis* showed that the percentage mortality was higher at 10^{11} and 10^{12} bacterial cells per ml (76 %) than 10^{10} bacterial cells per ml (72 %), though there was no significant difference in mortality at different concentrations ($P > 0.05$) (Fig. 1). The time to achieve 50 % mortality (LT_{50}) decreased as concentration increased and was estimated as 55 hours, 50 hours and 49 hours for concentrations at 10^{10},10^{11}, and 10^{12} bacterial cells/ml, respectively.

In *B. sphaericus*, the percentage mortality increased as the concentration increased while the LT_{50} decreased as the concentration increased. The concentration at 10^{12} bacterial cells/ml recorded the highest mortality (80 %) while the least was recorded at 10^{10} bacterial cells/ml (60 %) (Fig. 2). The LT_{50}s were 59 hours, 51 hours and 46 hours for 10^{10}, 10^{11} and 10^{12} bacterial cells/ml, respectively.

There were no significant differences in mortality recorded over the duration of the period of

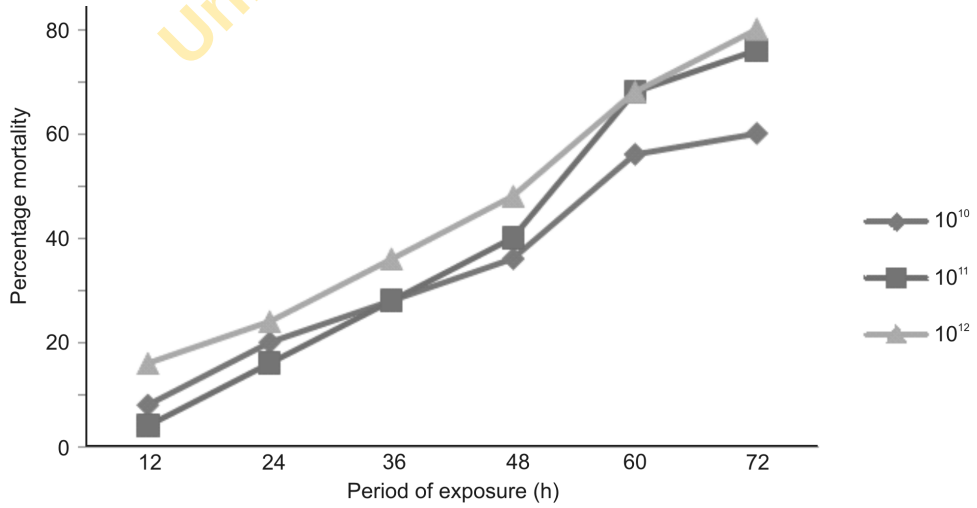

Fig. 2. Percentage mortality of *Culex quinquefasciatus* larvae exposed to different concentrations of *Bacillus sphaericus*.

exposure for different concentrations ($P > 0.05$). The comparison of the mortality and the LT_{50} of the two species did not show any significant difference ($P > 0.05$). However, there was no mortality in all the tadpoles exposed as they still remained active after 72 hours of exposure to the biolarvicides.

The results of physicochemical analysis of the breeding sites showed that the larval habitats were alkaline. This observation is contrary to the earlier report in the northern part of Nigeria where mosquito larval habitats were reported to range from slightly acidic to almost neutral (Adebote *et al*. 2008). The high alkalinity recorded in this study could be a reflection of large circulation of saturated ions at the breeding sites as indicated by the high electrical conductivity (above 100 μS) recorded in most of the larval habitats. The temperature above 25 °C conforms to the expectation of typical breeding sites of mosquitoes in Africa (Mwayangi *et al.* 2007).

The isolation of six microorganisms from mosquito larval habitats gives an indication that the habitats harbour a wide range of microorganisms which are also tolerant of the environmental conditions of the breeding sites. The bioassay of the two popular entomopathogenic bacteria (*B. thuringiensis* and *B. sphaericus*) against *Cx. quinquefasciatus* showed potency against the test mosquito larvae. The two biolarvicides achieved over 70 % mortality after 72 hours exposure which contradicts the reports of Manga *et al*. (2008) in Sokoto State, Nigeria, where it was reported that the imported strain of *B. sphaericus* has low potency in killing 60 % of larvae at the same period of exposure.

However, the two biolarvicides achieved LT_{50} beyond 24 hours and the LT_{50} decreased as concentration increased. The implication of this dose-dependent LT_{50} is that higher doses are required to achieve 50 % mortality within the shortest period. This information is germane for field evaluation in future; however, whether the high doses would also be safe for non-target organisms is a subject of further investigations. Thus, it could still be speculated that the high doses of the biolarvicides would be safe to non-target organisms, as previously postulated by literature (Anyaele & Amusan 2003; Schotle *et al*. 2004; Howard *et al*. 2010).

In conclusion, our study has shown that the locally isolated *B. sphaericus* and *B. thuringiensis* from the breeding sites of mosquitoes exhibited efficacy as biolarvicides to local species of *Cx. quinquefasciatus*. Further investigations exploring the use of locally isolated microorganisms for biocontrol of local mosquito vectors in the field will greatly help in containing the high spate of insecticide-resistant mosquitoes and ecological imbalances in the study area.

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