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Full Length Research Paper

Evaluation of the potency of some entomopathogenic bacteria isolated from insect cadavers on *Anopheles arabiensis* Giles (Order: *Dipthera*; Family: *Culicidae*) mosquito larvae in Nigeria

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The laboratory evaluation of larvicidal activities of some bacteria namely; *Pseudomonas aeruginosa, Bacillus polymyxa* and and *Bacillus subtilis* was assessed against the second and fourth instar of *Anopheles arabiensis* collected from South West Nigeria. Concentrations ranging from 1.3 x 10^7cfu/mL to 6.5 x 10^7 cfu/mL were tested on the larvae for a period of 48 h. The disparity in the activities was monitored with *B. subtilis* displaying the highest activity in both the second and fourth instar with LC_{50} of 0.865 and 2.361 mg/mL respectively. *P. aeruginosa* showed LC_{50} of 1.931 and 4.205 mg/mL while the least activity was recorded in *B. polymyxa* with LC_{50} of 5.776 and 7.403 mg/mL. There were significant differences in the LC_{50} value of the bacteria on the tested instars. Values obtained from the fourth instar group were significantly different from those obtained from the second instar group in all the treatments. Finding from this study show that *B. subtilis* may be a potential biocontrol agent of *A. arabiensis,* the main malaria vector in Nigeria.

Key words: Biocontrol, larvicidal activities, *Anopheles* mosquito larvae, malaria.

INTRODUCTION

Mosquito (Diptera) is one of the social insects that has posed serious problems to the health of man by serving as a vector to the etiologic agents of diseases such as malaria, dengue fever, filariasis and Japanese encephalitis which remain endemic in the sub-sahara region of developing countries (Omoya et al., 2009). Malaria is most challenging among the afore mention diseases considering the morbidity-mortality ratio. According to Breman et al. (2004), globally, 300 to 500 million cases of infection and over one million deaths are reported annually; 90% of these occur in tropical Africa. Nigeria is known for its high prevalence of malaria and available records show that about 50% of the population suffer from at least one episode of malaria each year. Malaria accounts for over 45% of all out-patient cases in Nigeria (Federal Ministry of Health, 2001). The global increase of malaria toward the end of the 1970s coupled with the inadequate results of malaria eradication strategies in many countries especially the tropical countries have led to concerted search for new methods of control. This was due largely to the emergence of strains of *Plasmodium falciparum* resistant to chloroquine and sulphadoxine-pyrimethamine (SP), the mainstays of treatment (Trape et al., 1998). The use of chemical insecticides as prophylaxis measure in applications such as coils, sprays, insecticide-treated nets (ITNs) have posed threat to human health and the ecosystem (Philip, 2001).

An important alternative measure to chemical insecticides is biological control measure which involves the regulation of pest population using natural control agents

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such as predators, nematodes and microbial insecticides (Merritt et al., 2005). It is the use of one biological organism to control another; releasing beneficial bacteria, fungi or arthropods to limit pest infestation. Weinzierl et al. (2005) noted that the organisms used in microbial insecticides are essentially non-toxic and non pathogenic to non-target organisms. The safety offered by microbial insecticides is their greatest strength. Bacteria and fungi have been shown to kill mosquitoes to varying degrees (Orduz and Axtel, 1991; Su et al., 2002). *Bacillus thuringiensis* var *isrealiensis* (BTI) and *B. sphaericus* are being used in worldwide field test designed to control mosquitoes' population (Philip, 2001). These microorganisms have their own limitations which include low persistence of the bacterial larvicidalcystal protein in warm environment as a result of sunlight inactivation. The direct use of BTI cells also has its drawbacks as its cells do not exhibit stable habitation in their environment. In addition, the strain of mosquito existing in a particular region appears to differ from that in another place. Thus, BTI may not be effective for use in all regions where mosquitoes are problematic. Therefore, the isolation and development of bacterial strains with larvicidal activity having a broad host range specifically, stable habitation and non-hazardous properties is desired. In order to achieve these, we investigate the larvicidal activities of *Pseudomonas aeruginosa, Bacillus polymyxa* and *Bacillus subtilis* on different instars of *Anopheles* mosquito larvae at varying concentrations under laboratory condition.

MATERIALS AND METHODS

Insect rearing

The mosquito larvae were collected from stagnant waters. They were selected and differentiated using both physical and molecular characterisations. The A. *arabiensis* larvae were reared in a meshed cage at 25°C and 70% relative humidity under 14L:10D photoperiod with slight modifications according to Zhong et al. (2006). They were fed daily with Tetramin® fish food. This allowed them to reach maturity stage and where offered blood meal. Eggs laid on wet filter papers were transferred to water trays. Larvae were fed and sorted for bioassays.

Entomopathogenic bacteria

This study was conducted at the Federal University of Technology Akure, Nigeria. Cockroaches and housefly were collected into sterile containers from their natural breeding habitats (cupboards for cockroach and housefly around the refuse dumps) in Akure, Nigeria. In the laboratory, adult cockroaches were placed inside a sterile Petri dish containing 10 mL of sterile water each (in triplicate). The Petri dish was properly shaken to ensure good washing away of particles that were on the cockroaches. 1 ml was taken from the wash water, serially diluted to 10^{-4} and 0.1 ml of the 10^{-4} serial dilution was pour plated using molten nutrient agar and potato dextrose agar. Incubation was done at 37°C for 24 h and the plates were observed for growth. Identification of the bacterial isolates was done using cultural, morphological and biochemical characteristics according to the methods of Holt et al. (1994). The identification of fungi was by comparison of the observed morphological characteristics with those described by Onions et al. (1995), after examination under the microscope. The same proce-dure was repeated using housefly. *B. subtilis, P. aeruginos*and *B. polymyxa* were selected for bioassay due to high larvicidal activity recorded on preliminary assay conducted in our laboratory.

Cultivation of bacteria

A basal medium containing K_2HPO_4 (17.4 g), NH_4SO_4 (1.98 g), $MgSO_4$ (0.48 g), $FeSO_4.7H_2O$ (0.0025 g) and glucose (2.0 g) in 100 mL of sterile distilled water was used. Each isolate was inoculated into10 mL of sterile basal medium, incubated at 37°C for 24 h. The cells were centrifuged at 12.168×10^3 g for 15 min (Centrifuge MSE Minor 35) and re-suspended into 2 mL sterile water. The cells were counted and diluted. At inoculation onto mosquito, the diluted cells were pour plated into nutrient agar, incubated and counted using colony counter.

Susceptibility of *A. arabiensis* larvae to *P. aeruginosa*, *B. polymyxa* and *B. subtilis*

One hundred (100) *A. arabiensis* larvae were used for each concentration in this experiment. The mosquito larvae were surface sterilised in separate Petri dishes using 75% alcohol and rinsing with sterile water. There were four replicates and control per treatments with 25 mosquito larvae in each container. The mosquito larvae were starved for 24 h prior to inoculation. Each mosquito larva was inoculated with the cells of bacterial isolates at varying cell loads. Incubation was carried out for 48 h. The cadavers were removed daily and counted.

Statistical analysis

All data were analysed using analysis of variance (ANOVA), Duncan Multiple Range Test was used to separate mean while Probit analysis (Finney, 1971) was used to determine the LC_{50} that will kill 50% of test population and resistance ratio.

RESULTS AND DISCUSSION

Based on our previous findings (Omoya et al., 2010) and by the new outcomes, we selected the tested microorganisms for evaluation in the successive bioassay. Cell population and incubation time were seen to affect the degradation and subsequently lead to increase in the percentage mortality (Figures 1 to 3). This implies that there is need to increase the contact time to effectively eradicate mosquito larvae particularly when low cell number is used. The degradation rate of mosquito larvae was rapid when sufficient high cell number was used. *B. subtilis* exhibited higher larvicidal activity than both *P. aeruginosa* and *B. polymyxa*. This observation was noticed after 24 h of infesting the larvae with the organisms. In all the treatments, the percentage mortality recorded was seen to increase with increase in concentration with *B. Subtilis* treatment showing the highest percentage mortality. Although, there was no significant

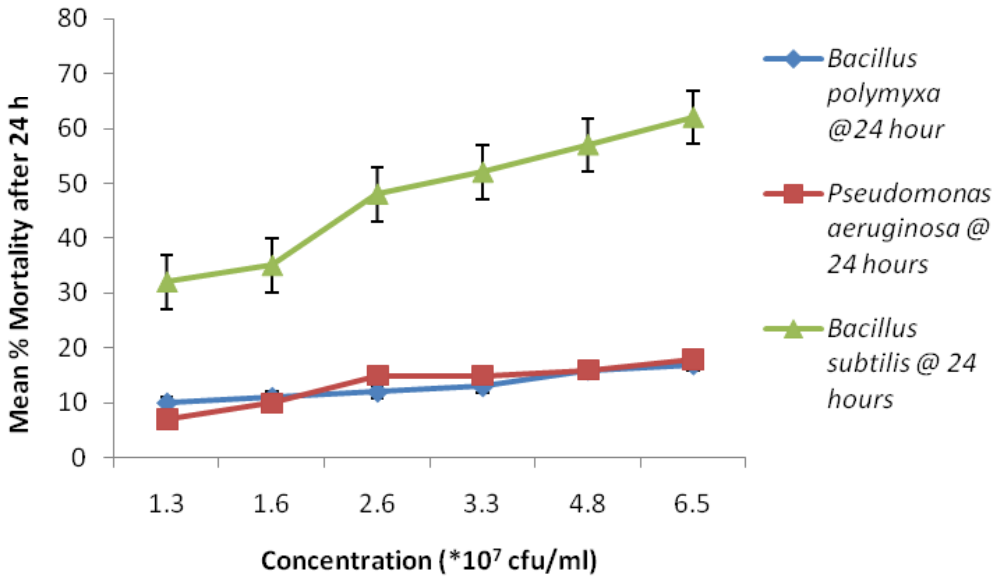

Figure 1. Effect of different concentration of bacterial isolateson the mortality of 2nd instar *Anopheles* mosquito larvae at 24 h.

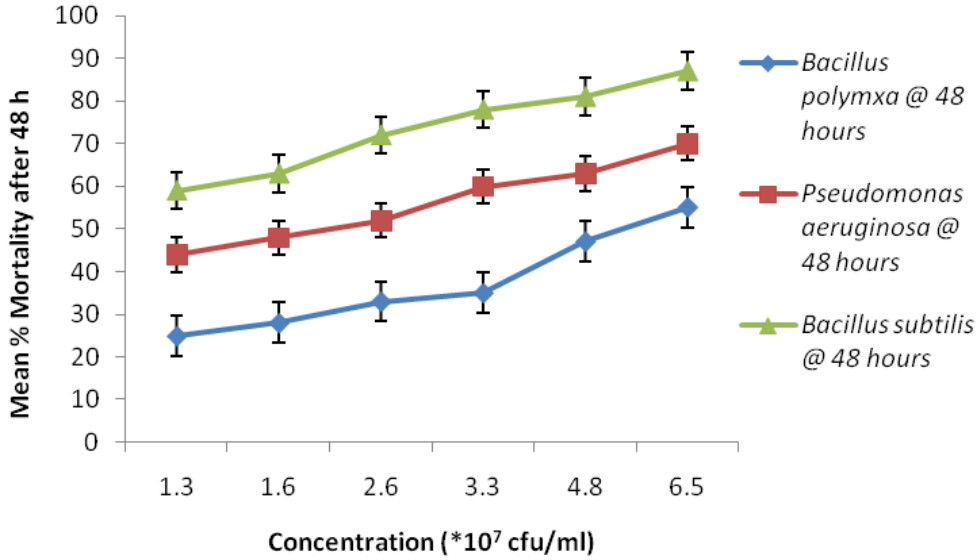

Figure 2. Effect of different concentration of bacterial isolateson the mortality of 2nd instar *Anopheles* mosquito larvae at 48 hours

difference in percentage mortality of *P. aeruginosa* and *B. polymyxa* at 24 h of exposure (Figure 1), at 48 h of exposure to all the treatments, gradual increase in percentage mortality was recorded and results from each treatment were significantly different from each other (Figure 2). This might be as a result of increase in the feeding rate of the larvae which is in agreement with the results of Gunasekaran et al. (2004). In Figure 3, decrease in percentage mortality of the inoculated mosquito larvae was observed. This could be as a result of decrease in the ingestion rate due to the age of the larvae. During this present study, the mosquito larvae showed greatest susceptibility to *B. Subtilis* when compared to the other tested bacterial isolates. Toxin concentration of 4 to 5 folds of *B. polymyxa* was necessary to induce the same effect of 50% mortality

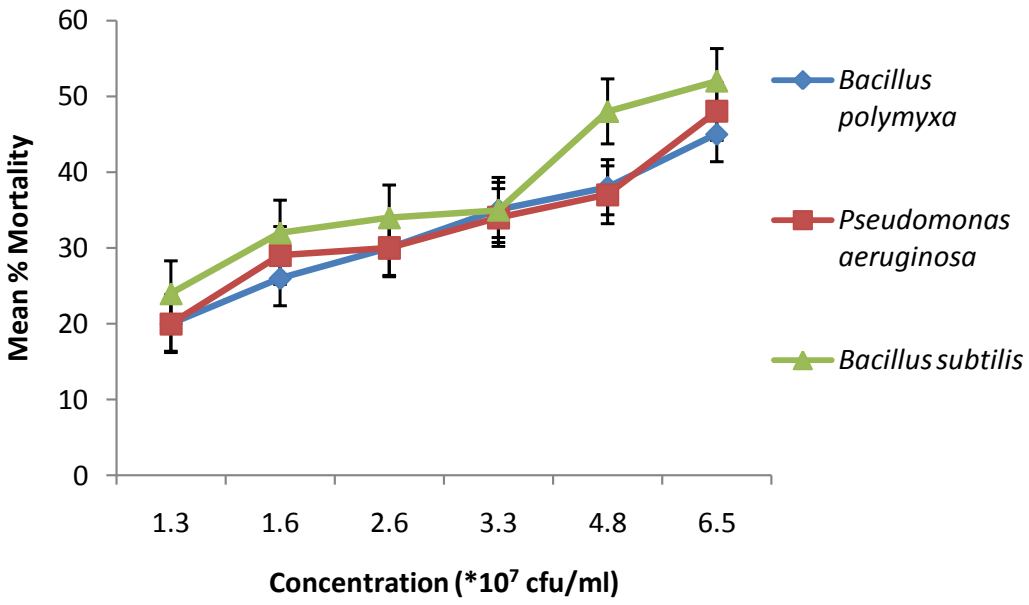

Figure 3. Effect of different concentration of bacterial isolateson the mortality of 4th instar *Anopheles* mosquito larvae.

Table 1. Relative potency of *Bacillus subtilis, Pseudomonas aeruginosa* and *Bacillus polymyxa* on second instar.

Line name	LC_{50}	Lower limit	Upper limit	1	2	3	Index	RR	Slope	LC_{50}
Bacillus subtilis	0.862	0.433	1.219	*			100	1	1.249	0.862
Pseudomonas aeruginosa	1.931	1.23	2.501		*		44.64	2.24	0.926	1.931
Bacillus polymyxa	5.776	4.442	9.415			*	14.92	6.701	1.116	5.776

LC_{50}, lethal concentration at which the extract kill 50% of the organisms at a given time.*1, 2 and 3 showed a significant difference in the test organisms; RR, resistance ratio: resistance ratio (RR) is calculated by dividing the LC_{50} of the most potent microorganisms with other susceptible strains.

Table 2. Relative potency of *Bacillus subtilis, Pseudomonas aeruginosa* and *Bacillus polymyxa*on fourth instar.

Line name	LC_{50}	Lower limit	Upper limit	1	2	3	Index	RR	Slope	LC_{50}
Bacillus subtilis	2.36	0.433	1.219	*			100	1	1.021	2.36
Pseudomonas aeruginosa	4.205	3.805	5.586		*		33.25	1.78	1.623	4.205
Bacillus polymyxa	7.403	4.442	9.415			*	16.78	3.34	1.862	7.403

LC_{50}, lethal concentration at which the extract kill 50% of the organisms at a given time.*1, 2 and 3 showed a significant difference in the test organisms; RR, resistance ratio: resistance ratio (RR) is calculated by dividing the LC_{50} of the most potent microorganisms with other susceptible strains.

(LC_{50}) on the larvae when compared to *B. Subtilis* while 2 folds of *P. aeruginosa* concentration will cause the same effect and resistance ratio (RR) showed that the three tested organisms in second instar varied. *P. aeruginosa* showed that the resistance ratio (RR) values was 2.24 folds above that of *B. substilis* while *B. Polymyxa* was 6.70 folds above *B. substilis* (Table 1). Variation in the LC_{50} and RR values was recorded in Table 2. Toxin concentration of above 3 folds of *B. polymyxa* was necessary to induce the same effect of 50% mortality (LC_{50}) on the larvae when compared with *B. subtilis* while 2 folds of *P. aeruginosa* concentration will cause the same effect and RR values of *P. aeruginosa* was 1.78 folds above that of *B. substilis* while *B. polymyxa* was 3.34 folds above *Bacillus substilis* in fourth instar. The observed difference in the susceptibility might be due to their ingestion rate (Sun et al., 1980). Hence more mortality of the mosquito larva was recorded

in the second instar treatments. Therefore, from the data obtained in this study, we conclude that the application of *B. Subtilis* to kill malaria vector larvae could significantly reduce parasite transmission, be economical and therefore lead to reduction of malaria risk. Hence this method of biological control has potential as a new strategy for malaria control in Nigeria.

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